REVERSED-PHASE HPLC METHOD DEVELOPMENT AND VALIDATION OF
LATANOPROST IN OPHTHALMIC SOLUTION AND IDENTIFICATION OF UNKNOWN
IMPURITIES BY LC-MS/MS

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Abstract

A simple, precise, accurate HPLC method is developed and validated for analysis of latanoprost in Ophthalmic Solution. Separation was achieved on a reversed-phase Waters Xterra RP18 (250 X 4.6 mm, 5 µm) column using a mobile phase consisting of 10mM ammonium formate with pH adjusted to 3.5 with formic acid and acetonitrile at a flow rate of 1.0ml/min and UV detection at 210 nm. The same analytical conditions were adopted for Mass spectrometry using HRMS and data acquisition by MassLynx software. This method is validated as per ICH guidelines, which include accuracy, precision, selectivity, robustness, linearity and range. The current method demonstrates good linearity over the range of 0.05–0.15 mg/ml of latanoprost with R² of 0.999. The average recovery of the method is 99.58% with a relative standard deviation of 0.2%. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust and rugged. The developed method is useful for specified, unspecified impurities as well as contaminants as Varnish impurities identification by mass spectrometer of latanoprost in eye drops.

Key Words: Latanoprost, Ophthalmic Solution, Varnish, impurity, HPLC and HRMS.

Introduction

Latanoprost is an antiglaucoma drug with the chemical name "Isopropyl-(Z)-7[(1R, 2R, 3R, 5S)3,5-dihydroxy-2-[(3R)-3-hydroxy-5-phenylpentyl]cyclopentyl]-5-heptenoate" and chemical structure is shown in Figure-1. Latanoprost is a prodrug of prostaglandin PGF2a approved for the therapy of glaucoma [Comparison of glaucomatous, (1998)] and ocular
hypertension [Camras et al, (1996)]. Glaucoma is a chronic disease requiring lifelong therapy [George et al, (2010), Quigley et al, (2006)]. Medical therapy is the first line treatment and prostaglandin (PG) analogues are the most effective drugs that increase uveoscleral outflow of aqueous humor [Maier et al, (2005), Husain et al, (2008)]. Intraocular pressure (IOP) reduced by latanoprost with minimal side effects [Stjernschantz et al, (1992)]. Carboxylic acid portion of latanoprost will be esterifies to increase the bioavailability of active drug in the eye [Basu et al, (1994)]. Latanoprost for IOP reduction is compared with other anti glaucoma drugs and found much greater [Ser et al, (1994), Watson et al, (1998), Higaki et al, (1995)]. Latanoprost was not been tested extensively for its IOP reducing action of other drugs. The reported methods are limited for drug evaluation of latanoprost by High Performance Liquid Chromatography (HPLC) [Patel et al, (2015)]. In this reference PDA used for latanoprost raw material analysis, is complicate and larger run times and not reported for formulations analysis. Therefore, a reverse phase liquid chromatographic method for selectively perform latanoprost in presence of impurities, degradation products as well as contaminants as Varnish impurities presented, Varnish chemical structure shown in Figure-1.

![Chemical Structure of Latanoprost and Varnish](image)

**Figure- 1. Chemical Structure of Latanoprost and Varnish.**

Impurity profiling in active pharmaceutical ingredients and formulation analysis is the prime challenge for analytical method finalization for scientists in the pharmaceutical industry [ICH guideline Q3B (R2), (2006), ICH guideline Q7A, (2005)] the presence of unwanted and unknown chemicals, at lower amount may work for therapeutic value but also safety of pharmaceutical formulation. The HPLC conditions were set in order to reach suitable operation for routine as well as impurity identification by mass spectrometry. The developed method conditions are mass compatible. The validation of method performance by checking response, linearity, precision, accuracy and ruggedness. The developed
method is simple and reproducible gradient RP-HPLC method for better resolution of all impurities and validated for the quantitative determination of Latanoprost and identification of new impurities in pharmaceutical dosage forms by LC-MS/MS, typical HPLC chromatogram is shown in Figure-2.

![HPLC chromatogram of Latanoprost.](image)

**Figure - 2. HPLC chromatogram of Latanoprost.**

**Experimental**

**Materials and reagents**

Class A glassware used for performing the analysis, analytical reagent grade ammonium formate (S. D. Fine Chem., India), HPLC grade and formic acid (Spectrochem, India), and acetonitrile and methanol from Rankem India, and HPLC grade Milli Q water collected in the laboratory was used for preparing mobile phase and filtered and degassed through 0.45µm membrane filter paper under vacuum, before its use as method mobile phase. To check the method suitability, stressed samples were subjected for use. Stress conditions, for which hydrogen peroxide, hydrochloric acid and sodium hydroxide (S. D. Fine India) were used. Prepared the reference and system suitability solution of pharmaceutical reference standards of latanoprost receive as gift from M/s Saimed labs limited, Erragadda, Hyderabad, India, were used.

**Stock and sample solutions.**

Stock solution of latanoprost: 1000 µg/ml in acetonitrile. LC-MS sample concentration: Stock solution of 1.25ml was diluted to 25 ml of acetonitrile, the resultant Solution is 0.005% w/v concentration.

**LC Conditions**

The method was developed using Waters alliance 2695 HPLC system, Xterra RP18, (250mm X 4.6 mm, 5 µm) column (Waters, Milford, USA). Mobile phase A containing buffer and acetonitrile in the ratio of 90:10 v/v, and Mobile phase B
consisting of buffer and acetonitrile in the ratio of 25:75 v/v. The buffer is 10mM ammonium formate, pH 3.5 was adjusted with formic acid. The mobile phase was filtered through 0.45 µm nylon 66 membrane filter. The gradient program (T/%B) was set as 0/35, 10/35, 50/50, 60/95, 80/95, 85/35 and 95/35. The flow rate of the mobile phase was 1.0 ml/min. The column temperature was maintained at 30°C and the wavelength was monitored at 210 nm. The injection volume was 50 µl. The retention time of latanoprost is about 48.8 min and the run time is 95 min.

**LC–MS/MS conditions**

LC–MS/MS system, Waters Acquity Ultra Performance Liquid chromatography coupled with Synapt™ MS System with MassLynx™ 4.1 Software with MassFragment™ (Waters Corporation, Milford, USA) was used for characterization of impurities. The analysis was performed in electro spray positive ionization mode (ESI+) with capillary and cone voltages were 3.5 kV and 15 V, respectively. Source and desolvation temperatures were 120°C and 350°C, respectively. Desolvation gas flow was 800 L/hr, extractor voltage: 3.0 V, cone gas flow:25 L/hr, acquisition energies: ramp from 10 to 30 eV, scan time: 1.0 second, lock mass: Leucine Enkelphalin 200 pg/µL @ 10 µL/min.

**Results and Discussion**

**Method Optimization**

Initially screened different C18 columns and testing several mobile phase compositions, temperature and different gradient programs for the separation of latanoprost with good chromatographic performance. Four different C18 columns, Agilent Eclips XDB C18 [Mansoor et al, (2014)], Inertsil C18 [Ankit et al, (2013)], Supleco C18 and Waters Xterra RP18 (250mm x 4.6mm, 5µ) were screened. The resolution and peak shapes were found to be good on Xterra RP18 column, hence Xterra RP18 column was selected for further optimization.

When methanol and acetonitrile were used in initial screening, it was observed that, acetonitrile was found to be better in terms of peak shape and resolution as compared to methanol. Hence acetonitrile as an organic modifier was selected. The column temperature was screened at 25, 30, 35 and 40°C and the peak tailing were found to be optimum at 30°C, therefore all further studies were carried out at 30°C of column temperature.

Ammonium formate was chosen as it is a mass compatible volatile buffer for LC-MS analysis. The effect of the concentration of ammonium formate was studied by varying its concentrations of 5, 10, 20 mM. The ammonium formate with 10mM was found to be highly suitable as the chromatographic peaks were well separated with symmetrical
peak shapes with good resolution. Gradient elution was selected for this separation as it improves peak shapes and suitable to elute all polar, medium polar and non polar analytes form the sample. Finally well separated peaks with acceptable peak shapes were achieved by employing the following mobile phase. Mobile phase A containing buffer (10mM ammonium formate, pH 3.5 was adjusted with formic acid ) and acetonitrile in the ratio of 90:10 v/v, and Mobile phase B consisting of buffer and acetonitrile in the ratio of 25:75 v/v. The mobile phase was filtered through 0.45 µm nylon 66 membrane filter. The gradient program (T/%B) was set as 0/35, 10/35, 50/50, 60/95, 80/95, 85/35 and 95/35. The flow rate of the mobile phase was 1.0 ml/min and a detection wavelength of 210 nm afforded the best separation of latanoprost free from interferences.

Method validation

After method development, validation of the current test method for latanoprost was performed in accordance with ICH requirements for assay determination which include accuracy, precision, selectivity, robustness, linearity and range [ICH guideline Q2 (R1), (2005)]

Linearity and range

The linearity was done for five point calibration of latanoprost containing 0.025, 0.050, 0.075, 0.100, 0.125, 0.150 mg/ml were analyzed. A plot of Latanoprost peak areas versus concentration was linear in the range from 0.05 to 0.15 mg/ml with a correlation coefficient of 0.999. Linearity was demonstrated over a range is shown in Table -1 and linearity plot shown in Figure -3.

Table -1. Linearity data of Latanoprost.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025</td>
<td>1253</td>
</tr>
<tr>
<td>0.050</td>
<td>2498</td>
</tr>
<tr>
<td>0.075</td>
<td>3766</td>
</tr>
<tr>
<td>0.100</td>
<td>5019</td>
</tr>
<tr>
<td>0.125</td>
<td>6101</td>
</tr>
<tr>
<td>0.150</td>
<td>7531</td>
</tr>
</tbody>
</table>
Accuracy and percentage recovery

Latanoprost drug added to the placebo for mimicking three different formulation concentrations (0.05, 0.1, 0.15 mg/ml of Latanoprost). Results have shown in Table -1 that the recovery of latanoprost is between 99.5–101.2%, and the RSD is less than 1.0% shown in Table-2.

Table -2 Accuracy (% recovery) of latanoprost.

<table>
<thead>
<tr>
<th>Latanoprost Accuracy (% recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mg/ml)</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>0.05</td>
</tr>
<tr>
<td>0.10</td>
</tr>
<tr>
<td>0.15</td>
</tr>
</tbody>
</table>

Precision

Repeatability

Percent RSD calculated as method repeatability for six injections of latanoprost standards found as 0.24% RSD and the recovery data referred in Table -2 as latanoprost analysis is repeatable.

Intermediate precision (ruggness):

Ruggness of the method is evaluated for six samples each by two analysts on different instruments and the resulting data is 0.8% RSD for twelve samples indicates ruggedness of the method.

Selectivity

Degraded sample was demonstrated for method selectivity of latanoprost under stress conditions (acid, base hydrolysis and oxidation), to show that latanoprost is separated from possible degradation products in stress condition. Results have
shown that latanoprost is stable in hydrogen peroxide solution for one week. Furthermore, it was also found that latanoprost solution was stable at 60°C temperature for one week storage. Latanoprost was degraded by 40% in acid and 35% in base was degraded in HCl and NaOH solutions respectively. No interferences were observed in the developed method.

**Robustness**

Robustness of the method was check by altering flow rate for Plus and minus 0.2ml/min of set method that is 1.0ml/min, detection at 205nm and 215nm and acetonitrile percentage variation by 2.0 in mobile phase A studied. The results are less than 1.0% RSD and tabulated in Table -3.

**Table -3. Robustness testing of the latanoprost.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Content Latanoprost Assay (%)</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample-1</td>
<td>Sample-2</td>
</tr>
<tr>
<td>Flow Rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>100.3</td>
<td>101.1</td>
</tr>
<tr>
<td>1.0</td>
<td>99.8</td>
<td>101.1</td>
</tr>
<tr>
<td>1.2</td>
<td>101.6</td>
<td>100.7</td>
</tr>
<tr>
<td>Acetonitrile (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>101.4</td>
<td>101.6</td>
</tr>
<tr>
<td>10</td>
<td>100.1</td>
<td>100.8</td>
</tr>
<tr>
<td>12</td>
<td>100.6</td>
<td>100.3</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>101.2</td>
<td>100.1</td>
</tr>
<tr>
<td>205</td>
<td>100.6</td>
<td>101.1</td>
</tr>
<tr>
<td>215</td>
<td>99.1</td>
<td>100.1</td>
</tr>
</tbody>
</table>
Identification of Impurities by LC-MS

The workflow approach provides a systematic data-driven association to correlate the variety of data acquired by the two scan functions generated by MS\(^E\) experiments. This is used for predicting the elemental composition of the chemical entities (low Collision Energy), and fragment ion data for structure confirmation (high Collision Energy) in the absence of pure standards and workflow schematic approach shown in Figure-4.

**Figure-4. Workflow for Impurity Identification in Formulation.**

Latanoprost sample analysis has shown two unspecified impurities by the developed mass compatible method and was subjected for identification. the relative retention times of two impurities are 0.38 and 0.40 with respect to latanoprost. Latanoprost and Varnish MS-MS\(^E\) positive mode low and high collision energy traces are shown in figure-5a and 5b. MS-MSE trace of RRT at 0.38 are shown in Figure-6a and 6b, and RRT 0.40 are shown in Figure-7a and 7b.
Figure-5a. UV&MS^E Trace of Latanoprost Ophthalmic Solution in Positive Mode at Low Collision Energy.

Figure-5b. UV&MS^E Trace of Varnish Sample in Positive Mode at Low & High Collision Energy.

Figure -6a. ESI-MS^E Spectrum at Low and High Collision Energy (impurity RRT 0.38).
Figure 6b. ESI-MS$^E$ Spectrum at Low Collision Energy (impurity RRT 0.38)

Figure 7a. ESI-MS$^E$ Spectrum at Low and High Collision Energy (impurity RRT 0.40).

Figure 7b. ESI-MS$^E$ Spectrum at Low Collision Energy (impurity RRT 0.40).
Based on the elemental composition proposed by the elemental predictor, search for the structures were done using ChemSpider database. There are one hundred hits obtained for the proposed elemental composition. Further doing advanced search, hits reduced down to one. Using the HRMS technology, identification of the source of the impurity at RRT 0.38 and 0.40 was made easy and use of software tools like elemental predictor and MassFragment help to determine the elemental composition and proposed Varnish impurities in latanoprost ophthalmic solution. Results obtained in both standard and the sample makes us to understand that Varnish is the source of the contaminant impurity present in the latanoprost ophthalmic solution.

**Conclusions**

To routine analysis a simple accurate and precise HPLC method was developed and validated latanoprost in eye drop formulations. The method is useful for routine impurities identification by mass spectrometer of latanoprost in eye drops. The method detected trace analytes in the sample therefore it is highly recommended, which is very useful for identifying the source of unknown impurities. This method is specific and no co-elusions of impurities, excipients and degradents observed. Method is accurate and linear over concentrations ranging from the 0.025 to 0.15 mg/mL. No major abnormalities were found using this method for every day analysis. Thus it is rugged and robust. Therefore this can agree that, the proposed method can be used in quality control as well as for the impurity profiling study of latanoprost.

**References**


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