Development and Validation of Forced Degradation Studies of Atazanavir Using RP-HPLC and Characterization of Degradants by LC-MS/MS

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ABSTRACT
The main objective of current study is to develop and validate RP-HPLC, simple, precise, accurate and specific chromatographic method for the determination of atazanavir and degradation products are characterized by LC-MS/MS in tablets. A high performance liquid chromatography instrument and Hypersil BDS, 150 x 4.6 mm, 5 were used for determination of atazanavir. Mobile phase as a acetonitrile and buffer in the ratio of 1:1V/V. The flow rate of 1.0mL/min was set with isocratic, the temperature of column compartment maintained at 30°C and Ultra violet detection done at 248nm wavelength. The atazanavir eluted at 3 minutes and then run time was set as about 10 minutes. The correlation coefficient (≥ 0.999) shows the linearity of response against concentration over the range of 20 to 150%. The observed result shows that the method is rapid, precise, accurate and simple. The method was validated as per ICH guidelines.

Keywords: Atazanavir, Hypersil BDS, RP-HPLC, Validation.

INTRODUCTION
Atazanavir is an antiretroviral drug of the protease inhibitor class. Like other antiretrovirals, it is used to treat infection of human immunodeficiency virus (HIV). Atazanavir is distinguished from other protease inhibitors in that it can be given once-daily. The IUPAC name of atazanavir is methyl N-[(2S)-1-[(2S,3S)-2-hydroxy-3-[(2S)-2-(methoxycarbonylamino)-3,3-dimethylbutanoyl]amino]-4-phenylbutyl]-2-[(4-pyridin-2-ylphenyl)methyl]hydrazinyl]-3,3-dimethyl-1-oxobutan-2-yl]carbamate. Atazanavir is metabolized by CYP3A4 in the liver and is 86% bound to human serum proteins. 1-3

Figure 1: Structure of Atazanavir

In recent years several methods have been developed to quantify antiretroviral drugs in plasma. Literature survey reveals that there was so many spectrophotometric and HPLC methods were available for determination of Atazanavir in bulk and formulations. 4-12. But only a few LC-MS/MS methods 13-18 allow the simultaneous monitoring of atazanavir in human plasma. The major disadvantages of all these methods include complicated and expensive extraction procedures, long chromatographic run time, typical mobile phase, and polarity switching. Moreover these methods are not specific for the determination of atazanavir; hence it may create conflicts in final results due to improper characterization of selectivity. The chemical structure of atazanavir shown below figure-1. The present work was validated as per ICH guidelines. 19-25

MATERIALS AND METHODS

Chemicals
Qualified standards for drug substance and samples were obtained from Local analytical Laboratory and were used without any further purification.

The chemicals like potassium dihydrogen phosphate, triethylamine, orthophosphoric acid, acetonitrile were purchased from Merck, Mumbai. Millipore water generated from TKA system.

The analytical column used was Hypersil BDS, 150 X 4.6 mm, 5μ.

Instruments

A Waters prominence HPLC system equipped with a quaternary UFLC LC-20AD pump, a DGU-20A5 degasser, a SPD-M20A diode array detector, a SIL-20AC auto sampler, a CTO-20AC column oven and CBM-20A communications bus module was used for method development and validation studies.

Standard Stock preparation

Accurately Weighed and transferred 10mg of Atazanavir working standards into a 10 ml clean dry volumetric flask, add 7ml of water, sonicated for 5 minutes and make up to the final volume with water.
Preparation of sample for 150 mg/ml
5 tablets were weighed and calculated the average weight of each tablet. Then the weight equivalent to 5 tablets were transferred into a 100mL volumetric flask, 50mL of water added and sonicated for 25 min, further the volume made up with water and filtered.

From the filtered solution 0.2ml pipette out into a 10 ml volumetric flask and made up to 10ml with water.

Chromatographic conditions
The chromatographic column used was Hypersil BDS column with dimensions of 150 mm X 4.6 mm with 5µm particle size.

The column temperature was maintained at 30°C and detection was monitored at a wavelength of 248 nm. Injection volume was 10 µl and the mobile phase flow was set at 1.0mL/min.

The water, acetonitrile in the ratio 30:70 v/v was used as diluents for preparation of solutions.

Method Validation
The developed method for determination of was validated for system suitability along with method selectivity, specificity, linearity, range, precision (Repeatability and Intermediate precision), accuracy, limits of detection and Limit of quantification according to the ICH guidelines.

System Suitability
The system suitability was conducted using diluted standard preparation and evaluated by injecting six replicate injections.

Specificity
Specificity is the ability of analytical method to assess un equivocally the analyte in the presence of component that may be expected to be present, such as impurities, degradation products and matrix components.

Linearity
Performed the linearity with atazanavir in the range of 20 to 150% of specification limit. Recorded the area response for each level and calculated slope, intercept & correlation coefficient.

Precision and Accuracy
The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample.

LOD and LOQ
The LOD and LOQ were determined for atazanavir based on the standard deviation of (SD) of the response and slope (S) of the regression line as per ICH guidelines.

Forced degradation studies
Forced degradation of atazanavir was carried out according to ICH guidelines Q1A (R2).

About 1.0 ml of atazanavir was subjected to forced degradation under acidic, basic and neutral conditions by refluxing with each 1.0mL of 2N hydrochloride, 2N sodium hydroxide and water at 60°C for 4 Hours.

Oxidation of atazanavir was carried out using 20% H2O2 for 30 minutes. Thermal stress studies conducted in hot air oven at 105°C for 6 hours.

RESULTS AND DISCUSSION
Optimization of chromatographic conditions
Method development includes selection of appropriate chromatographic conditions/factors like detection wave length, selection and optimization of stationary and mobile phases.

The wavelength of 248 nm was selected since it produces less noise, which minimizes problems that may exhibit around the active ingredient when attempting to quantify of atazanavir. Preliminary development trials were performed with various BDS columns of different types and dimensions from different manufacturers were tested for the peak shape and the number of theoretical plates for specification concentrations. Finally by switching to Hypersil BDS 150 X 4.6 mm, 5µ column there was a substantial increase in the theoretical plates (4573) with a significant improvement in the peak shapes with 1.04 tailing factor.

System suitability
The RSD from six replicate injections of diluted standard preparation was 0-3 %. Theoretical plates and Tailing factor for atazanavir peak 4573 and 1.04

Selectivity
Performed the specificity parameter of the method by injecting diluent, standard preparation, and sample preparation into the chromatographic system and recorded the retention times. Specificity results of atazanavir given in the below table 1. The selectivity Chromatogram Shown in the Fig.2.

<table>
<thead>
<tr>
<th>Table 1: Selectivity results of atazanavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Diluent</td>
</tr>
<tr>
<td>Placebo</td>
</tr>
<tr>
<td>Standard preparation</td>
</tr>
<tr>
<td>Sample as such</td>
</tr>
</tbody>
</table>
Figure 2: Chromatogram of atazanavir

**Linearity**

To demonstrate the linearity with atazanavir sample in the range of 20 to 150% of specification limit. Correlation coefficient of haloperidol and its related compounds was 1.000.

**Table 2: Linearity results of atazanavir**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration in ppm</th>
<th>Area Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>320848</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>781806</td>
</tr>
<tr>
<td>3</td>
<td>105</td>
<td>1107831</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>1609560</td>
</tr>
<tr>
<td>5</td>
<td>180</td>
<td>1928225</td>
</tr>
<tr>
<td>6</td>
<td>225</td>
<td>2424609</td>
</tr>
</tbody>
</table>

**Accuracy**

Accuracy study found that the mean % of recovery was more than 85% and less than 115% at each level 50 to 150% of concentration levels, hence method is accurate. The accuracy results are given table 3:

**Table 3: Accuracy results of atazanavir**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Level in %</th>
<th>% Mean Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>99.518</td>
</tr>
<tr>
<td>2</td>
<td>100%</td>
<td>99.8389</td>
</tr>
<tr>
<td>3</td>
<td>150%</td>
<td>100.2069</td>
</tr>
</tbody>
</table>

**Precision**

**Table 4: Precision results**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Area response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1608685</td>
</tr>
<tr>
<td>2</td>
<td>1602861</td>
</tr>
<tr>
<td>3.</td>
<td>1608639</td>
</tr>
<tr>
<td>4.</td>
<td>1603466</td>
</tr>
<tr>
<td>5.</td>
<td>1603962</td>
</tr>
<tr>
<td>6.</td>
<td>1613002</td>
</tr>
<tr>
<td>Mean</td>
<td>1605466</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The % RSD of the Retention time for peaks obtained from 6 injections of standard preparation 0.3. Precision results are given in the table 4.

**LOD and LOQ**

The limit of detection and limit of quantification of test method was validated based on signal to noise ratio method. The LOD and LOQ data are given in table 5.

**Table 5: LOD and LOQ establishment data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S/N Value</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>9.2</td>
<td>1.63</td>
</tr>
<tr>
<td>LOQ</td>
<td>3.2</td>
<td>0.54</td>
</tr>
</tbody>
</table>

**Degradation of Atazanavir**

The degradation behavior of atazanavir under various stress conditions was investigated by LC.

**Oxidation**

To 1 ml of stock solution of Atazanavir, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60°C. For HPLC study, the resultant solution was diluted to obtain 150µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Alkali Degradation Studies**

To 1 ml of stock solution Atazanavir, 1 ml of 2 N sodium hydroxide was added and refluxed for 30mins at 60°C. The resultant solution was diluted to obtain 150µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Acid Degradation**

To 1 ml of stock solution Atazanavir, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60°C .The resultant solution was diluted to obtain 150µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Neutral Degradation Studies**

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60°C. For HPLC study, the resultant solution was diluted to 150µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

**Thermal/Dry Heat Degradation Studies**

The standard drug solution was placed in oven at 105°C for 6 hr to study dry heat degradation. For HPLC study, the resultant solution was diluted to 100 µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.
Photo Stability Studies

The standard drug solution was placed in oven at 105°C for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 150 µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Mass spectral fragmentation

The analysis of the degradation products was carried by LC and LC-MS. atazanavir was subjected to LC-MS/MS with atmospheric pressure chemical ionization (APCI) to know the fragmentation pattern of drug. The different structures of fragmented molecules based on the M/Z values are shown in the below figure-3.

Figure 3: Different structures of molecules based on the mass fragmentation

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CONCLUSION

A simple LC-MS/MS method has been developed and validated for the forced degradation studies of atazanavir. The developed method has been found to selective, sensitive, precise, robust, and stability indicating. The method can be directly adopted in quality control laboratories for routine analysis.

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