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A R T I C L E   I N F O

Article history:
Received 27 September 2012
Received in revised form 30 September 2013
Accepted 9 December 2013

Keywords:
Atazanavir sulfate
RP–HPLC
Isocratic elution
Validation
Stability indicating

A B S T R A C T

A stability-indicating reverse phase–high performance liquid chromatography (RP–HPLC) method was developed and validated for the determination of atazanavir sulfate in tablet dosage forms using C18 column Phenomenix (250 mm × 4.6 mm, 5 μm) with a mobile phase consisting of 900 mL of HPLC grade methanol and 100 mL of water of HPLC grade. The pH was adjusted to 3.55 with acetic acid. The mobile phase was sonicated for 10 min and filtered through a 0.45 μm membrane filter at a flow rate of 0.5 mL/min. The detection was carried out at 249 nm and retention time of atazanavir sulfate was found to be 8.323 min. Linearity was observed from 10 to 90 μg/mL (coefficient of determination R2 was 0.999) with equation, y = 23.427x + 37.732. Atazanavir sulfate was subjected to stress conditions including acidic, alkaline, oxidation, photolysis and thermal degradation, and the results showed that it was more sensitive towards acidic degradation. The method was validated as per ICH guidelines.

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1. Introduction

Atazanavir sulfate, chemically known as 3,12-bis([1,1-dimethyllethy])-8-hydroxy-4,11-dioxo-9-[(phenylmethyl)-6-[(4-[(2-pyrrolidinyl)phenyl]methyl)]-dimethyl ester, [1] (Fig. 1) is a white to pale yellow powder, slightly soluble in water. It, in combination with other antiretroviral agents, is used in the treatment of human immunodeficiency virus (HIV-1) infection. Atazanavir formulated as 1:1 sulfate salt is the most recently introduced azapeptide inhibitor of HIV-1 protease. The drug was approved by the United States Food and Drug Administration (USFDA) in June 2003. Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily HIV. When several such drugs, typically three or four, are taken in combination, the approach is known as a highly active antiretroviral therapy (HAART). The American National Institutes of Health and other organizations recommend offering antiretroviral treatment to all patients with AIDS [2]. Structure activity studies with a series of azidipeptides were designed to mimic the transition state of the peptide-cleavage reaction catalyzed by HIV-1 protease identified lead compounds that have either potent antiviral activity against mutant HIV-1 strains or good oral bioavailability [3]. Lead optimization using X-ray structural data from an inhibitor–protease complex [4] led to the discovery of atazanavir sulfate, which showed an excellent antiviral activity with high oral bioavailability and could be used in combination with other antiretroviral agents [5]. Recently, a new method has been developed for the estimation of in vitro dissolution assessment of atazanavir and ritonavir in combined tablet dosage forms by the RP–HPLC method [6,7]. An literature survey reveals that atazanavir is quantitatively assayed in biological fluids either individually [8] or in the presence of other retroviral drugs using liquid chromatography [9,10]. The literature survey also indicates that its pharmacokinetics studies in healthy volunteers have been done [11]. However, some UV–vis spectrophotometric methods were proposed for the estimation of atazanavir in bulk and pharmaceutical dosage forms [12–14]. According to ICH guidelines [15,16], our present work not only developed and validated a method, but also was a stability-indicating study. A degradation product is an impurity resulting from a chemical change in the drug substance brought during manufacture and/or storage of the new drug product by factors such as light, temperature, pH, water or by the reaction with an excipient and/or the immediate container closure system.

2. Materials and methods

2.1. Chemicals

The reagents used in this work were methanol (HPLC grade – Lichrosolv), acetic acid, HCl (AR), NaOH (AR), and hydrogen...
2.4.1. Selection and preparation of mobile phase

Various mobile phases containing methanol, water, acetonitrile, and glacial acetic acid in different ratios were tried with different flow rates. Good symmetrical peak was found with the mobile phase comprising methanol and water in the ratio 90:10 (v/v) (pH adjusted to 3.55 with glacial acetic acid).

Mobile phase was prepared by mixing 900 mL of HPLC grade methanol with 100 mL of water of HPLC grade, and the pH was adjusted to 3.55 with acetic acid. The mobile phase was sonicated for 10 min and filtered through the 0.45 μm membrane filter.

2.4.2. Preparation of standard stock solutions

The standard stock solutions of 100 μg/mL of the drug were prepared by dissolving 50 mg of pure drug in the mobile phase in a 50 mL volumetric flask and the volume was made up to the mark. Resulting solutions were further diluted with mobile phase to obtain a final concentration of 100 μg/mL and stored under refrigeration.

2.4.3. Preparation of calibration curve

Aliquots of standard stock solutions were put in a 10 mL volumetric flask and diluted up to the mark with mobile phase. In such a way, the final concentrations of the drug were in the range of 10–90 μg/mL. Triplicate injections of 20 μL were made and analyzed by chromatograph under the conditions as described above. Evaluation of the drug was performed and peak areas were recorded. Calibration curves were constructed by plotting the peak area on the y-axis against respective concentration of the drug on the x-axis. The calibration curve was evaluated by its coefficient of determination (R²).

2.5. Method validation

The developed method was validated by evaluating linearity, accuracy, precision, robustness, detection limit, quantification limit and stability. Coefficients of variation and relative errors of less than 2% were considered acceptable, except for the quantification limit, for which these values were established at 2%, as recommended in the literature [15].

2.5.1. Linearity

A stock solution of atazanavir sulfate of 1000 μg/mL was prepared with mobile phase. From it, various working standard solutions were prepared in the range of 10 to 120 μg/mL and injected into HPLC. It was shown that the selected drug had linearity in the range of 10–90 μg/mL. The calibration plot (peak area ratio of atazanavir sulfate versus atazanavir sulfate concentration) was generated by replicate analysis (n=9) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel™ program.

2.5.2. Accuracy

The accuracy of the method was carried out using one set of different standard addition methods at different concentration levels, 80%, 100% and 120%, and then comparing the difference between the spiked value (theoretical value) and actual found value.

2.5.3. Precision

The precision of the method was ascertained from the peak area obtained by actual determination of six replicates of a fixed amount of the drug (50 μg/mL). The precision of the assay was also determined in terms of intra- and inter-day variation in the peak areas of a set of drug solutions on three different days. The intra- and inter-day variation in the peak area of the drug solution was calculated in terms of relative standard deviation (RSD).

2.5.4. Robustness

Robustness of the proposed method for atazanavir sulfate was carried out by the slight variation in flow rate, pH and mobile phase ratio. The percentage recovery and RSD were noted for atazanavir sulfate.

2.5.5. Ruggedness

The test solutions were prepared as per test method and injected under variable conditions. Ruggedness of the method was studied by different analysts.

2.5.6. Detection limit and quantification limit

The limit of detection (LOD) and limit of quantification (LOQ) were established based on the calibration curve parameters, according to the following formulas:

\[ \text{LOD} = 3.3 \sigma / \text{slope} \]
\[ \text{LOQ} = 10 \sigma / \text{slope} \]

or detection limit = \(3.3\sigma/s\), quantification limit = \(10\sigma/s\), where \(\sigma\) is the standard deviation of \(y\)-intercept of regression line, and \(s\) is the slope of the calibration curve.

2.6. Forced degradation studies

The specificity of the method can be demonstrated through forced degradation studies conducted on the sample using acid, alkaline, oxidative, thermal, photolytic, and ultra violet (UV) degradations. The sample was exposed to these conditions, and the main peak was studied for the peak purity, thus indicating that the method effectively separated the degradation products from the pure active ingredient [16].

2.6.1. Hydrolytic degradation

Hydrolytic stress testing was performed to force the degradation of the drug substance to its primary degradation products by exposure to neutral, acidic and basic conditions over time. Functional groups likely to undergo hydrolysis are amides (lactams), esters (lactones), carbamates imides, imines, alcohols (epimerization for chiral center) and aryl amines.

To initiate hydrolytic studies, a preliminary solubility screen of the drug substance was performed. Solubility of at least 1 mg/mL in neutral, acidic and basic conditions was recommended for the neutral/acid/base stress testing. However, concentration less than 1 mg/mL can be used, if solubility is an issue. In some cases, a co-solvent may be necessary to achieve the target concentration. Special attention should be given to the drug substance structure when an appropriate co-solvent was chosen.

2.6.2. Oxidative degradation

Oxidative degradation of drug substances in pharmaceutical formulation is well documented. Although exact mechanistic details about what promotes reaction between drug substance and molecular oxygen in pharmaceutical formulations are not fully understood, such reactions are generally thought to be in the category of auto-oxidation process.

There are three major pathways: (i) autooxidation or radical-mediated oxidation, (ii) peroxide-mediated oxidation and (iii) photochemically induced oxidation. Traditionally, dilute aqueous peroxide solutions have been used for oxidative stress testing of pharmaceuticals. In addition to the auto-oxidation processes, peroxide-mediated oxidative degradation can occur, which may not be observed using radical indicator.

The process of oxidation depends on the amount of oxygen in the air and the nature of the material it touches. True oxidation happens on the molecular level. We can only see the large scale effects as the oxygen causes free radicals on the surface to break away.

2.6.3. Photolytic degradation

For photolytic degradation, the drug was exposed to the direct sunlight. Sufficient amount of the drug was taken in a closed petri-dish and exposed to sunlight. At different time intervals, the drug was taken out, diluted appropriately and injected into HPLC to determine the amount of degradation of the drug.

2.6.4. UV-degradation

The goal of UV-degradation studies is to force the degradation of drug substance via UV and fluorescent condition over time to determine the primary degradation products. A molecule absorbs light when an absorption band overlaps to some extent with the incident light energy and a valence electron in the relevant atmosphere rose to an excited state.

A near UV fluorescent lamp has a spectral distribution from 320 nm to 400 nm with a maximum energy emission between 350 nm and 370 nm, and the significant proportion of UV should be in both bands of 320–360 nm and 360–400 nm.

2.6.5. Thermal degradation

To evaluate therolytic pathways, evaluated temperatures (e.g. 50 °C and –80 °C) in the solid state and/or in solution can be used. Many compounds began to degrade via different mechanisms above 80 °C, giving rise to degradation products. To solid-state stressing, the use of high and low humidity atmosphere of the evaluated temperatures is appropriate.

To evaluate stability under the temperatures above, stress conditions were selected based on a conservative estimate of the Arrhenius expression, a quantitative relationship of reaction rate and temperature using average activation energy.

\[ K_{ob} = A \exp\left(-\frac{E_a}{RT}\right) \]

3. Results

3.1. Method development

3.1.1. Chromatographic separation

A number of HPLC chromatographic systems were investigated to optimize the separation of atazanavir sulfate. Retention time for atazanavir sulfate function of stationary phase (Phenomenex C18 reversed-phase column), the mobile phase and the other optimized chromatographic conditions are shown in Table 1.

3.1.2. Calibration curve

The coefficient of determination (R²), slope and intercept for atazanavir sulfate were 0.999, 23.427 and 37.732, respectively. The retention time for atazanavir sulfate was 8.323 min. HPLC overlay chromatogram of atazanavir sulfate at 249 nm and calibration curve are shown in Fig. 2.

3.2. Method validation

3.2.1. Linearity, accuracy and precision

The coefficient of determination (R²) for atazanavir sulfate was 0.999 as shown in Fig. 2. The accuracy of the method was determined and indicated by the recovery. The results are shown in Table 2.

Intra- and inter-day precision data of the RP–HPLC method for atazanavir sulfate are presented in Supplementary Table 1 and 2.
3.2. Robustness and ruggedness

Robustness of the method was studied by deliberate variations of the analytical parameters such as flow rate (0.5 ± 0.1 mL/min) and different pH values. The results are given in Table 3 and 4.

Ruggedness of the method was carried out by different analysts. The results are displayed in Table 5.

3.2.3. LOD and LOQ

The LOD and LOQ of atazanavir sulfate were determined to be 0.09 µg/mL and 0.23 µg/mL, respectively.

3.3. Forced degradation studies

3.3.1. Degradation in neutral condition

Neutral degradation of atazanavir sulfate was performed using distilled water. Ten micrograms of the atazanavir sulfate bulk was weighed accurately and transferred into a 10mL clean volumetric flask. The content of the volumetric flask was dissolved in 5 mL of distilled water. Then the volumetric flask was heated on a water bath at 80 °C. Samples were prepared for different time intervals such as 0 min, 30 min, 1 h, 2 h, and 4 h. At different time intervals,

---

Table 2

<table>
<thead>
<tr>
<th>No. of preparations (%)</th>
<th>Concentration (µg/mL)</th>
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Table 3

Robustness data of the RP–HPLC method at different flow rates for atazanavir sulfate.

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<td>Calc. amt. (µg/mL)</td>
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<td>1211.27</td>
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<td>50.09</td>
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different sample solutions were taken out and 5 mL of HPLC grade methanol was added. The sample solution was then sonicated for 5 min and diluted to yield a concentration of 50 μg/mL in the mobile phase. It was then filtered through a 0.22 μm filter and 20 μL was injected into the HPLC for analysis. The obtained chromatogram was observed for any degradation occurred during the time. The results are given in Table 6 and Supplementary Fig. S1.

3.3.2. Degradation in acidic condition
Acid degradation of atazanavir sulfate was performed using 0.1 M HCl. Ten micrograms of the atazanavir sulfate bulk was weighed accurately and transferred into a 10 mL clean volumetric flask. The content of the volumetric flask was dissolved in 5 mL of 0.1 M HCl and then was subjected to heat on a water bath at 80 °C. Samples were prepared for different time intervals such as 0 min, 30 min, 1 h, 2 h, and 4 h.

At different time intervals, different sample solutions were taken out and 5 mL of HPLC grade methanol was added. The sample solution was then sonicated for 5 min and diluted to obtain a concentration of 50 μg/mL in the mobile phase. It was then filtered through a 0.22 μm filter and injected into the HPLC. The obtained chromatogram was analyzed for any degradation happened during the time. The results are given in Table 7 and Supplementary Fig. S2.

3.3.3. Degradation in basic condition
Alkaline degradation of atazanavir sulfate was performed using 0.01 M NaOH. Ten micrograms of the atazanavir sulfate bulk was weighed accurately and transferred into a 10 mL clean volumetric flask. The content of the volumetric flask was dissolved in 5 mL of 0.01 M NaOH, and then was heated on a water bath at 80 °C.

Samples were prepared for different time intervals such as 0 min, 30 min, 1 h, 2 h, and 4 h.

At different time intervals, different sample solutions were taken out and 5 mL of HPLC grade methanol was added. The sample solution was then sonicated for 5 min and diluted to obtain a concentration of 50 μg/mL in the mobile phase. It was then filtered through a 0.22 μm filter and injected into the HPLC. The obtained chromatogram was studied for any degradation happened during the time. The results are given in Table 8 and Supplementary Fig. S3.

3.3.4. Oxidative degradation
For oxidation, the reagent chosen was hydrogen peroxide (3%). Ten micrograms of the atazanavir sulfate bulk was weighed accurately and transferred into a 10 mL clean volumetric flask. The content of the volumetric flask was dissolved in 5 mL of H2O2. Then it was placed at room temperature for degradation. Samples were prepared for different time intervals such as 0 min, 30 min, 1 h, 2 h, and 4 h. At different time intervals, different sample solutions were taken out and 5 mL of HPLC grade methanol was added. The sample solution was then sonicated for 5 min and diluted to yield a concentration of 50 μg/mL in the mobile phase. It was then filtered through a 0.22 μm filter and injected into the HPLC. The obtained chromatogram was studied for any degradation underwent during the time given. The results are shown in Table 9 and Supplementary Fig. S4.

3.3.5. Photolytic degradation
For photolysis, 100 mg of the atazanavir sulfate bulk was weighed accurately and transferred into a clean petridish. Then the closed petridish was placed under direct sunlight for degradation. At different time intervals, 10 mg of sample was taken out. From it, a stock solution of 1000 μg/mL was prepared. Then it was sonicated for 5 min and diluted to obtain a working solution of 50 μg/mL in the mobile phase. It was then filtered through a 0.22 μm filter and injected into the HPLC. The obtained chromatogram was observed for any degradation occurred during the time. The results are given in Table 10 and Supplementary Fig. S5.
gradation. After 3 h, the UV lamp was switched off and 10 mg of the UV lamp. The cover of the petridish was removed for de-
petridish was placed under a UV chamber 30 cm at distance from
weighed accurately and transferred into a clean petridish. Then the
3.3.6. UV-degradation
Results of oxidative degradation of atazanavir sulfate.
Table 9
Results showing alkali degradation of atazanavir sulfate.
Table 8
Results showing acidic hydrolysis of atazanavir sulfate.
Table 7

<table>
<thead>
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<th>Sl. no.</th>
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<th>Area (mV s)</th>
<th>Height (mV)</th>
<th>Area (%)</th>
<th>Height (%)</th>
<th>W0.5 (min)</th>
<th>Results</th>
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<th>Height (mV)</th>
<th>Area (%)</th>
<th>Height (%)</th>
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3.3.6. UV-degradation

For UV degradation, 100 mg of the atazanavir sulfate bulk was weighed accurately and transferred into a clean petridish. Then the petridish was placed under a UV chamber 30 cm at distance from the UV lamp. The cover of the petridish was removed for degradation. After 3 h, the UV lamp was switched off and 10 mg of sample was taken out. From it, a stock solution of 1000 μg/mL was prepared with the mobile phase, from which 50 μg/mL of working solution was prepared. It was sonicated and filtered through a 0.22 μm filter. Twenty microlitres of the sample was injected into the HPLC.

The degradation of atazanavir sulfate under UV light is given in Table 11 and Supplementary Fig. S6.

3.3.7. Thermal degradation

Thermal degradation was performed by placing the atazanavir sulfate bulk in the incubator at 40 °C. Samples were drawn at definite time intervals. The weighed amount of sample was added to 5 mL of HPLC grade methanol and sonicated for 5 min. Volume was made up to mark with methanol, which is the bulk standard stock solution of 1000 μg/mL. From the stock, a working standard solution of 50 μg/mL was prepared with the mobile phase. It was sonicated and filtered through a 0.22 μm filter, and 20 μL of the sample was injected into the HPLC. The obtained chromatogram was analyzed for any degradation undergone during the time, and the results are given in Table 12 and Supplementary Fig. S7.

From the neutral hydrolytic degradation study of atazanavir sulfate, it was found that no degradation took place over 4 h in neutral condition.

The results of oxidative degradation showed degradation peaks at 4.77 min and 5.31 min along with the drug peak. The peak area showed that 51.62% of degradation of the drug occurred when the drug was kept in 0.1 M HCl at 80 °C up to 4 h.

Atazanavir sulfate upon alkaline degradation in 0.01 M NaOH at 80 °C up to 4 h underwent degradation showing degradation peak at 5.18 min in the chromatogram. The peak area of the drug showed that the percentage degradation is 30.64% in the above condition.

Atazanavir sulfate did not degrade after it was kept under direct sunlight for 21 days. No peak other than the drug peak was found in the chromatogram of that sample.

Atazanavir sulfate was not degraded after it was kept in the UV chamber for 48 h.

In the oxidative degradation study atazanavir sulfate showed no degradation after 4 h of exposure.

The thermal degradation study showed that atazanavir sulfate was degraded when kept at 40 °C for 15 days. The degradant was retained at 7.29 min in the chromatogram along with the drug peak. The drug peak area showed that the degradation of the drug was 18.97%.

5. Conclusion

The developed RP–HPLC method was found to be suitable for the analysis of atazanavir sulfate in bulk form, and was found to be simple, reliable, sensitive, economical and precise.

The drug atazanavir sulfate was found to be more degraded when exposed to acidic hydrolysis as it degraded by 51.62% and least degraded when exposed to neutral hydrolysis, UV-degradation and oxidative degradation. Therefore, this RP–HPLC method for estimation of atazanavir sulfate can be used in various laboratories for its quantitative determination in bulk and pharmaceutical dosage forms.

Acknowledgments

The authors are grateful to the principal of Royal College of Pharmacy and Health Sciences, Berhampur, Orissa, for providing the facilities for working and also to the laboratory assistance.

Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jpha.2013.12.002.

Table 12

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