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Method Development And Validation for the Simultaneous Estimation of Atazanavir And Ritonavir in Tablet Dosage Form By Rp-Hplc And Uv Spectrophotometry

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Abstract: *An accurate, precise, rapid and robust RP-HPLC and UV methods were developed and validated for the simultaneous estimation of Atazanavir and Ritonavir in tablet dosage forms. The chromatographic separation was carried out on X-tera C_{18} (100mmX4.6mmX3.5µ) by using mobile phase of Potassium Phosphate Buffer 0.05M at a pH of 2.5 and Acetonitrile in the ratio of 40:60 at a flow rate of 1.2 ml/min. The detection was carried out at 236 nm. The retention time of Atazanavir and Ritonavir was found to be 1.982 and 2.576 respectively. UV method involves solving simultaneous equations based on measurement of absorbance at two wavelengths 247 nm and 239 nm λmax of Atazanavir and Ritonavir respectively. Beer's law was obeyed in the concentration range of 6-30 µg/ml and 2-10 µg/ml for Atazanavir and Ritonavir respectively. These methods were statistically validated and recovery studies confirmed the accuracy of the proposed methods.*

Keywords: *Atazanavir, Ritonavir, Potassium Phosphate Buffer, Acetonitrile, RP-HPLC, UV Spectrophotometry, Validation.*

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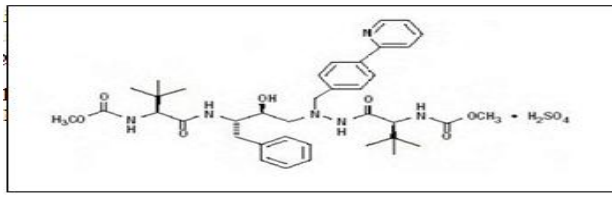
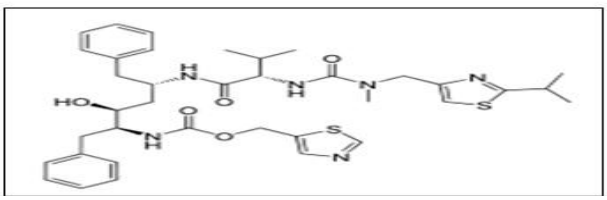
I. Introduction

The chemical name for Atazanavir Sulfate is (3S,8S,9S,12S)-3,12-Bis(1,1-dimethylethyl) 8hydroxy4,11dioxo9(phenylmethyl)6[[4(2pyridinyl)phenyl]methyl]2,5,6,10,13pentaazatetradecanedioic acid dimethylester Sulfate. Atazanavir Sulphate is an azapeptide inhibitor of HIV-1 protease1[1-2]. It is a white to pale yellow crystalline powder and slightly soluble in water. Its molecular weight is 802.9 (sulfuric acid salt). The free base molecular weight is 704.9. Its molecular formula is $C_{38}H_{52}N_6O_7 \bullet H_2SO_4$. The brand name of which is Ryataz and its structure is

Ritonavir is chemically designated as 10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)- 2,4,7,12- tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*,8R*,10R*,11R*)].Ritonavir is an inhibitor of HIV protease with activity against the Human Immunodeficiency Virus[3-5]. Ritonavir is a white-to-light tan powder. It has a bitter metallic taste and freely soluble in methanol and ethanol, soluble in isopropanol and practically insoluble in water. Its molecular weight is 720.95. Its molecular formula is $C_{37}H_{48}N_6O_5S_2$ .The brand name of which is Norvir and its structure is





Combination therapy of Atazanavir and Ritonavir is used in treatment of HIV infection[6-9]. Various methods such as High Performance Liquid Chromatography (HPLC)[10-13], High Performance Thin Layer Chromatography (HPTLC)[14], UV methods [15-18] have been reported in the literatures for the determination of these compounds in various biological samples and pharmaceutical preparations. The aim of present work is to develop a simple,precise ,accurate,and rapid method with less run time for the determination of Atazanavir and Ritonavirin combined dosage form without lack of interference.

II. Materials And Methods:

Instruments

HPLC equipped with Quaternary pump, Auto sampler, UV Detector and Empower 2 software, make-WATERS. A double beam UV-visible spectrophotometer LAB INDIA-3000 series with UV –win software and 1cm quartz cell.

Chemicals and Reagents

The solvents used were of HPLC/AR grade. Double distilled water was used in preparation of mobile phase. Pure drug sample of Atazanavir and Ritonavir were obtained as a gift sample from Emcure Pharmaceuticals Limited, Pune.

Chromatographic Conditions

When several mobile phases were tried, the mobile phase containing acetonitrile and buffer (0.05M KH_2PO_4, pH 2.5±0.02, 60:40 v/v) was considered appropriate. The Column X-tera (C_{18}, 100mm x 4.6mm, 3.5µ) was selected for the quantitative determination. The mobile phase was filtered through 0.45µm membrane filter and then ultrasonicated for 15 minutes. The flow rate was set to 1.2ml/min and UV Spectro photometric detection was carried out at 236 nm.

Preparation of mobile phase

Mix a mixture of 0.05M KH_2PO_4 buffer 400 ml (40%) and 600 ml of Acetonitrile HPLC (60%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45µ filter under vacuum filtration.

Method development

Method development by using HPLC and UV Spectrophotometry was initiated by taking λmax and isoabsorptive point.

Determination of λmax and isoabsorptive point

Standard stock solutions was prepared by dissolving accurately weighed 10 mg of Atazanavir and Ritonavir in Methanol and the volume was made up to 10 ml with methanol in 10 ml volumetric flasks (Stock solution-I, 1000 mcg / ml). 1 ml of stock solution-I was diluted to 10ml with methanol (Stock solution-II, 100 mcg / ml). 1 ml of stock solution-II was taken in 10 ml standard flask diluted to 10 ml with methanol to get the concentration 10 mcg / ml. The absorbance of resulting solutions was measured against respective blank solution in the UV region of 200-400 nm, which shows maximum absorbance at 247nm and 239nm for Atazanavir and Ritonavir respectively. The isoabsorptive point was determined.

Preparation of standard and sample solutions of Atazanavir & Ritonavir

Preparation of Standard Solution

Accurately weighed and transferred 30mg of Atazanavir and 10mg of Ritonavir working standard into a 10ml clean dry volumetric flask and added about 7ml of diluent. It was sonicated to dissolve completely and made volume up to the mark with the same diluent. (Stock solution)(3000, 1000 µg/ml). From the above stock solution, 1ml of the a solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent. (300, 100 µg/ml). From this, 4ml of the solution was pipetted into another 10ml volumetric flask and diluted up to the mark with diluent.

Sample Solution Preparation

Accurately weighed and transferred 49.8mg of Atazanavir and Ritonavir tablet powder (SYNTHIVAN, Cipla Pharmaceuticals Ltd.) into a 10ml clean dry volumetric flask and added about 7ml of diluent. It was sonicated to dissolve it completely and made volume up to the mark with the same diluent (Stock solution). From the above stock solution, 1ml of the solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent. From this, 4 ml of the solution was pipetted into another 10ml volumetric flask and diluted up to the mark with diluent.

III. Results And Discussion

The main aim of present investigation is to develop a new method and validate Atazanavir and Ritonavir in pharmaceutical dosage forms by UV and RP-HPLC methods. The individual UV-Spectrum was recorded using Atazanovir and Ritonavir both standard and samples at its individual maximum wavelengths (λmax) of Atazanavir at 247 nm and Ritonavir at 239 nm. From the individual scan spectra, the isoabsorptive point was found to be 247 nm.RP-HPLC spectrum developed for both standard and sample solutions of Atazanavir and Ritonavir at isoabsorptive point (247 nm), the retention time was found to be 1.982, 2.576 in standard and 2.009, 2.577 in sample respectively (Fig. 1&2).

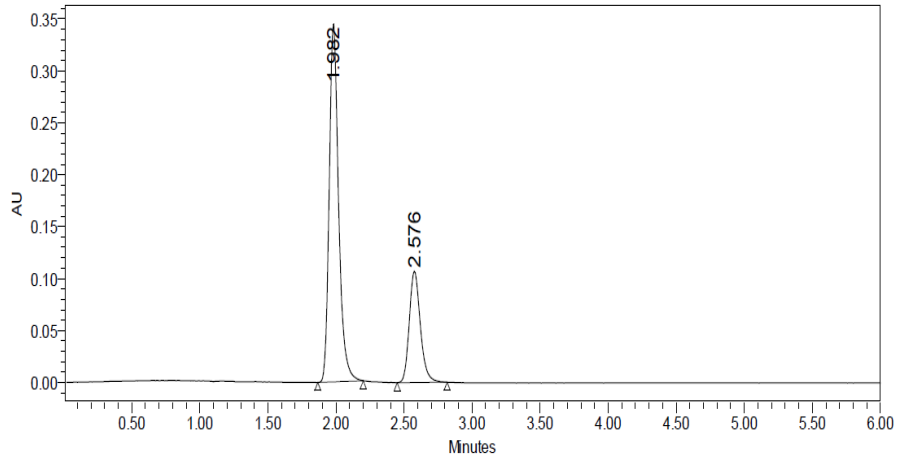


Figure 1: Atazanavir and Ritonavir standard

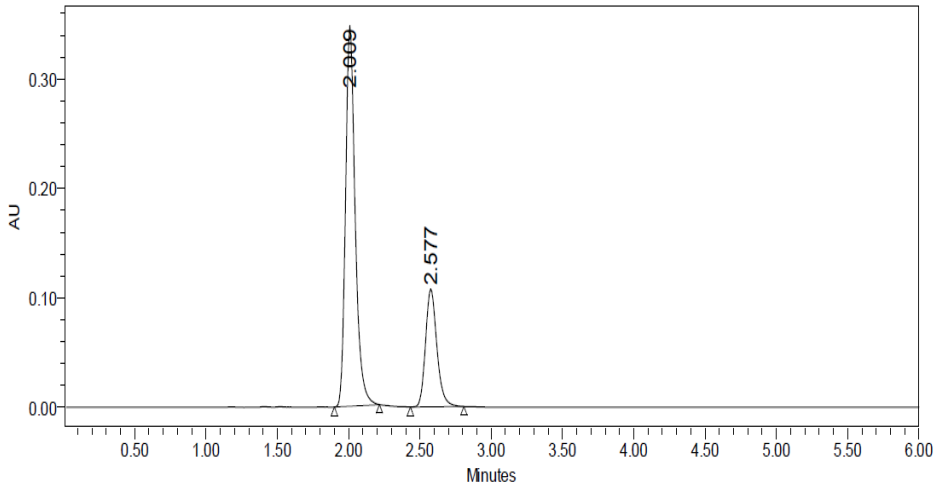


Figure 2: Atazanavir and Ritonavir sample

Method validation

Specificity

To check the specificity, standard and sample solutions were injected ,verified that there is no interference of tablets excipients.

Linearity

The calibration curve was obtained with five concentrations of the standard solution for 60-180ppm of Atazanavir and 20-60ppm for Ritonavir HPLC method. Then five concentrations of stanadard solution of 6-30mcg/ml for Atazanavir and 60-30mcg/ml for Ritonavir for UV-spectro photometric method. The linearity was evaluated by linear regression analysis,which was calculated by the least square regression method.

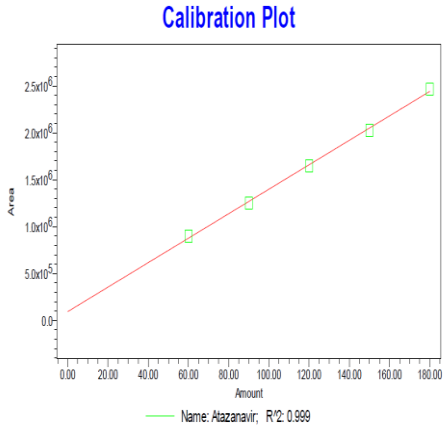


Figure 3: Linearity graph of Atazanavir by HPLC method

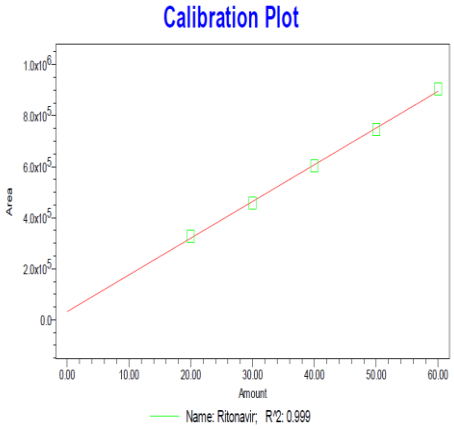


Figure 4: Linearity graph of Ritonavir by HPLC method

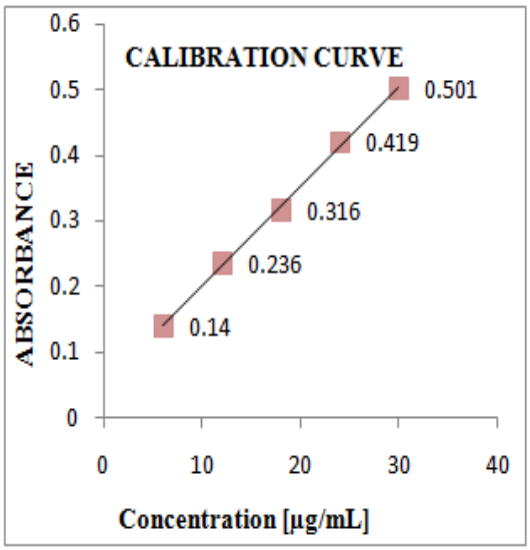


Figure 5: Linearity graph of Atazanavir by U.V method. method.

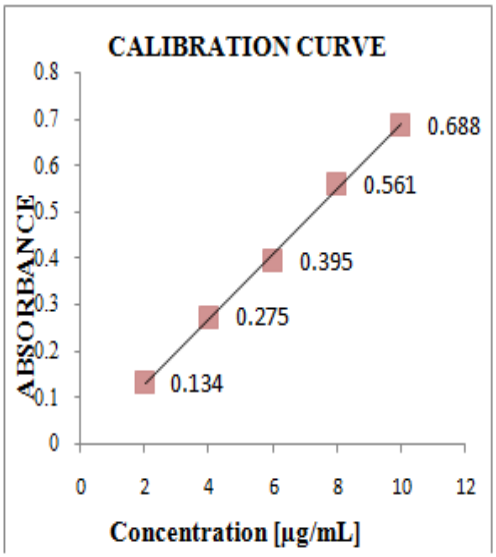


Figure 6: Linearity graph of Ritonavir by U.V

Precision

The assay precision was carried out by repeatability (within -day) and intermediate precision (inter-day). Five sample solutions (30 mcg/ml) of Atazanavir and 10mcg/ml of Ritonavir for HPLC and UV method were prepared and assayed **.**

Table

Drug	AZATANAVIR		RITONAVIR	
	Hplc Method	Uv Method	Hplc Method	Uv Method
Precision Analyst -1%	0.17	0.14	0.20	0.22
Intermediate Precision -2%RSD	0.10	0.26	0.14	0.17

1: Precision

Accuracy

Sampe solutions were prepared at three different concentrations (50%, 100 and 150%) and the amount of sample was added to this solution and recovery of added sample was studied.

Table 2: Accuracy Recovery studies

Concentration Added	Azatanavir Mean% Recovery		Ritonavir Mean % Recovery	
	Hplc Method	Uv Method	Hplc Method	Uv Method
50%	100.7%	99.07	99.8%	99.24
100%	99.9%	99.59	99.9%	100.79
150%	99.7%	100.65	100.4%	100.05

Robustness

To evaluate the robustness of the developed method a small deliberate varaiations in optimized method parameters were done, such as small change in the percentage of organic composition in the mobile phase (10%) , flowrate(1.0-1.4ml/min) .Effect of these changes on retention time ,peak symmetry , resolution and theoretical plates were evaluated.

Limit of detection and Quantificaton

LOD was determined using the signal to noise ratio and then comparing the test results from the sample with known concentrations. The analyte concentration that produced a signal to noise ratio of 3:1 was accepted as LOD. Limit of quantification is defined as the lowest concentration of the analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions. To develop a simple, precise , accurate, and rapid reverse phase high performance liquid chromatographic method and U.V spectrophotometric for the simultaneous estimation of Azatanovir and Ritonavir ,different chromatographic conditions were tried .The symmetry X-tera c_{18} column , mobile phases containing acetonitrile and buffer 0.05m KH_2PO_4 60:40v/v and the flow rate of 1.2ml/min found to resolve all two components with good peak symmetry and theoretical plates.

The retention times for Azatanovir and Ritonavir were found to be 1.982 and 2.576min respectively. The specificity of the method was assessed by comparing the retention time of standard Azatanovir and Ritonavir and sample, good correlation was obtained between the retention time of standard and sample, blank were injected and there were no peaks. There are no interferences. Hence method is specific ,the linearity range for Azatanovir and Ritonovir were found to be 60-180ppm, 20-60ppm respectively and correlation coefficient was found to be 0.999%and relative standard deviation (%RSD) was found to be less than 2 for sample analysis ,that proves method is precise.

The recovery studies shown recovery of the sample is between 99-100% that proves method is accuracy. The analysis of sample by second analyst did not shown any effect on its performance. The small deliberate changes in mobile phase composition, pH of the buffer and flow rate did not show any impact on retention time, peak symmetry, resolution and theoretical plate count. The limit of detection for Azatanovir and Ritonavir were found to be 2.95µg/ml and 2.97µg/ml. The limit of quantification for Azatanovir and Ritonavir was found to be 9.97µg/ml. and 10.0µg/ml respectively. A good accuracy method was verified with mean recovery of 100% within day and interday. Finally the method showed to the specific for the determination of Azatanovir and Ritonavir in tablets.

IV. Conculsion

The proposed methods was simple, specific, acurate and precise. Hence can be used in routine for estimation of Azatanovir and Ritonavir in tablets dosages. Statical analysis of the result has been carried out revealing high accuracy and good precision. The %RSD for all parameters was found to be less than 2, which indicates the validity of the methods in the assay results obtained by this method are in fair agreements.

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