ISSN 2349-7203

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals

HUMAN

Human Journals
**Research Article**
July 2015 Vol.:3, Issue:4


# Simultaneous Quantification of Atazanavir and Ritonavir in Pharmaceutical Dosage Form by Validated RP-HPLC Method

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

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**Submission:** 26 June 2015
**Accepted:** 1 July 2015
**Published:** 25 July 2015



**Keywords:** Atazanavir, Ritonavir, ICH guidelines

**ABSTRACT**

A simple, precise, accurate, isocratic RP- HPLC method have been developed by the author for the simultaneous estimation of atazanavir and ritonavir in marketed formulations on X-Bridge $C_{18}$ column (150×4.6 mm, 5µ) by using optimized mobile phase containing phosphate buffer (pH-3.4) and acetonitrile in the ratio of 45:55% V/V with a flow rate of 1.0mL/min and detection wavelength at 240nm. The linear regression analysis data for the calibration plots showed a good linear relationship over the concentration range of 37.5 - 375µg/ml for atazanavir and 12.5 - 125µg/ml for ritonavir, respectively. The method was validated for precision, robustness and recovery. Statistical analysis showed that the method is repeatable and selective for the estimation of atazanavir and ritonavir.

## A. INTRODUCTION

Atazanavir is chemically [1,2] (3S, 8S, 9S, 12S)-3,12-bis(1,1-dimethylethyl)-8-hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)phenyl]methyl]-2,5,6,10,13 Pentaazatetradecanedioic acid dimethyl ester, sulfate (1:1) is a selective inhibitor of the HIV-1 aspartic protease enzyme that cleaves viral Gag and Gag-Pol polyproteins, preventing the formation of infectious virons. Its molecular formula is $C_{38}H_{52}N_6O_7 \bullet H_2SO_4$, which corresponds to a molecular weight of 802.9 (sulfuric acid salt). The free base molecular weight is 704.9.

Ritonavir [3] is chemically 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}

butanamido]-1,6-diphenylhexan-2-yl]carbamate is an antiretroviral (anti-HIV) drug. Its molecular formula is $C_{37}H_{48}N_6O_5S_2$, and its molecular weight is 720.95.

A very less number of HPLC methods were reported for the determination of atazanavir and ritonavir in combination and the other methods reported were meant for the determination of the two drugs singly or in combination with other drugs [4-19]. Basing on the above literature study it made essential to develop a new suitable RP-HPLC method for routine analysis of the above said drugs in combined formulations, and in this accord attempts were made by the author to develop simple, precise and accurate RP-HPLC method for the simultaneous assay of the titled drugs and extended it for their determination in formulations.

## B. MATERIALS AND METHODS

**a. Instrumentation:** The chromatographic analysis in the present study was made by using Water's 2695 HPLC system provided with Hamilton Syringe, auto sampler and 2996 Photodiode array detector. Data acquisition, analysis and reporting were performed by Empower 2 (waters) chromatography software. X- Bridge $C_{18}$ column (150×4.6 mm, 5µ) was used as stationery phase for the separation of atazanavir and ritonavir. Shimadzu (Tokyo, Japan) electronic weighing balance [Model BL 220 H] was used for weighing the samples. Elico pH meter (Hyderabad, India) LI 120 model was used for pH measurements.

**b. Chemicals and Reagents:** Pharmaceutically pure sample of atazanavir and ritonavir were obtained from Hetero Drugs as gifted samples and commercial tablets of atazanavir (300mg) and

ritonavir (100mg) in the brand name of VIRATAZ-R film-coated tablets were procured from the local market**.** Milli-Q water, acetonitrile and methanol (HPLC Grade), Orthophosphoric acid (GR Grade), potassium dihydrogen orthophosphate monohydrate (GR Grade) was obtained from Qualigens Ltd., Mumbai. All other chemicals of analytical grade were procured from local sources unless specified. All dilutions were performed in standard class-A, volumetric glassware.

**c. Preparation of Phosphate Buffer:** The buffer solution was prepared by dissolving accurately weighed 6.8grams of potassium dihydrogen orthophosphate and transferred into a clean and dry 1000ml volumetric flask, dissolved and diluted with 1000ml water [HPLC Grade]. The final pH of the buffer was adjusted to 3.4 by using Orthophosporic acid.

**d. Mobile Phase Preparation:** Prepare a filtered and degassed mixture of phosphate buffer and acetonitrile in the ratio of 45:55 v/v respectively.

**e. Diluent Preparation:** Mobile phase is used as diluent in the present assay.

**f. Preparation of Stock & Working Standard Solutions:** The stock solution was prepared by weighing accurately 150mg of atazanavir and 50mg of ritonavir and transferred into a clean and dry 100ml volumetric flask. About 70ml of diluent was added and sonicated for five minutes. Later, the volume was made unto the mark with the same diluent. From the above prepared stock solution pipette out suitable aliquots and transferred into a clean and dry 10ml volumetric flask, the diluent was added up to the mark to get final concentration of 37.5 - 375µg/ml for atazanavir and 12.5 - 125µg/ml, for ritonavir respectively.

**g. Preparation of Sample Solution:** Ten tablets of VIRATAZ-R film-coated tablets for oral usage [Atazanavir (300mg) and Ritonavir (100mg)] procured from the local market were powdered to fine powder. Then sample solution was prepared by weighing and transferring equivalently 100mg of the fine powder of formulation mixture into a 100ml clean and dry volumetric flask containing 70ml of diluent and sonicated to dissolve it completely and the volume made up to the mark with the same solvent. From above prepared stock solution pipette out aliquots of the above solution and transferred into a clean and different dry 10ml volumetric flasks, the diluent was added up to the mark 10ml to get final concentration of 237.5 - 375µg/ml for atazanavir and 12.5 - 125µg/ml, for ritonavir respectively. 10µL volumes of these standard

and sample solutions were injected five times and the peak areas were recorded. The mean and percentage relative standard deviation were calculated from the peak areas.

## C. RESULTS AND DISCUSSION

**i. HPLC Method Development:** In the development of the present method for the selected drugs a number of trials were made by changing the columns and mobile phase by varying its composition as well as by changing the solvents. All these trials have resulted either in low resolution or asymmetric peaks or peaks with more tailing factors or longer time of elution. However, finally the X-Bridge $C_{18}$ column (150×4.6 mm, 5µ) with a flow rate of 1.0mL/min of mobile phase and UV detection at a wavelength of 240nm and column temperature at 25°C with mobile phase of phosphate buffer and acetonitrile in the ratio of 45:55 v/v had resulted in excellent elution of the two drugs with low retention and run times. With the above optimized conditions atazanavir and ritonavir gave acceptable retention time (2.7min and 3.9min for atazanavir and ritonavir respectively), plates and good resolution at 240nm respectively (**Figure 2)**. This developed method was further validated in pharmaceutical dosage forms with satisfactory precision and accuracy for both the drugs in its solid combined dosage forms.

**ii. Method Validation:** The developed RP-HPLC method was validated in accordance with ICH guidelines [20,21] using the following parameters.

**a. System Suitability:** System suitability parameters like number of theoretical plates, HETP and peak tailing were determined for the proposed method and the values for the parameters of atazanavir and ritonavir were tabulated in **Table 1**. It was found from above data that all the system suitability parameters for developed method for atazanavir and ritonavir were within the limit.

**b. Specificity:**

**i. Blank and Placebo Interference:** The specificity of the proposed method was established by injecting blank and placebo using the above chromatographic conditions. The chromatogram of blank for atazanavir and ritonavir showed no peaks at the retention time of atazanavir and ritonavir peak revealing that the diluent solution used in sample preparation do not interfere in estimation of atazanavir and ritonavir in tablets. Similarly the chromatogram of placebo solution

showed no peaks at the retention time of atazanavir and ritonavir peak indicating that the placebo used in sample preparation do not interfere in estimation of atazanavir and ritonavir in their formulations.

**c. Linearity of Detector Response:** The linearity of the proposed method was ascertained in the concentration range of 37.5 - 375µg/ml for atazanavir and 12.5 - 125µg/ml for ritonavir. Evaluation of the two drugs was performed with PDA detector at 240nm and the respective peak areas were recorded for all the peaks. Further, standard curves were plotted for atazanavir and ritonavir (**Figure 3a & 3b)** and linear regression analysis was calculated respectively for the determination of slope, intercept and correlation coefficient [r2] (**Table 2 & 3)**. The slope and intercept value for calibration curve were **y = 6532.x – 5720 ($r^2$ = 0.999)** for atazanavir and **y= 16633.x - 5132 ($r^2$ = 0.999)** for ritonavir respectively. From the data obtained it is revealed that an excellent correlation exists between response factor and concentration of cited drugs within the concentration range indicated as above respectively.

The LOD values for atazanavir and ritonavir were found to be 0.009µg/mL and 0.00145µg/mL, respectively and the LOQ values were 0.0326µg/mL and 0.00484µg/mL and are reported in **Table 4** respectively.

**d. Precision:** The precision of the developed method was evaluated by carrying out inter-day and intra-day analysis by injecting six replicate injections of 100% test concentration of the above mentioned drugs and results were expressed in terms of standard deviation and %RSD. The results were given in **Table 5**. From the results [%RSD were 0.053 & 0.229 for atazanavir and 0.118 & 0.139 for ritonavir], it was revealed that the developed method was found to be precise as the %RSD values for repeatability and intermediate precision studies were < 2 %, respectively.

**e. Accuracy:** The accuracy of the method were carried out in triplicate preparations on composite blend collected from 10 tablets of atazanavir and ritonavir, analyzed as per the proposed method. The %RSD was ranged from 0.053-0.253 for atazanavir and 0.008-0.053 for ritonavir with percentage recoveries ranged from 99.65-99.90% for atazanavir and 99.50-99.90% for ritonavir respectively. From the data reported in **Table 6,** revealed that the developed RP-HPLC method was found to be accurate for atazanavir and ritonavir assay.

**f. Robustness Studies:** The robustness study of the developed assay method for atazanavir and ritonavir were established in all variance conditions and it was revealed that the assay values of the test preparation solution of the cited drugs was not affected and it was in accordance with that of actual. And moreover the system suitability parameters were also found satisfactory thereby concluding the proposed method to be robust (**Table 7**).

**g. Analysis of Marketed Formulation:** Analysis of marketed tablets was carried out using the above said optimized HPLC conditions. The % drug content of tablets obtained by the proposed method for atazanavir and ritonavir was found to be 99.99% and 99.98%, respectively. The results revealed that the estimation of dosage forms was accurate within the acceptance level of 95% to 100%. The results are given in **Table 9**.

## D. CONCLUSION

A simple, precise, accurate, isocratic RP- HPLC method have been developed by the author for the simultaneous estimation of atazanavir and ritonavir in marketed formulations by using optimized mobile phase containing phosphate buffer (pH-3.4) and acetonitrile in the ratio of 45:55% V/V and detection wavelength at 240nm. In the present assay the mobile phase preparation was easy and the solvents used were of low cost making the method more economical. The % RSD for all validation parameters were found to be less than two, which indicated the validity of developed method. Therefore, it is concluded that this developed RP-HPLC method can be conveniently used in future as alternative method for the pharmacokinetic studies and bioanalytical assays of the above cited drugs in combined dosage forms.

## ACKNOWLEDGEMENTS

The author would like to thank to M/s Spectrum Pharma Research Solutions, Hyderabad, India, for providing reference samples and other technical support.

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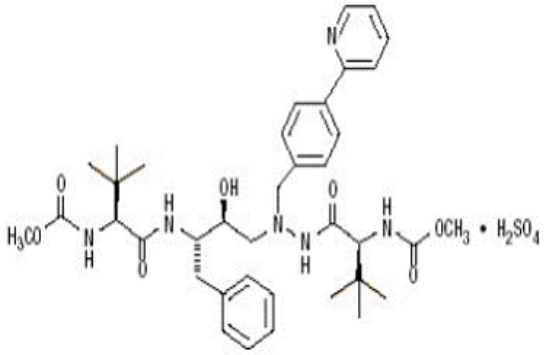

**Figure 1.a. Chemical structure of Atazanavir**

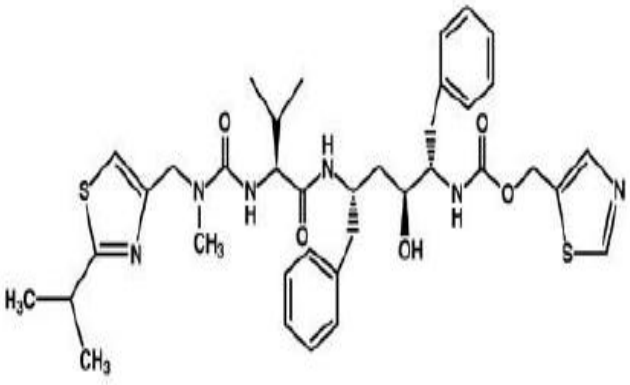

**Figure 1.b. Chemical structure of Ritonavir**

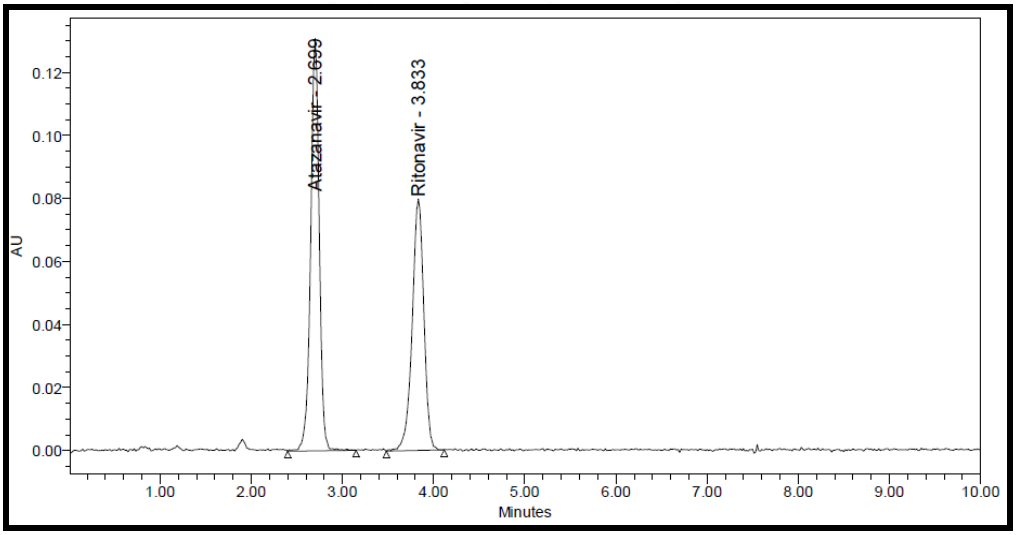


**Figure 2. System suitability Chromatogram of Atazanavir and Ritonavir**

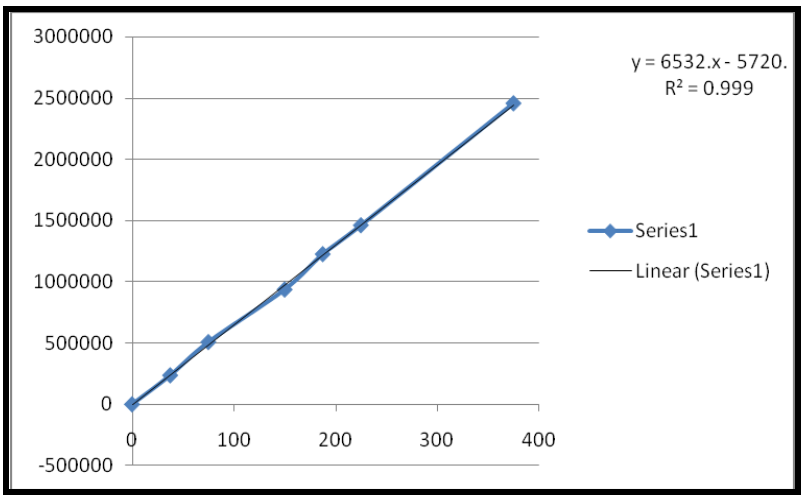


**Figure 3.a. Calibration curve for Atazanavir**

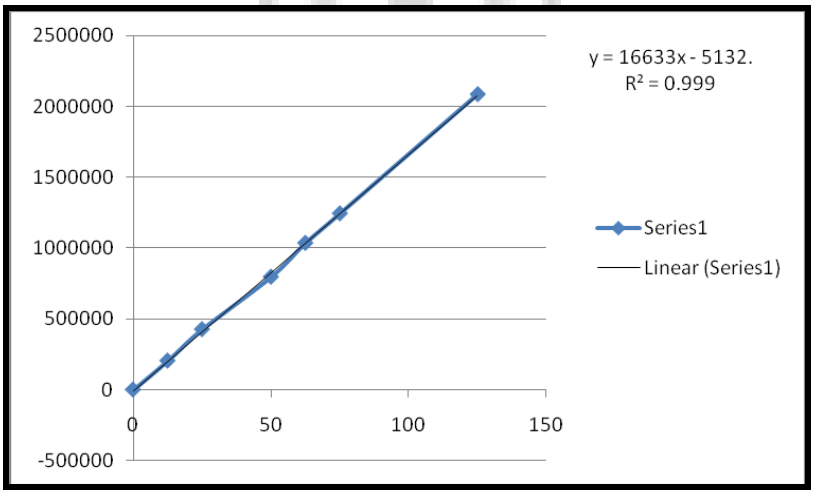


**Figure 3.b. Calibration curve for Ritonavir**

**Table 1. System Suitability of Atazanavir and Ritonavir**

| **PARAMETERS** | ATA | RIT |
|---|---|---|
| No. of theoretical plates | 4136 | 4598 |
| Tailing factor | 0.95 | 0.93 |
| Area | 865683 | 722544 |

**Table 2. Results of Linearity of Atazanavir**

| PPM | Set-1 | Set-2 | Set-3 | AVERAGE |
|---|---|---|---|---|
| 37.5 | 236413 | 236220 | 236431 | 236355 |
| 75 | 511773 | 497591 | 512324 | 507229 |
| 150 | 944591 | 942611 | 921869 | 936357 |
| 187.5 | 1222793 | 1214318 | 1237001 | 1224704 |
| 225 | 1441769 | 1457611 | 1480648 | 1460009 |
| 375 | 2449361 | 2451355 | 2462604 | 2454440 |
| **Slope,b** | **6532** | | | |
| **Intercept,a** | **-5720** | | | |
| **Correlation, $r^2$** | **0.999** | | | |

**Table 3. Results of Linearity of Ritonavir**

| PPM | Set-1 | Set-2 | Set-3 | AVERAGE |
|---|---|---|---|---|
| 12.5 | 203636 | 200719 | 207699 | 204018 |
| 25 | 416259 | 429792 | 431272 | 425774.3 |
| 50 | 795295 | 797416 | 794797 | 795836 |
| 62.5 | 1029216 | 1035810 | 1038202 | 1034409 |
| 75 | 1236057 | 1236354 | 1255084 | 1242498 |
| 125 | 2074134 | 2085045 | 2089701 | 2082960 |
| **Slope,b** | **16333** | | | |
| **Intercept,a** | **-5132** | | | |
| **Correlation, $r^2$** | **0.999** | | | |

**Table 4. LOD & LOQ Values of Atazanavir and Ritonavir**

| | ATA | RIT |
|---|---|---|
| **LOD** | 1.219849 | 0.326839 |
| **LOQ** | 3.696513 | 0.99042 |

**Table 5. Results of Precision of Atazanavir and Ritonavir**

| | Repeatability (%Assay) | | Day to Day (%Assay) | |
|---|---|---|---|---|
| | **ATA** | **RIT** | **ATA** | **RIT** |
| Sample 1 | 98.48 | 99.3 | 99.19 | 98.79 |
| Sample 2 | 99.4 | 99.19 | 100.76 | 101.9 |
| Sample 3 | 100.53 | 100.07 | 99.76 | 99.2 |
| Sample 4 | 100.1 | 100.79 | 99.49 | 98.3 |
| Sample 5 | 101.3 | 99.7 | 100.93 | 101.21 |
| Sample 6 | 100.16 | 100.92 | 99.83 | 100.57 |
| %Mean | 99.995 | 99.995 | 99.99333 | 99.995 |
| SD | 0.967135 | 0.73639 | 0.699276 | 1.441732 |
| %RSD | 0.967183 | 0.736427 | 0.699322 | 1.441804 |

**Table 6. Results of Accuracy of Atazanavir and Ritonavir**

| | Spiked amount (ppm) | | Standard drug solution (ppm) | | % Recovered | |
|---|---|---|---|---|---|---|
| | **ATA** | **RIT** | **ATA** | **RIT** | **ATA** | **RIT** |
| **50%** | 50 | 500 | 25 | 250 | 98.31496 | 96.69597 |
| | 50 | 500 | 25 | 250 | 98.10227 | 97.25029 |
| **100%** | 50 | 500 | 25 | 250 | 98.18718 | 101.4593 |
| | 50 | 500 | 50 | 500 | 99.97836 | 101.5531 |
| | 50 | 500 | 50 | 500 | 100.5208 | 100.3748 |
| **150%** | 50 | 500 | 50 | 500 | 98.70178 | 100.9372 |
| | 50 | 500 | 75 | 750 | 99.90604 | 100.9597 |
| | 50 | 500 | 75 | 750 | 99.78411 | 100.6552 |
| **MEAN** | | | | | 99.29235 | 99.98568 |
| **SD** | | | | | 0.952039 | 1.781539 |
| **%RSD** | | | | | 0.958824 | 1.781794 |

**Table 7. Results of Robustness Studies of Atazanavir and Ritonavir**

| | Changed value | Retention time | | Tailing factor | | % assay | |
|---|---|---|---|---|---|---|---|
| | | ATA | RIT | ATA | RIT | ATA | RIT |
| **Column** | 25 | 2.566 | 3.56 | 0.96 | 0.92 | 99.1 | 99.5 |
| **Temperature** | 35 | 2.63 | 3.59 | 0.95 | 0.94 | 101 | 101.4 |
| **Flow Rate** | 1.1 | 2.915 | 4.113 | 0.95 | 0.91 | 99.5 | 99.2 |
| | 1.3 | 2.538 | 3.549 | 0.97 | 0.92 | 100.2 | 101 |
| **Mobile Phase** | 40:60 | 2.591 | 3.513 | 0.95 | 0.91 | 98.9 | 100.2 |
| **Composition** | 50:50 | 2.62 | 3.68 | 0.96 | 0.93 | 100.6 | 100.9 |
| **Mean** | | | | | | 99.88333 | 100.3667 |
| **SD** | | | | | | 0.847152 | 0.882421 |
| **RSD** | | | | | | 0.848142 | 0.879197 |

**Table 8. Stability Data of Atazanavir and Ritonavir**

| Drug | % Assay at 0 hr | % Assay at 24 hr | % Deviation |
|---|---|---|---|
| **ATA** | 99.40 | 99.73 | 0.99 |
| **RIT** | 99.91 | 100.17 | 0.99 |

**Table 9. Results for HPLC Analysis of Tablets**

| Sample | PEAK AREA | | % ASSAY | |
|---|---|---|---|---|
| No. | ATA | RIT | ATA | RIT |
| **1** | 876850 | 728897 | 99.25482 | 99.67915 |
| **2** | 878473 | 734421 | 99.43854 | 100.4346 |
| **3** | 877422 | 727165 | 99.31957 | 99.44229 |
| **4** | 878972 | 725728 | 99.49502 | 99.24578 |
| **5** | 878131 | 731353 | 99.39982 | 100.015 |
| **6** | 879229 | 736326 | 99.52411 | 100.6951 |
| **AVG** | | | 100.7502 | 100.2688 |
| **SD** | | | 0.994865 | 0.604502 |
| **%RSD** | | | 0.987457 | 0.602882 |