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A modified liquid chromatographic method development and validation for simultaneous estimation of atazanavir and ritonavir in bulk and tablet dosage form

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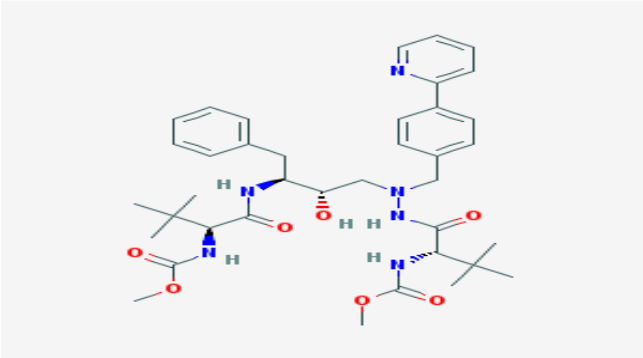
ABSTRACT

A simple, precise, accurate, reproducible and economical reverse phase liquid chromatography method was developed and validated for the quantitative simultaneous estimation of Atazanavir and Ritonavir in bulk and marketed formulations. Estimation of drugs in this combination was done with a C18 column Kromasil 100-5C_{18} column [250mm x 4.6mm].using mobile phase of composition Acetonitrile and phosphate buffer (50:50 v/v, pH 5).The flow rate was 1 ml/min and the effluents were monitored at 212nm. The retention time of Atazanavir and Ritonavir were3.3 min and 6.2 min respectively. The method was found to be linear over a concentration range of 20-100 µg/ml for both Atazanavir and Ritonavir. The established method proved as reproducible one with a %RSD value of less than 2 and having the robustness and accuracy within the specified limits. Assay of marketed formulation was determined and find with 99.48% and 98.7% for Atazanavir and Ritonavir respectively. The method was validated according to the guidelines of International Conference on Harmonization (ICH) and was successfully employed in the estimation of commercial formulations. This liquid chromatographic method can be applied for the qualitative and quantitative determination of selected drugs by the modern chemist.

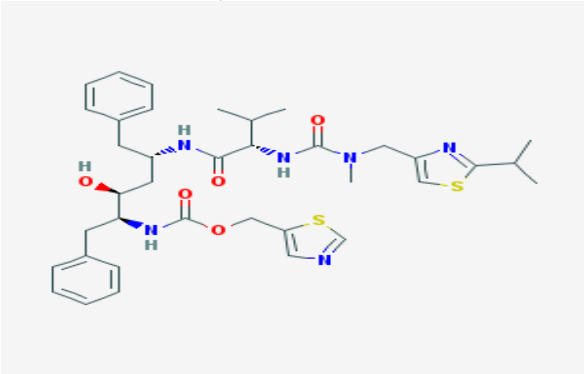
Keywords*:* Atazanavir, Ritonavir, RP-HPLC and Method validation.

INTRODUCTION

Atazanavir sulfate, chemically known as 3, 12-bis (1, 1-dimethylethyl)-8-hydroxy-4, 11-dioxo-9-(phenyl methyl)-6-((4-(2-pyridinyl) phenyl) methyl)-, dimethyl ester. The chemical structure was shown in Figure 1a. It is an oral antiretroviral Protease inhibitors used in the treatment of HIV/AIDS. ATV is an antiretroviral drug specifically belongs to protease inhibitors class. Ritonavir is (5S, 8S, 10S, 11S)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3, 6-dioxo-8, 11-bis (phenyl methyl)-2, 4, 7, 12-tetraazatridecan-13-oicacid 5-thiazolyl methyl ester Figure.1b. It is official in Indian Pharmacopoeia and United States Pharmacopoeia. Ritonavir is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. Ritonavir is frequently prescribed with Highly Active Anti-Retroviral therapy, not for its antiretroviral action, but as it inhibits the same host enzyme that metabolizes other protease inhibitors [1-3].



a) ATAZANAVIR



b) RITONAVIR

FIG 1: CHEMICAL STRUCTURES OF a) ATAZANAVIR b) RITONAVIR

Extensive literature survey proved that very few methods [4-19] were reported for the determination of Atazanavir and Ritonavir by RP-HPLC. So we attempted to develop an accurate, rapid, precise, stable, sensitive and economically viable liquid chromatographic method for the simultaneous determination of selected drugs in the present research.

MATERIALS AND METHODS

Equipment used

The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20µl fixed loop. A reverse phase C18[Kromasil 250mm × 4.6 mm]was used. Lab India 3000^{+}double beam UV visible spectrophotometer and Axis AGN204-PO electronic balances were used for spectrophotometric determinations and weighing purposes respectively.

Reagents and chemicals

Pharmaceutical grade pure Atazanavir and Ritonavir gift samples were procured from Mylan Laboratories, Hyderabad. Marketed tablet formulations (ATOZOR-R) with of 300mg of Atazanavir and 100mg of Ritonavir were procured from local market. (Mfd.by Emcure Pharmaceuticals ltd). HPLC grade Acetonitrile and Water were commercially procured from Merck specialties private limited, Mumbai.

Chromatographic conditions

Kromasil 100-$5C_{18}$ column [250mm x 4.6mm] was used for the chromatographic separation at a detection wave length of 212 nm. Mobile phase composition of Acetonitrile and Phosphate buffer pH 5 in a ratio of 50:50 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was adjusted to 1ml/min and the injection volume was 20µl.

Preparation of Mobile phase
Phosphate buffer pH 5 was prepared by dissolving 0.136gm of Potassium dihydrogen phosphate and 2 ml of Triethyl amine in 80ml of HPLC grade water and adjusts the pH to 5.0 with orthophosphoric acid and volume was adjusted with water to produce100 ml, which is then filtered through 0.45µ membrane filter and sonicated for 20 minutes.

Preparation of Standard solutions
25mg each of Atazanavir and Ritonavir were accurately weighed and transferred into two 25ml volumetric flasks respectively and dissolved in mobile phase as mentioned above and the volume was made up with the same solvent to obtain primary stock solutions A (Atazanavir) B (Ritonavir) to achieve standard of concentrations of 1000µg/ml of each drug. From the primary stock solutions , 1 ml of each solution was pipette out and transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 100 µg/ml of Atazanavir and Ritonavir respectively and this solution is (working stock solution A).

Preparation of Sample Solution
Twenty tablets of Atazanavir and Ritonavir were weighed and crushed. Tablet powder equivalent to 300mg of Atazanavir and 100mg of Ritonavir was weighed accurately and transferred to a 25ml volumetric flask. The content was dissolved with 10ml of mobile phase and then sonicated for 15min. The volume was made up with the mobile phase and filtered with 0.45µmembrane filter and sonicated for 20min.0.1 ml of this solution was pipette out and transferred to a 10mlvolumetric flask and the volume was made up with the mobile phase to obtain a concentration of 120µg/ml of Atazanavir and 40µg/ml of Ritonavir(working stock solution B).

Optimization of RP-HPLC method
The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Atazanavir and Ritonavir. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with Acetonitrile ,Phosphate buffer pH5 (50:50 v/v) using Kromasil 100-5C_{18} column [250mm x 4.6mm].

Validation of the RP-HPLC method
Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability
System suitability was carried out with five injections of solution of 100% concentration having100 µg/ml of Atazanavir and Ritonavir of each in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factors (T) were reported in table 1.

Linearity
For the determination of linearity, appropriate aliquots were pipetted out from working stock solution A to a series of 10ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 20-100µg/ml of Atazanavir and Ritonavir. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Atazanavir and Ritonavir were shown in figure 3 and figure 4 their corresponding linearity parameters were given in table 2.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)
The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in table 2.

Precision
The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (100µg/ml of Atazanavir and Ritonavir respectively) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were given in table 3.

Accuracy
Standard addition method was followed to ensure the reliability and accuracy of the method recovery studies. A known quantity of pure drug was added to pre-analyzed sample and contents were reanalyzed by the proposed method and the percent recovery was reported. The results were given in table 4.

Specificity
Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients. The optimized chromatogram of Atazanavir and Ritonavir without any interference was shown in figure 2.

Robustness
Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, wave length detection, flow rate, etc. and the % RSD should be reported. Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ±2nm in the detection wave length and ±0.2ml/min in the flow rate, were tried individually. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. %RSD was reported in the table 5.

Assay of Marketed Formulations
20µl of sample solution of concentration 120 µg/ml of Atazanavir and 40µg/ml of Ritonavir was injected into chromatographic system and the peak responses were measured. The solution was injected three times in to the column. The amount of drug present and percentage purity was calculated by comparing the peak areas of the standards with that of test samples. A typical chromatogram for assay of marketed formulation was shown in figure 5and the obtained values were reported in the table 6.

RESULTS AND DISCUSSION

After a number of trials with mobile phases of different composition, Acetonitrile, Phosphate buffer pH 5 in the ratio 50:50v/v was selected as mobile phase because of better resolution and symmetric peaks. Atazanavir and Ritonavir were found to show appreciable absorbance at 212nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Atazanavir and Ritonavir at different R_Ts was shown in figure 2.

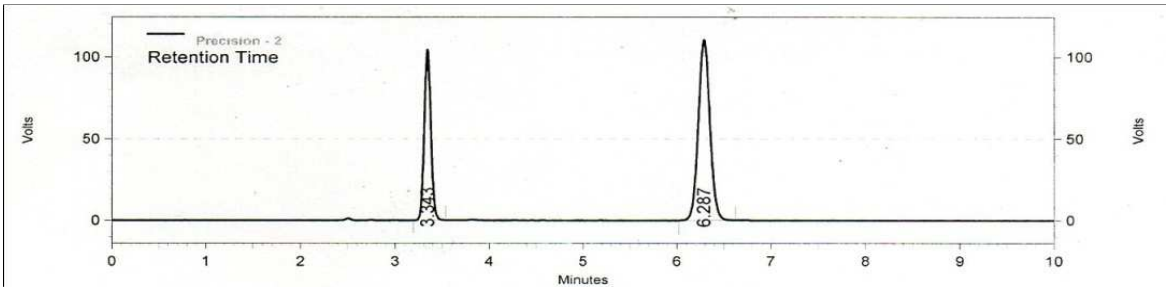


FIG 2: OPTIMIZED CHROMATOGRAM OF ATAZANAVIR AND RITONAVIR

System suitability was carried out by injecting 5 replicate injections of 100% test concentration, number of theoretical plates, HETP and resolution were satisfactory. The chromatograms confirm the presence of Atazanavir and Ritonavir at 3.3min and 6.2min respectively without any interference. The parameters were given in table 1.

TABLE 1: SYSTEM SUITABILITY PARAMETERS (n=5)

Parameters	Atazanavir	Ritonavir
Retention time (min)	3.3	6.29
Theoretical plates (N)	11456	10366
Tailing factor (T)	1.2	1.4
Resolution (R_s)	2.99	

**n= No. of determinants*

Concentration range of 20-100µg/ml for Atazanavir and Ritonavir were found to be linear with correlation coefficients 0.998 and 0.999 for Atazanavir and Ritonavir respectively. The respective calibrations curve was shown in Figure 3 and 4 respectively. The results were given in table 2.

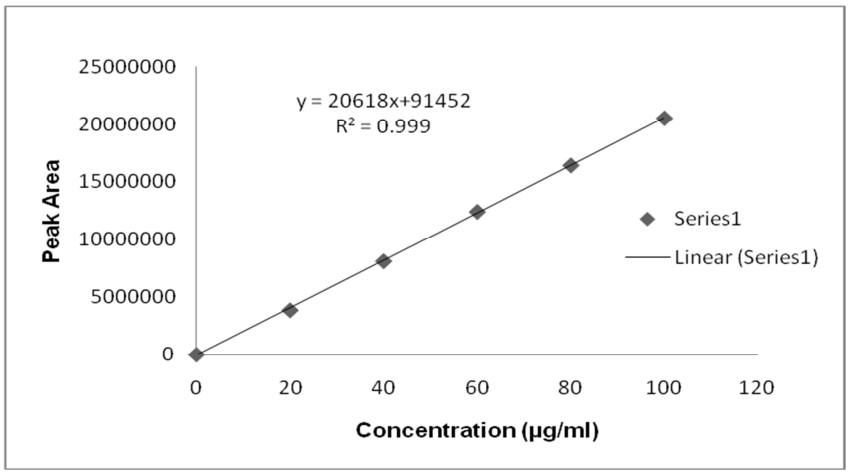


FIG 3: CALIBRATION PLOT OF ATAZANAVIR

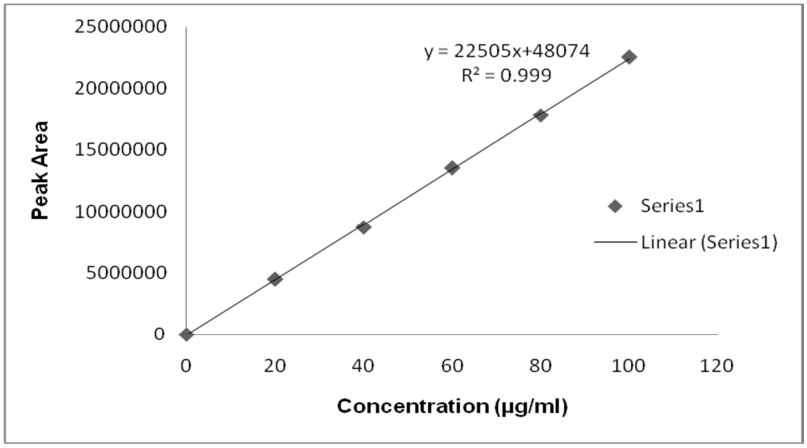


FIG 4: CALIBRATION PLOT OF RITONAVIR

The limits of detection for Atazanavir and Ritonavir were found to be 0.65µg/ml and 0.54µg/ml respectively and the limits of quantitation were 1.99µg/ml and 1.64µg/mlrespectively. Values were represented in table 2.

TABLE 2: RESULTS FOR LINEARITY (n=3)

Parameter	Atazanavir	Ritonavir
Linearity Range (µg/ml)	20-100	20-100
Regression Equation	y = 16324x+12346	y = 86676x + 37329
Slope (m)	16324	86676
Intercept (c)	12346	37329
Regression Coefficient (r^2)	0.998	0.999
Limit of Detection (µg/ml)	0.65	0.54
Limit of Quantitation (µg/ml)	1.99	1.64

**n= No. of determinants*

The proposed method was found to be precise and reproducible with %RSD of 0.42 and 0.19for Atazanavir and Ritonavir respectively. %RSD was reported in table 3.

TABLE 3: RESULTS OF PRECISION (n=6)

Drug	Intraday Precision (%RSD)	Interday Precision (%RSD)
Atazanavir	0.42	0.34
Ritonavir	0.19	0.11

**n= No. of determinants*

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 98.6% to 99.4% for Atazanavir and 98.4 to 99.3% for Ritonavir. This indicates that the method was accurate. Values obtained were given in table 4.

TABLE 4: RESULTS FOR ACCURACY (n=3)

**n= No. of determinant*

Recovery level	Atazanavir				Ritonavir			
	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery
	std	test			std	Test		
80%	20	60	78.9	98.6	20	60	79.5	99.3
100%	40	60	99.4	99.4	40	60	98.4	98.4
120%	60	60	119.1	99.2	60	60	118.7	98.9
Mean recovery	98.6-99.4				98.4-99.3			

The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in table 5.

TABLE 5: RESULTS FOR ROBUSTNESS (n=3)

Parameters (n=3)	%RSD	
	Atazanavir	Ritonavir
Detection wavelength at 210nm	0.12	0.38
Detection wavelength at 214nm	0.36	0.66
Flow rate 0.8ml/min	0.34	0.52
Flow rate 1.2ml/min	0.31	0.35

**n= No. of determinant*

The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 99.48% for Atazanavir and 98.7% for Ritonavir. The typical chromatogram for assay of marketed formulations was shown in figure.5 and Values obtained were given in table 6.

FIGURE 5: A TYPICAL CHROMATOGRAM FOR ASSAY OF MARKETED FORMULATION CONTAINING 120µg/ml OF ATAZANAVIR AND 40µg/ml OF RITONAVIR

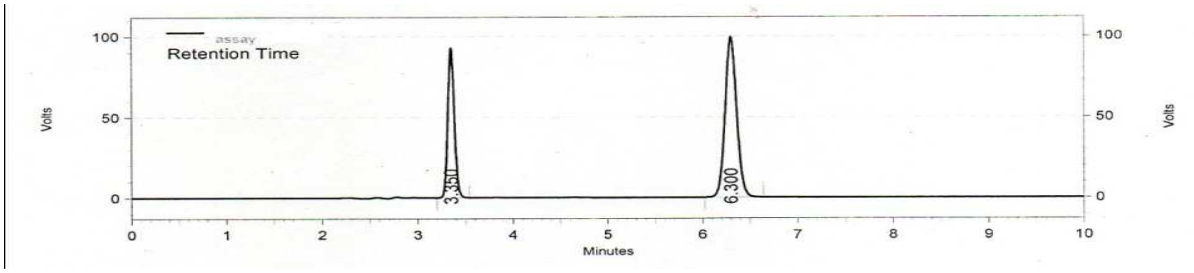


TABLE 6: RESULTS FOR ASSAY (n=3) OF MARKETED FORMULATION (ATAZOR-R)

Drug	Label claim (mg/tab)	Amount recovered	% Amount found in drug
Atazanavir	300	298.44	99.48
Ritonavir	100	98.7	98.7

**n= No. of determinants*

CONCLUSION

The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Atazanavir and Ritonavir from their formulations. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the method was found to be simple, accurate, precise, rugged and robust. So the established method can be employed in the routine analysis of the marketed formulations.

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