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METHOD DEVELOPMENT AND VALIDATION OF RP HPLC METHOD FOR ESTIMATION OF ANTIVIRAL COMBINATION AS ATAZANAVIR AND RITONAVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: This study was conducted to develop a validated liquid chromatographic method for the simulteneous determination of atazanavir and ritonavir in bulk and pharmaceutical dosage form.

Method: The method employed RP-Purosnosphere C_{18} column as the stationary phase while acetonitrile:water (80:20) pH adjusted to 3.0 was used as mobile phase and 273 nm has been selected as detection wavelength for HPLC method.

Results: The method showed good sensitivity within linearity range of atazanavir and ritonavir with correlation coefficients of 0.9998 and 0.999 respectively observed at 273 nm wavelength. Best resolution was obtained with mean retention time of 2.95 and 6.86 minute respectively for atazanavir and ritonavir at flow rate of 1.0 ml per minute. Mean percent recovery of samples at each level for drugs were found in the range of 99.77% to 100% with RSD of less than 2.0%.

Conclusion: The method was validated according to the guidelines of International Conference on Harmonisation and was successfully employed in the estimation of commercial formulation.

Key words: RP-HPLC, Atazanavir , Ritonavir, Combined dosage forms, Simultaneous estimation, Validation.

INTRODUCTION

Atazanavir is in a class of medications called protease inhibitors and chemicaly designated as (3S-(3R*,8R*,9R*,12R*))-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. Atazanavir is used along with other medications, such as ritonavir to treat human immunodeficiency virus (HIV) infection.

Atazanavir selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells by binding to the active site of HIV-1 protease, thus preventing the formation of mature virions and it is not active against HIV-2. [1,2]

Ritonavir chemicaly is 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 peptidomimetic and an inhibitor of HIV protease with activity against the HIV. Ritonavir is an orally active peptidomimetic inhibitor of both the HIV-1 and HIV-2 proteases. This protease acts at last step in HIV replication i.e. at the time of maturation of new virus particles when RNA genome acquires core proteins and enzymes. Protease inhibitor bind with the protease molecule and interferes with its cleaving function as they are effective at the last step of viral cycle they are useful for both newly and chronically infected cells. [3,4]

The combination of AZV and RTV was first approved by US Food and Drug Administration (FDA) Mylan's product was approved under the tentative approval scheme in November 2011 for the treatment of HIV infection in adults. [5]

Literature survey revealed that AZV and RTV were estimated individually by UV; reverse phase-high performance liquid chromatography (RP-HPLC) in tablet formulation,bulk or in biological fluids, other than these there are several analytical methods reported for the above drugs with other class of anti-retroviral drugs. [6-14]

The purpose of this study was to develop a more simple, rapid, precise, and accurate RP-HPLC method for the simultaneous estimation of these drugs in combined tablet dosage form.

Figure 1. Chemical structure of atazanavir

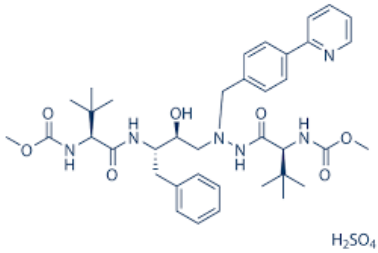
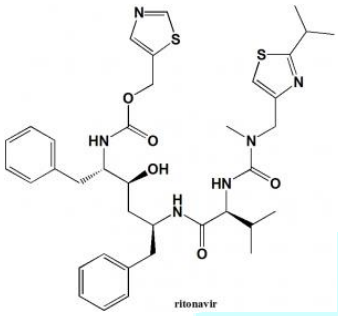


Figure 2. Chemical structure of ritonavir



MATERIALS AND METHODS

HPLC instrumentation and conditions

The chromatographic separation was performed on Systronics LC 8600 liquid chromatographic system integrated with an injector equipped with 20µl fixed loop. The chromatographic system operated using Chemitochrom 2000 software and separate programmable UV detectorsystem coupled with Purosphere (Merk, Germany) RP-18 column (5 µm, 4.6mm, 250 mm) was used at ambient conditions. Shimadzu UV 1800 double beam UV visible spectrophotometerused for spectrophotometric estimation, sansui-vibra DJ-150S-S electronic balance for weighing purposes and Fast clean ultrasonicate cleaner (India) was used for degassing the mobile phase.

Reagents and chemicals

Pharmaceutical grade pure ritonavir (RTV) were obtained as generous gift from Matrix Laboratories Limited, Nasik and atazanavir (AZV) from Cipla Pvt. Ltd., Kurkumbh., Acetonitrile, methanol and water used were of HPLC grade (Merck Specialties Private Ltd., Mumbai, India). A nylon filter (Pall life Sciences, Mumbai, India) was used. All other chemicals and reagents used were analytical grade unless otherwise indicated.

Preparation of stock and standard solutions

The standard stock solutions containing 1 mg/ml each of AZV and RTV were prepared separately by dissolving reference standards in acetonitrile:water (80:20) and diluting with the same diluent. Aliquots from the standard stock solutions of AZV and RTV were transferred to 10 ml calibrated volumetric flask and the volume was made up to the mark with acetonitrile:water (80:20) to prepare standard preparations

having a desired concentration. Calibration curve solutions containing 10 to 30 µg/ml of RTV and 30 to 70 µg/ml for AZV were prepared by diluting the stock solution to the appropriate volume with the same diluent.

Preparation of test Solution

Mixed standard solution: Transferred aliquot volume of AZV and RTV of above stock solution to a 10 ml volumetric flask and made up to volume with acetonitrile:water (80:20) to attain a concentration of RTV (20 µg/mL) and AZV (60 µg/mL) in solution.

Standard Formulation solution

Twenty QVIR Kit tablets (Atazanavir 300 and Ritonavir 100) were weighed and finely powdered in a mortar. Tablet powder equivalent to 60 mg of AZV and 20 mg RTV was accurately weighed and transferred to a 100 ml calibrated volumetric flask. Around 60 ml of mobile phase mixture was added, and the solution sonicated for 10 min. Volume wasmade up to the mark with the same solvent mixture. Thesolution was filtered through 0.45 mm membrane filter. This solution contains RTV (20 µg/mL) and AZV (60 µg/mL). The chromatogram is shown in Figure 6.

RESULTS AND DISCUSSION

Selection of mobile phase

The pure drugs of AZV and RTV were injected into the HPLC system and run in different solvent systems. Different mobile phases like methanol and buffer, acetonitrile and buffer, methanol, acetonitrile and water were tried. It was found that acetonitrile:water in composition of 80:20 gives satisfactory results as compared to other mobile phases. Finally, by using systematic approach, the optimal composition of the mobile phase was determined to be acetonitrile:water in composition of 80:20 pH adjusted to 3.0 with diluted orthophosphoric acid.

Selection of Wavelength

Appropriate dilution was prepared using standard stock solution to get desired concentration of, AZV and RTV in 80:20 pH adjusted to 3.0 with diluted orthophosphoric acid. The solutions were scanned over range of 200-400 nm, using medium scan speed. Considering the spectra 273 nm has been selected as detection wavelength for HPLC method.

Chromatographic conditions

Chromatographic separations were performed on HPLC system with Chemitochrom 2000 (Systronics, India) separation module equipped with a UV 8600 detector. The separation was performed on a Purospher RP-18 column (5 µm, 4.6mm, 250 mm) at an ambient temperature with acetonitrile:water in composition of 80:20 pH adjusted to 3.0 with diluted orthophosphoric acid was selected as mobile phase for elution with optimized flow rate of 1ml/min. The injection volume and detection wavelength were fixed at 20 µL and 273 nm respectively. The elution was monitored by injecting the 20µl and the flow rate was adjusted to 1.0 ml/min.

METHOD VALIDATION PARAMETERS

Validation of the optimized RP HPLC method was performed as per the ICH guidelines.[15-16]

System suitability

System suitability was carried out with six injections of solution of AZV and RTV in to the chromatographic system. Number of theoretical plates (N) obtained and tailing factor (T) was reported in table 1.

The retention times of AZV and RTV was 2.95 and 6.86 respectively with a resolution of above 2. All the components showing tailing factor bellow 2 and the theoretical plates numbers were above 2000. These values are within the acceptable range of United state pharmacopoeia. The results obtained are shown in Table 1.

Linearity

Calibration curves were obtained from the peak area and concentration of the drug were subjected to regression analysis and correlation coefficients. Table 2 represents the linearity of the proposed methodwhich shows the responses for the drugs was strictly linear ($r^2>0.99$) in the concentration range of 30 to 70 µg/ml for AZV and 10-30 µg/ml for RTV. The slope for AZV and RTV was found to be 5.191 and 5.222 where as intercept for AZV and RTV was found to be 3.991 and 19.90 respectively.

The LOD and LOQ were determined from the calculated standard deviations of each calibration standard. LOD was found to be 2.8000 and 2.8184 µg/ml and LOQ was found to be 8.5407 and 8.4954 µg/ml for

AZV and RTV respectively. The calculated LOQ and LOD concentrations confirmed that the method is sensitive.

Accuracy and precision

Accuracy data of analytical method in the present recovery study ranged from 99.90 - 99.93% for AZV and 99.79 to 99.86 % for RTV which (Table 3) indicates that there was no interference from excipient components of market formulation and the method is specific and accurate.

Accuracy and precision were determined by elaboration of three standard calibration curves, two from the same day (intra-day) and third one from a different day (inter-day). The intra-day and inter-day precisions (% RSD) at different concentration levels were found to be less than 2 % (Table 4). Moreover, the % RSD (less variation) showed good stability andprecision of the developed HPLC method.

Specificity

The specificity test of the proposed method demonstrated that the excipients from sample do not interfere with the drug peak. A specific method should can measure the analytes accurately in the presence of interferences such as solvent, excipients and other active ingredients with their chemical groups. Figure 5 and 6 shows chromatographic base-line separation of AZV and RTV in their standard mixture and formulation respectively considering which one should conclude that there are no interferences found at the retention times of AZV and RTV in their dosage forms due to excipients.

Assay

The percentage assay was found to be 99.74 and 99.50 for AZV and RTV respectively with a percentage RSD well below 2. The results obtained are shown in Table 5.

CONCLUSION

A RP-HPLC method was developed for anti-retroviral drugs, which can be conveniently employed for routine quality control in pharmaceutical dosage forms. The chromatographic conditions were optimized in order to provide a good performance of the assay. The mobile phase for each drug was selected based on its polarity. Different ratios of acetonitrile: water compositions were tried for AZV and RTV the fixed mobile phase was 80:20 pH adjusted to 3.0. The flow rate was optimized to reduce the extent of longitudinal broadening which is inversely related to flow rate of mobile phase. Best resolution was obtained mean

retention time of 2.95 and 6.86 min respectively for AZV and RTV at flow rate of 1.0 ml per minute.The chromatogram shown in Figure 6 confirms that the method was specific as none of the excipients interfered with the analytes of interest. The recoveries achieved are good for all the molecules hence; the method was suitably employed for assaying the commercial anti-retroviral formulations.

The proposed RP-HPLC is simple, reliable and selective. It also provides satisfactory accuracy and precision with lower limits of detection and quantification. Moreover, the shorter duration of analysis for AZV and RTV make these reported methods suitable for routine quantitative analysis in pharmaceutical dosage forms.

CONFLICT OF INTERESTS

Hereby, the authors declare that there is no conflict of interests for current publication.

Table 1: System suitability parameters

System Suitability Parameters	Proposed Method	
	RTV	AZV
Retention Time (t_R)	6.86	2.95
Area (%)	46.47	53.53
Number of Theoretical Plates	6536.6	2714.5
Tailing Factor	1.1364	1.3333
Resolution Factor (R)	11.7500	

* An average of six determinations

Figure 3: Calibration curve of Ritonavir

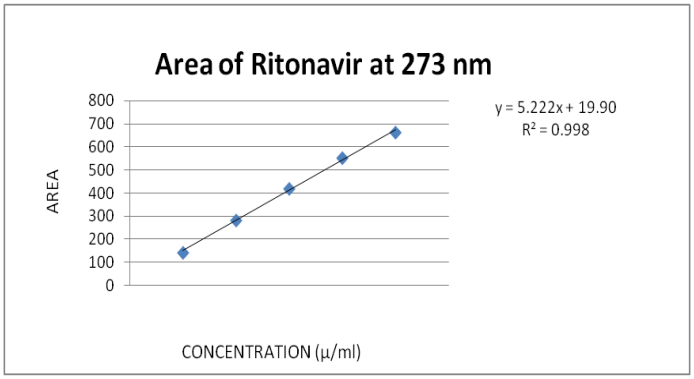


Figure 4: Calibration curve of Atazanavir.

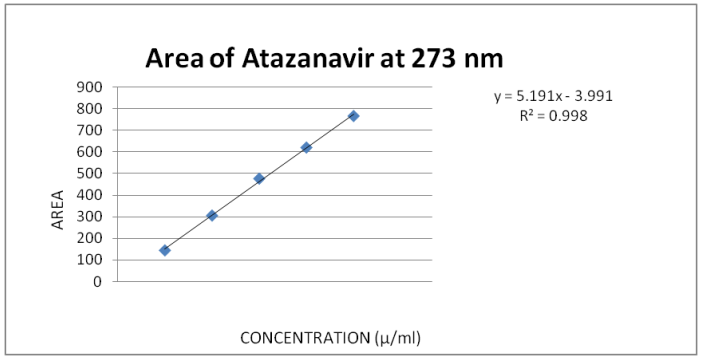


Table 2: linearity of the proposed method.

Sr. No.	Parameter	RTV	AZV
1.	Linearity range	10-30 µg/ml	30-70 µg/ml
2.	Regression equation	y = 5.222x + 19.90	y = 5.191x - 3.991
3.	Slope	5.222	5.191
4.	Intercept	19.90	3.991
5.	Regression Coefficient (r^2)	0.998	0.998
6	LOD	2.8184	2.8184
7	LOQ	8.5407	2.8000

* An average of six determinations

Table 3: Recovery studies by RP-HPLC

Drug	(mg/tablet)	Percentage (%)	Amount Added mg	Total Amount Found (mg)	Recovery (%) %RSD
RTV	100	80	80	180	99.90 ± 0.876
		100	100	200	99.96 ± 0.973
		120	120	220	99.93 ± 0.786
AZV	300	80	240	540	99.79 ± 0.894
		100	300	600	99.85 ± 1.073
		120	360	660	99.86 ± 0.998

* An average of three determinations

Table 4: Precision data for the analysis of RTV and AZV

Drug	Conc. [µg/ml]	Intra-day Amount Found			Inter-day Amount Found		
		Amount found [*n* = 3]	S.D	% R.S.D.	Amount found [*n* = 3]	S.D.	% R.S.D.
RTV	10	9.94	1.5050	1.5894	9.96	1.5050	1.5894
	15	14.98	1.9148	1.6953	14.97	1.9148	1.6953
	20	19.96	1.4583	1.2545	19.97	1.4583	1.2545
AZV	40	40.60	0.6822	0.1419	40.85	0.6822	0.1419
	50	49.74	1.6683	0.9051	49.83	1.6683	0.9051
	60	59.84	1.5677	1.7564	59.82	1.5677	1.7564

* An average of six determinations

Table 5: Assay of tablet dosage form

Sr. No.	Amount Present (µg/ml)		% Drug Estimation	
	RTV	AZV	RTV	AZV
1	20	60	98.99	99.98
2	20	60	99.98	99.55
3	20	60	99.23	99.70
4	20	60	99.90	99.61
5	20	60	99.43	99.87
MEAN			99.50	99.74
SD			0.4246	0.1796
%RSD			0.4267	0.1801

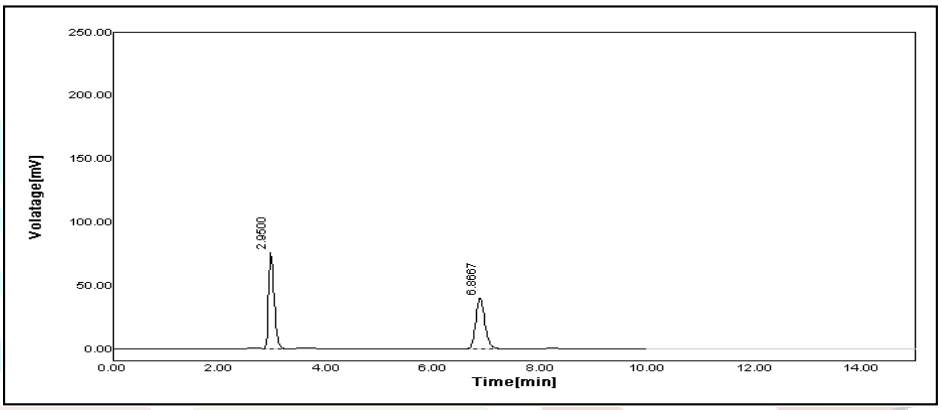


Figure 5: Typical chromatogram showing the elution of pure mixed standards at their respective retention time in laboratory mixed standards.

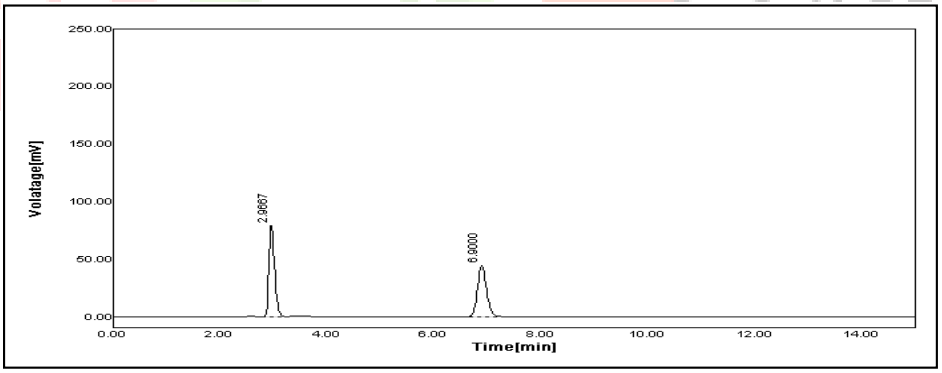


Figure 6: Typical chromatogram showing the elution of RTV and AZV at their respective retention time in tablet formulation.

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