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SIMULTANEOUS ESTIMATION OF METHOD DEVOLOPMENT AND VALIDATION OF ATAZANAVIR AND RITONAVIR BY RP-HPLC METHOD

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ABSTRACT

A new, simple, sensitive, precise and accurate High-performance thin-layer chromatographic method for simultaneous determination of Ritonavir and Atazanavir in their combined tablet dosage form has been developed, validated and used for determination of the compounds in commercial pharmaceutical products. Chromatographic separation was achieved on Eclipse C_{18} column (100 mm × 4.6 mm, 3.5 µm particle size) as the stationary phase and Acetonitrile and acetate buffer in the ratio of 60:40. With mean recoveries of 98.20 to 100.03% for atazanavir and 99.73 to 99.98% for ritonavir respectively. Limit of detection for ritonavir and atazanavir were found to be 1.717µg/ml and 0.646µg/ml respectively.

KEYWORDS

High-performance thin-layer chromatography, Ritonavir and Atazanavir.

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INTRODUCTION

Ritonavir (RTV) chemically (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-oic acid 5-thiazolyl methyl ester. Atazanavir (ATV) chemically (3S,8S,9S,12S) -3,12-Bis (1,1- dimethylethyl) -8-hydroxy-4,11-dioxo-9- (phenyl methyl) -6- [4-(2-pyridinyl) phenyl]methyl]-2,5,6,10,13-penta aza tetradecanedioic acid dimethyl ester, sulphate (1:1). Ritonavir and Atazanavir inhibit HIV protease, enzyme required to form functional proteins in HIV infected patients. Ritonavir is available in combination of Atazanavir, which inhibits the

CYP3A4-mediated metabolism of Lopinavir, increasing the Atazanavir concentrations[1-2].

Literature review reveals that methods have been reported for analysis of RTV and ATV in pharmaceutical formulations and in human plasma individually or in combination with other antiviral drugs using liquid chromatography (LC) [3-14], Thin Layer Chromatography (TLC) [15] and UV spectroscopy[16-19] and LC/MS[20-22]. As per literature review, there is no single HPTLC method has been reported for simultaneous determination of RTV and ATV in combination tablets. Hence, aim of the present study to develop and validate accurate method and determine both drugs concurrently by simple, rapid, and selective HPTLC method that could be used for quality control and routine analysis. The proposed method was validated in accordance with International Conference on Harmonization (ICH) guideline[23].

EXPERIMENTAL

Materials and Reagents

RTV and ATV powders were procured from Hetero Drugs Ltd. (Hyderabad, India). VIRATAZ-R Tablets was manufactured by Hetero Drugs Ltd. Each tablets claimed to contain 100 mg RTV and 300 mg ATV. Methanol was purchase from Loba Chemie (Mumbai, India). Toluene and Chloroform were purchased from Rankem (RFCL Ltd, New Delhi, India). Ethyl acetate and Acetone were purchased from Finar (Ahmadabad, India). All experiments were performed with analytical grade chemicals.

Instrumentation and Chromatographic Conditions

Optimization of Chromatographic Conditions

Selection of chromatographic method

Proper selection of the method depends on the nature of the sample (ionic or ionisable or neutral molecules), its molecular weight, pka value and stability. The drug selected in the present study is polar and so reverse phase or ion exchange chromatography can be used. The reversed phase HPLC was selected for the initial separation because of its simplicity and suitability. From the literature survey and with the knowledge of properties of the selected drug, Eclipse C18- 100mm x 2.5mm column was chosen as stationary phase and mobile phase with different compositions such as acetonitrile and acetate buffer were used.

Optimized conditions

The following optimized parameters were used as a final method in estimation of ritonavir in bulk and pharmaceutical formulations.

Instrument : Agilent model 1220
Column : Eclipse C18- 100mm x 2.5mm
Column Oven Temperature : Ambient
Detection wave length : 250nm
Flow rate : 1ml/min
Injection volume : 20µl
Runtime : 5mins
Elution type : Isocratic
Mobile phase : Acetonitrile: Acetate buffer
Solvent ratio : 60:40 v/v

Preparation of Standard Stock Solutions

Standard stock solution of 30 mg of atazanavir and 10mg ritonavir dissolved in acetonitrile and made the volume up to 10 ml with same solvent.(3000µg/ml), (1000µg/ml. From the above stock solutions 1ml of the each aliquots were pipette out in a 10 ml volumetric flask separately and the volume was made up to the mark with same solvent to obtain the final concentration of 300µg/ml of atazanavir sulfate and 100µg/ml of ritonavir(stock B solution)

Preparation of Sample Solution

Twenty Tablets were taken and their average weight was determined they were crushed to fine powder. The powder equivalent to 30mg of atazanavir and 10 mg of ritonavir was taken in 100ml volumetric flask and sonicated for about 25 min. Dissolved in 100ml acetonitrile solvent with vigorous shaking for 5-10 minutes The solution was then filtered through what man filter paper No.41 µm. The filtrate contains 300 µg/ml of atazanavir and 100 µg/ml ritonavir. Determine the amount of % Atazanavir and Ritonvir according to the following formula.

Results and Discussion

Selection of wavelength

The working standard solutions of 30µg/ml of atazanavir sulfate 10µg/ml of ritonavir were prepared by appropriate dilution of standard stock solutions. Overlain spectra of atazanavir sulfate and

ritonavir were scanned which is shown in Figure No.1, from which the wavelengths of 250nm, 239.4nm were selected for atazanavir sulfate and ritonavir respectively for further studies.

System Suitability Testing (SST)

System suitability tests are an integral part of chromatographic method (Figure No.2). They were used to verify that the reproducibility of the chromatographic system is adequate for the analysis. It is defined as tests to measure that the method can generate result of acceptable accuracy and precision (Table No.2).

Specificity

The analyte was assessed in the presence of the components and it was found that there was no interaction with the analyte (Figure No.3). Hence the method is said to be specific.

Linearity

To check the linearity various concentrations (3-150µg/ml) were prepared. Each of these drug solutions (20µL) was injected into the chromatographic system for three times. The peak area and retention time were recorded and the mean values of peak areas were plotted against concentrations (Figure No.4 and 5).

Accuracy

Accuracy of the proposed method was determined using recovery studies by standard addition method. The recovery studies were carried out by adding known amounts (50, 100 and 150%) i.e. 15, 30 and 45 µg/ml to the standard concentration of 30 µg/ml for atazanavir and 5, 10 and15 µg/ml to the standard concentration of 10 µg/ml for ritonavir. The solutions were prepared in triplicates and the % recovery was calculated. % recovery was found to between 99.1 to 99.6 (atazanavir) 98.5 to 99.1 (ritonavir). Results are shown in the Table No.4.

Acceptance criteria

A method is said to be accurate if the % recovery studies are in the range of 98-102. The results for accuracy indicate that the % recovery values are within the range which indicates that the method is accurate as it meets the necessary criteria.

Precision

Repeatability of the method was determined by analyzing five samples of same concentrations of drug i.e. 15 µg/ml for atazanavir and 5 µg/ml Chromatographs were recorded and area of each chromatograph was measured and the values are represented in the Table No.5.

Acceptance criteria

A method is said to be precise if the % RSD is < 2 %, the results show % RSD for repeatability studies was 1.94 for atazanavir and 1.535 for ritonavir. This indicates the results meet the acceptance criteria and hence the method is said to be precise.

Intraday precision

The intra-day precision of the assay method was evaluated by carrying out 9 independent assays of a test sample at three concentrations against a qualified reference standard. The % RSD of three obtained assay values at three different concentration levels was calculated.

Interday precision

The inter-day precision study was performed at three different concentration levels and each value is the average of three determinations (n=3). Record the chromatograms and measure the peak response. The results were reported as % RSD.

Acceptance criteria

A method is said to be precise if the % RSD is < 2 %, the results show % RSD for the intraday and interday were within the limits which and hence the method is said to be precise.

Robustness

Robustness studies were carried out by changing one factor at a time to study the effect. Variation of wavelength by 2nm (248nm, 267nm and 252nm) and mobile phase flow rate by 0.2ml (0.8ml, 1ml and 1.2ml/min) has no significant effect on the retention time and chromatographic response of the 20 µg/ml solution, indicating that the method was robust.

Acceptance criteria

A method is said to be robust if the % RSD is <2. The above results show that the % RSD for varied wavelength and varied flow rates were found to be 1.098 and 0.845 for atazanavir and for ritonavir 1.190 and 1.999 were respectively. This means that the method is robust as it meets the acceptance criteria.

Ruggedness

To determine ruggedness, two different analysts performed assay on marketed tablets of the drug in similar operational and environmental conditions using developed method.

Procedure

Standard solution of mixture of atazanavir and ritonavir were analyzed by analyst 1 and analyst 2 at similar operational and environmental conditions using developed method.

Acceptance criteria

A method is said to be robust if the %RSD values is <2%. The results indicate that the %RSD values for different analysts were found to be in the range of atazanvir0.709 and for ritonavir 1.543 were respectively observed. This indicates that they meet the acceptance criteria.

Application to pharmaceutical dosage forms

Assay studies were carried out by weighing twenty tablets of atazanavir and ritonavir tablets and are powdered. The powder equivalent to 30 mg and 10mg was taken and the solution equivalent to 1000µg/ml was prepared and was used for further dilutions. The results of the assay are shown in Table No.11.

Acceptance criteria

A method should have the % purity in the range of 98-102%. The results show that the % purity meets the acceptance criteria.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The Limit of Quantification (LOQ) and Limit of Detection (LOD) were based on the residual standard deviation of the response and the slope of the constructed calibration curve (n=3), as described in International Conference on Harmonization guidelines Q2 (R1).

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where,

σ = the standard deviation of the response and

S = slope of the calibration curve.

Optimum conditions, optical characteristics and Statistical data of the Regression equation in HPLC method

The optical characteristics such as Beer's law limits molar absorptivity, LOD and LOQ in each method were calculate and the results are displayed in Table No.13 also the regression equation like slope (b), intercept (a) and correlation, coefficient (R^2) using the method of least squares were calculated and are presented. The results show that the methods are reasonably precise.

Table No.1: System Suitability Parameters

S.No	Drug name	Retention time	Resolution	Theoretical plates (N)	Tailing factor
1	Atazanavir	2.497	1.8	3984	1.6
2	Ritonavir	2.953	1.4	3825	1.8

Table No.2: Linearity for Atazanavir at 250nm

S.No	Concentration (µg/ml)	Peak area
1	3	893888
2	6	1787776
3	9	2519710
4	12	3575552
5	15	4075305
6	30	8271276
7	45	17877513
8	60	23395026
9	90	24386292
10	120	33812451
11	150	41337196

Table No.3: Linearity Studies of Ritonavir

S.No	Concentration (µg/ml)	Peak area
1	1	210520
2	2	536875
3	3	1073751
4	4	1073850
5	5	1091256
6	10	2182492
7	15	3055771
8	20	4251387
9	30	6356650
10	40	8502775
11	50	10786730

Table No.4: Accuracy Results for Atazanavir (250nm) and Ritonavir (239.4nm)

S.No	Drug name	Spiked level (%)	Formulation Conc. (µg/ml)	Pure Drug Conc. (µg/ml)	Amount found	% Recovery	% Mean recovery ± SD	% RSD
1	Atazanavir	50	30	15	44.54	99.8	99.6±0.40	0.404
		100	30	30	59.50	99.16	99.1±0.568	0.566
		150	30	45	59.90	99.86	99.6±0.305	0.304
2	Ritonavir	50	10	5	14.75	98.50	98.5±0.475	0.481
		100	10	10	19.44	99.60	99.1±1.409	1.422
		150	10	15	24.78	98.80	98.8±0.056	0.056

Table No.5: Repeatability Results of Atazanavir

S.No	Concentration (µg/ml)	Peak area (250nm)	Area mean ±S.D (n=6)	% RSD
1	15	3733331	38267±74582	1.948
2	15	3733331		
3	15	3826036		
4	15	3817327		
5	15	3962625		
6	15	3962824		

Table No.6: Repeatability Results of Ritonavir

S.No	Concentration (µg/ml)	Peak area (250nm)	Area mean ±S.D (n=6)	% RSD
1	5	942366	969834±14889	1.535
2	5	968326		
3	5	971020		
4	5	983574		
5	5	971254		
6	5	967452		

Table No.7: Intraday Precision studies of Atazanavir and Ritonavir

S.No	Drug Name	Conc. (µg/ml)	Peak Area			Area mean ±S.D (n=3)	% RSD
			1	2	3		
1	Atazanavir sulfate	15	4055232	4024650	4175246	4065118±142828	0.351
2	Ritonavir	5	942366	971243	971020	971989±155501	1.59

Table No.8: Interday Precision Studies of Atazanavir and Ritonavir

S.No	Drug name	Conc (µg/ml)	Peak Area			Area mean ± S.D (n=3)	% RSD
			1	2	3		
1	Atazanavir sulfate	15	4125654	4076307	4056231	4074727±288965	0.709
2	Ritonavir.	5	1054781	1073641	1045674	1050069±16206	1.543

Table No.9: Results for Change in Flow Rate and Wave Length for Atazanavir and Ritonavir

S.No	Parameter	Atazanavir RT	Atazanavir Area mean ± S.D (n=3)	Atazanavir % RSD	Ritonavir RT	Ritonavir Area mean ± S.D (n=3)	Ritonavir % RSD
1	Flowrate 0.8ml/min	0.846	8271065±695227	0.845	1.090	2451245±286866	1.190
2	Flowrate 1.2ml/min	1.092	6421635±706121	1.098	1.356	2085461±280148	1.356
3	Wavelength 248nm	0.907	7875395±707185	0.903	1.999	2471254±494974	1.999
4	Wavelength 252nm	0.855	8484905±721277	0.855	1.236	2412546±273643	1.236

Table No.10: Ruggedness Results for Different Instruments

S.No	Drug name	Parameter	Concentration (µg/ml)	Peak Area	Area mean ± S.D (n=3)	% RSD
1	Atazanavir	Analyst 1	15	4125654	4039567±28896	0.709165
2	Ritonavir	Analyst 2	5	1054781	1024561±16206	1.543393

Table No.11: Assay results of Atazanavir and Ritonvir

S.No	Drug name	Formulation	Label claim	Amount found	Mean % purity± S.D (n=3)	% RSD	% Purity
1	Atazanavir	SYNTHAVIN (Tablets30+10mg)	30mg	29.24	98.45±0.03	0.038	98.45
2	Ritonavir		10mg	9.83	99.23±0.913	0.920	99.23%

Table No.12: Determination of LOD and LOQ Results for Atazanavir and ritonavir at 250nm

S.No	Parameter	Atazanavir at 250nm	Ritonavir at 239.4
1	Limit of detection	1.717	0.646
2	Limit of quantification	2.15	0.90

Table No.13: Summary of HPLC validation parameters

S.No	Parameter	Atazanavir	Ritonavir
1	Linearity range (µg/ml)	3-150	1-50
2	Regression equation (y= mx + c)	y = 27737x - 14395	y = 21362x - 3629
3	Slope	27737	21362
4	Intercept	14395	3629
5	Correlation coefficient (r^2)	0.999	0.999
6	Accuracy	98.20-100.03	99.73-99.98
7	Precision (%RSD)	0.3513	1.599
8	Limit of detection	1.717	0.646
9	Limit of quantification	2.150	0.90

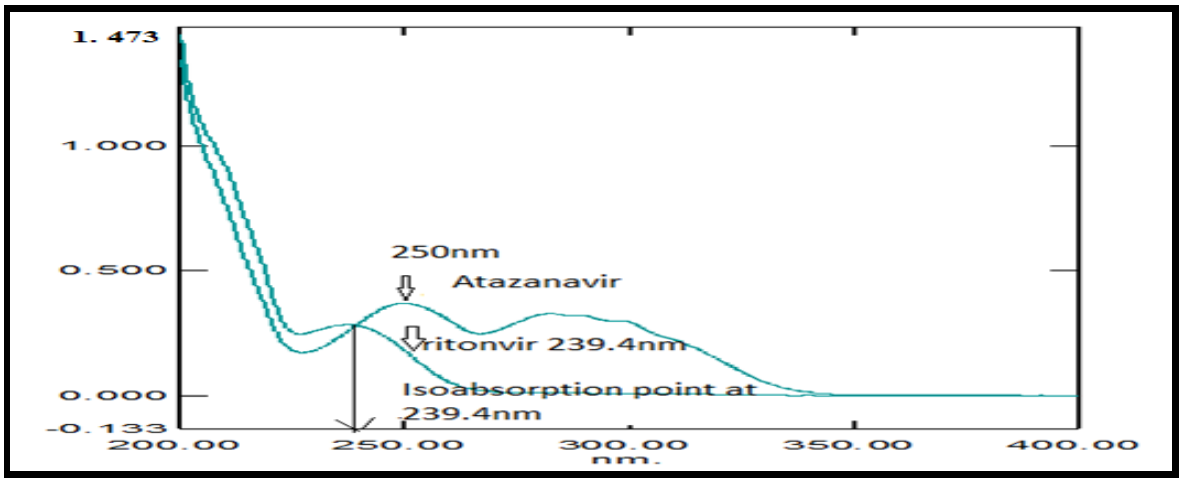

Figure No.1: Overly spectra of Atazanavir and Ritonavir in acetonitrile

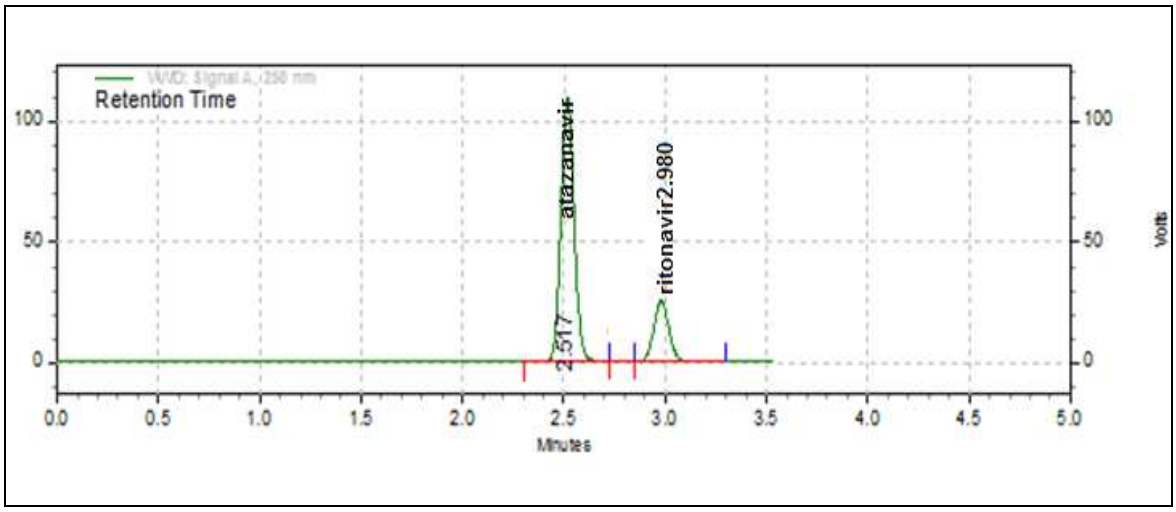

Figure No.2: Typical Chromatogram for Atazanavir and Ritinovir Standard

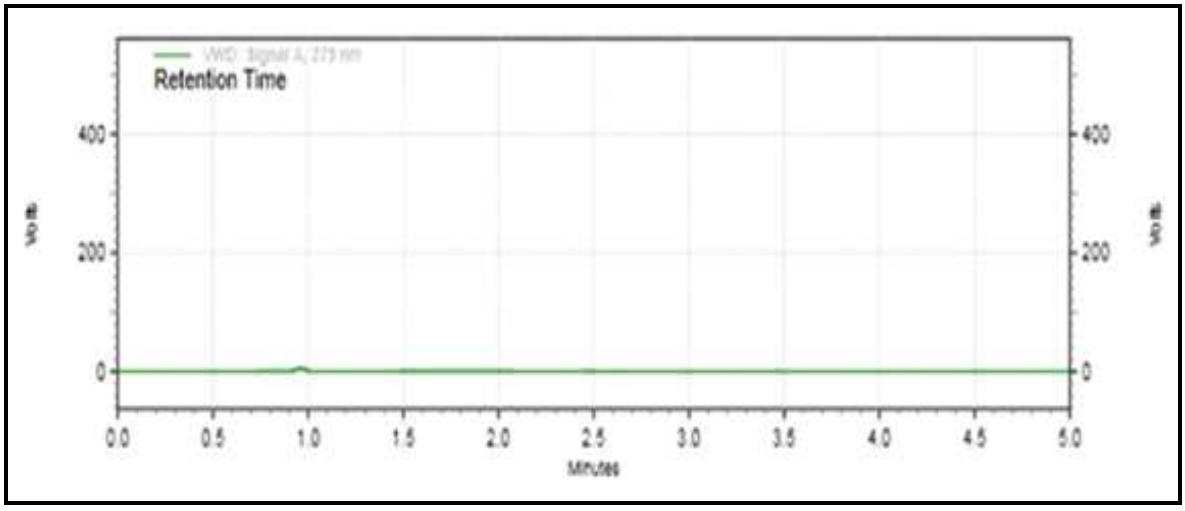

Figure No.3: Chromatogram of specificity

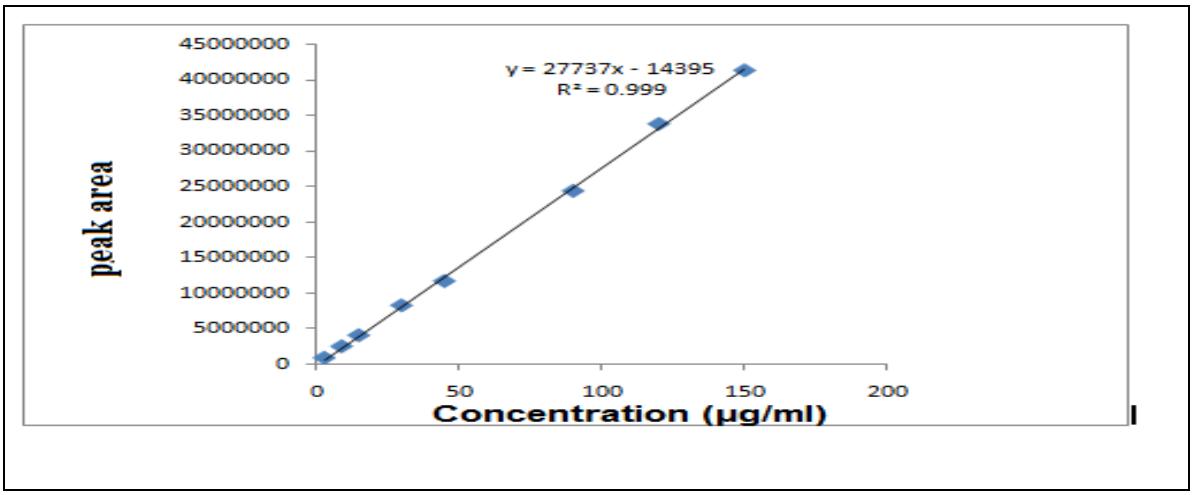

Figure No.4: Calibration curve for Atazanavir at 250nm

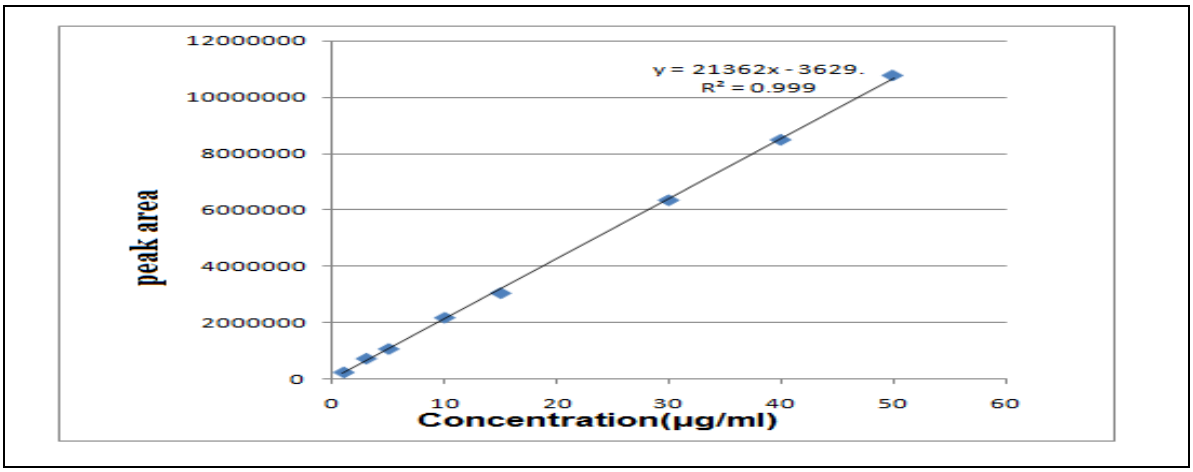

Figure No.5: Calibration Curve for Ritonavir at 250nm

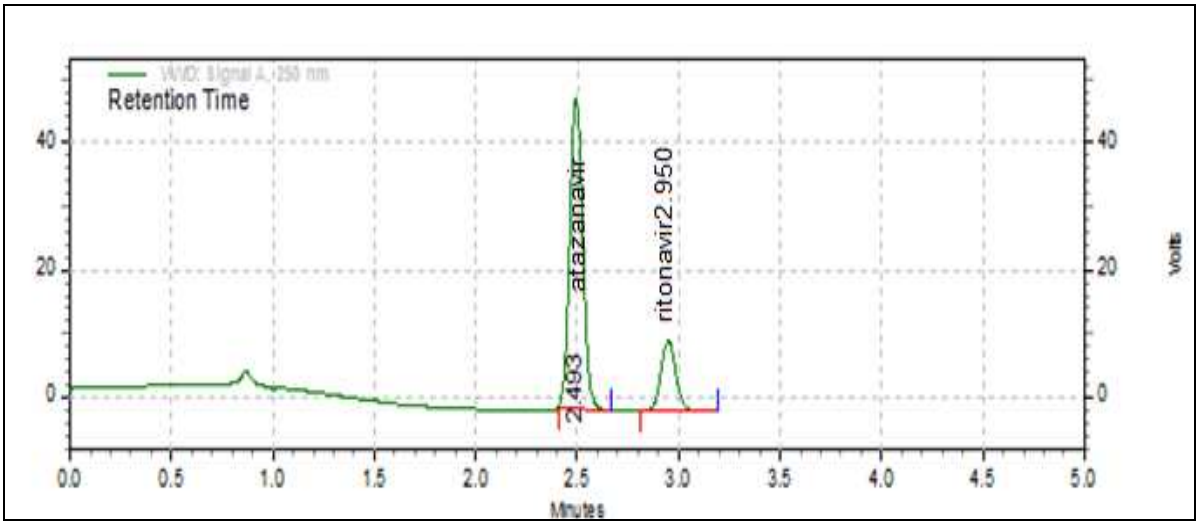

Figure No.6: Sample Chromatogram of Atazanavir and Ritinovir (300µg/ml)

CONCLUSION

A new simple, sensitive, accurate, reproducible and precise HPTLC method for determination of RTV and ATV in combination tablets has been developed and validated. Statistical analysis proves that the method is suitable for analysis of RTV and ATV in pharmaceutical formulation without any interference from excipients. The proposed HPTLC method is less expensive, simple, rapid, and more flexible than HPLC.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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