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A Validated RP-HPLC Method for the Simultaneous Estimation of Atazanavir and Ritonavir in Pharmaceutical Dosage Forms

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Abstract: *A rapid, precise and accurate reverse phase high performance liquid chromatographic method have been developed for the validated of Atazanavir and Ritonavir, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Phenomenex Gemini C18 (4.6 x 150mm, 5µm) column using a mixture of Methanol: Water (90:10% v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 249nm. The retention time of the Ritonavir and Atazanavir was 2.256, 5.427 ±0.02min respectively. The method produce linearity responses in the range of 5-25mg/ml of Ritonavir and 15-75mg/ml of Atazanavir. The method precision for the determination of assay was below 2.0%RSD. The method is useful for the quality and quality control of bulk and pharmaceutical formulations.*

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

1. INTRODUCTION

Atazanavir Sulphate Methyl is a Antiretroviral drug N- [(1S)-1-{ [(2S,3S) - 3 - hydroxy-4- [(2S)-2-[(methoxycarbonyl) amino] - 3, 3 – dimethyl - N' - {[4-(pyridin-2-yl)phenyl]methyl} butanehydrazido]-1- phenylbutan-2-yl] carbamoyl}-2, 2 - dimethylpropyl] carbamate sulphate is a azapeptide HIV-1 protease inhibitor The compound selectively inhibits the virusspecific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells, thus preventing formation of mature virions. Ritonavir is a Antiretroviral drug 1,3-thiazol- 5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2 {[methyl({[2-(propan-2-yl)-1,3-tiazole-4- yl]methyl})carbamoyl]amino} butanamido]-1,6-diphenylhexan-2-yl] carbamate. Ritonavir inhibits the HIV viral protease enzyme. This prevents cleavage of the gag-pol polyprotein and, therefore, improper viral assembly results. This subsequently results in noninfectious, immature viral particles. Literature survey revealed that very few methods have been reported for the analysis of Atazanavir and Ritonavir combinational dosage forms which include UV spectroscopy, Reverse Phase High performance Liquid Chromatography, Densitometric method, HPTLC methods

2. EXPERIMENTAL

Reagents and Chemicals

Ritonavir API and atanavir API were obtained as gift sample from sura labs. Acetonitrile,Water, methanol is used of HPLC grade and purchased

Instrumentation

Chromatographic separation was performed on a Waters HPLC with auto sampler and PDA Detector 996.variablewavelength programmable UV/VIS detector, Phenomenex Gemini C18 (4.6×150mm, 5µ) with10µl fixed loop.

Chromatographic conditions

Phenomenex Gemini C18 (4.6×150mm, 5µ) were the column used for separation. Mobile phase

Containing a mixture of Methanol: Water (90:10% v/v) in 1000 ml of water and pH was adjusted to 4.2 in the ratio 50:50 v/v was delivered at a flow rate of 1.0 ml/min with detection at 249nm. The mobile phase is filtered through a 0.45 nylon filter and sonicated for 20 min.

Method development

Acetonitrile, methanol and water in different proportions were tried and finally Methanol: Water = 90:10v/v was selected appropriate mobile phase which gave good resolution, retention time and acceptable system suitability parameters.

Procedure

Preparation of standard solution

Accurately weighed and transfer 10 mg of Ritonavir and Atazanavir working standard into a 10ml of dry volumetric flasks add about 7ml of Methanol and 3ml of water sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol and water.

Further pipette 0.15ml of the above Ritonavir and 0.45ml of Atazanavir stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples to the Rp-Hplc by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution and retention for performing validation parameters as per ICH guidelines

Linearity

Accurately measured volumes of working standard solution of Ritonavir and Atazanavir was transferred into a series of 10ml volumetricflasks and diluted appropriately with mobilephase. 10µl of each solution was injected under chromatographic conditions described above. Calibration curves were obtained by plotting the response (area of drug peak) versus concentration of drug. Regression were calculated. The method was found linear over a concentration range of 5-25 µg/mL for **Ritonavir** and 15-75 µg/mL for **Atazanavir** respectively.

Procedure for analysis of tablets

15 tablets were weighed and powdered. Accurately weighed portion of this powder equivalent to 10 mg of **Ritonavir** and 10 mg of **Atazanavir** was transferred to a 100 ml volumetric flaskcontaining 80 ml of mobile phase. The contents of the flask were allowed to stand for15 minutes with sonication toensure that to complete solubility of the drugs and make up the volume with mobile phase. The above solution was filtered through 0.45µmnylon filter. From this solution appropriate dilutions were made with mobile phase to obtain concentration in calibration range for both the drugs and this solution was used for estimation.With the optimized conditions , a steady baseline was recorded, the mixed working standard solution were injected and the chromatogram was record. The retention times of Ritonavir and Atazanavir were found. The proposed method was found to be specific and no interference from common tablet excipients like starch etc. The response factors of the standard solutions and sample solutions were calculated. The assay was calculated from the equation of regression line for each drug. The assay procedure was repeated for 6 times and the percentage of individual drug in the formulation was calculated. The results of analysis shows that the amount of drug was in good

3. METHOD VALIDATION

SYSTEM SUITABILITY

Accurately weigh and transfer 10 mg of Ritonavir and 10mg of Atazanavir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15 ml of Ritonavir and 0.45ml of Atazanavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

SPECIFICITY STUDY OF DRUG:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Ritonavir and 10mg of Atazanavir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15 ml of Ritonavir and 0.45ml of Atazanavir from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution:

Take average weight of the Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Ritonavir and Atazanavir sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.15 ml of Ritonavir and 0.45ml Atazanavir above stock solution into a 10ml volumetric flask and dilute up to the mark with diluen

Linearity

The method was linear in the range of150-450µg/mL and 37.5-112.5 µg/mL for ATZ and RIT respectively. Linear regression data was given

Precision

The precision of the method was demonstrated by inter day and intraday studies. In the intraday studies, solutions of standard and sample were repeated 3times in a day and percent relative standard deviation (%RSD) was calculated. The intraday %RSD of Ritonavir and Atazanavir were foundto be 0.54 and 0.8 respectively.In the interday variation studies, injections of standard and sample solutions were made on two days and %RSD was calculated. The interday %RSD for Ritonavir and Atazanavir were found to be 0.5 to0.63 respectively. From the data obtained the developed RP-HPLC method was found to be precise.

Accuracy

The accuracy of the method was determined by recovery experiments. A known amount of concentration of working standard was added to the fixed concentration of the pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of working standard. Forboth the drugs, recovery was performed in the same way. The recovery studies were performed in 3 times This standard addition method was performed at 50%, 100%, 150%level and the percentage recovery wascalculated. Percent recovery was within the range of 98.2 to 99.6 for Ritonavir and 98.2 to99.6 for Atazanavir that indicates method was accurate.

Limit of detection and limit ofquantification

The Limit of detection and quantification were calculated using standard deviation of the response and slope of calibration curve. The LOD for Ritonavir and Atazanavir was found to be 0.54µg/ml and 1.4 µg/mlrespectively. The LOQ is the smallest concentration of the analyte, which gives response that can be accurate. The LOQ was 1.6 µg/ml and 4.4µg/ml for Ritonavir and Atazanavir respectively.

Robustness

Robustness of the method was checked by making slight changes in chromatographic conditions like mobile phase ratio, pH of buffer, flow rate. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed RP-HPLC method is robust.

4. RESULTS AND DISCUSSION

The proposed method was found to be linear in the concentration range of 5-25µg/mland 15-75 µg/ml for Ritonavir and Atazanavir. The method was specific since excipients in the formulation did not interferein the estimation of Ritonavir and Atazanavir. Accuracy of the method was indicated by recovery values from 98.2 to 99.6 for Ritonavir and 98.2 to99.6 for Atazanavir. Precision is reflectedby %RSD values less than 2. The LOD for Ritonavir and Atazanavir was found to be

0.54µg/ml and 1.4 µg/mlrespectively LOQ was 1.6 µg/ml and 4.4µg/ml for Ritonavir and Atazanavir respectively .Validation parameters were summarized

System suitability:

Table. *Results of system suitability for Ritonavir*

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Ritonavir	2.247	136092	14051	5506	1.36
2	Ritonavir	2.246	135626	14025	5674	1.2
3	Ritonavir	2.248	135557	14132	5298	1.2
4	Ritonavir	2.252	136141	14306	5032	1.0
5	Ritonavir	2.248	136557	14152	5812	1.33
Mean			135994.6			
Std. Dev			410.662			
% RSD			0.3			

Table. *Results of system suitability for Atazanavir*

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Atazanavir	5.452	636065	39373	5146	1.04	4.0
2	Atazanavir	5.484	633325	39429	5024	1.20	4.5
3	Atazanavir	5.491	633435	39403	5167	1.2	4.3
4	Atazanavir	5.482	625113	39745	5076	1.1	4.1
5	Atazanavir	5.491	633435	39403	5327	1.2	4.2
Mean			632274.6				
Std. Dev			4166.895				
% RSD			0.6				

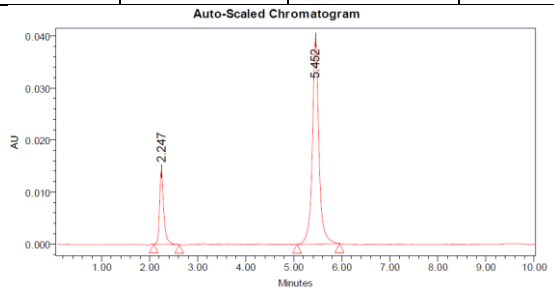

Fig: *Chromatogram showing injection -1*

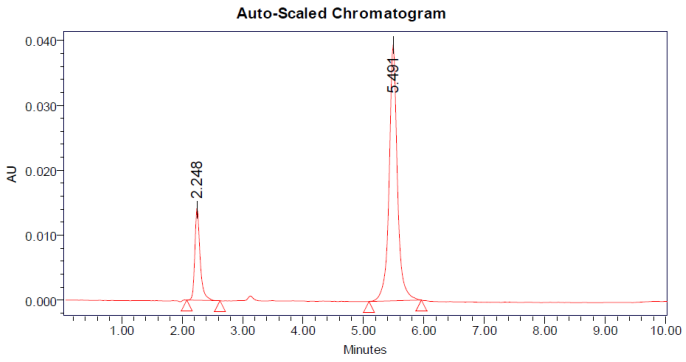

Fig: *Chromatogram showing injection -5*

SPECIFICITY:

Table. *Peak results for assay standard*

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Ritonavir	2.256	134994	13905		1.32	7535	1
2	Atazanavir	5.427	627906	39948	4.27	1.03	5101	1

3	Ritonavir	2.249	136394	14163		1.38	7701	2
4	Atazanavir	5.430	636779	39935	4.13	1.05	5360	2
5	Ritonavir	2.248	135870	14082		1.40	7684	3
6	Atazanavir	5.443	635760	39609	4.19	1.05	5228	3

Assay (Sample):

Table. *Peak results for Assay sample*

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Ritonavir	2.247	136092	36065		1.26	7251	1
2	Atazanavir	5.452	636779	37984	4.42	1.28	5023	1
3	Ritonavir	2.246	136052	33061		1.22	7605	2
4	Atazanavir	5.461	614678	39373	4.42	1.19	5146	2
5	Ritonavir	2.243	134182	39537		1.28	7228	3
6	Atazanavir	5.466	635423	39457	4.48	1.20	5247	3

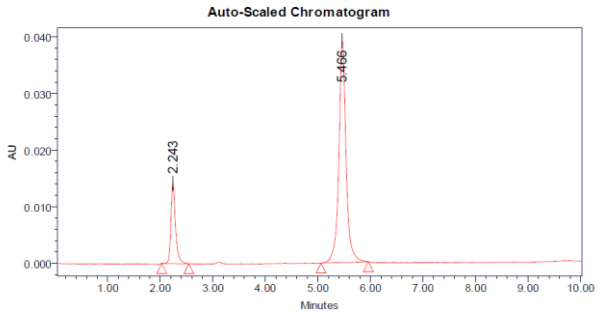

Fig: *Chromatogram showing assay of sample injection-3*

Assay (Standard):

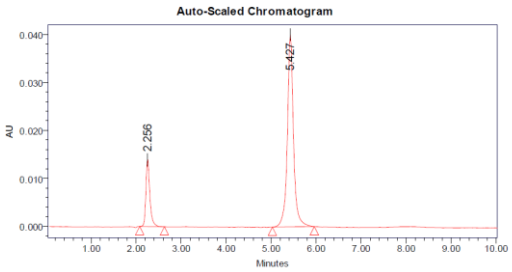

Fig: *Chromatogram showing assay of standard injection -1*

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

= 628960/633481.7×10/45×45/0.0603×99.7/100×2.4122/400×100

= 98.9%

The % purity of Ritonavir and Atazanavir in pharmaceutical dosage form was found to be 98.9%.

LINEARITY:

Ritonavir:

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33.3	5	51080
66.6	10	92208
100	15	139140
133.3	20	180998
166.6	25	223920

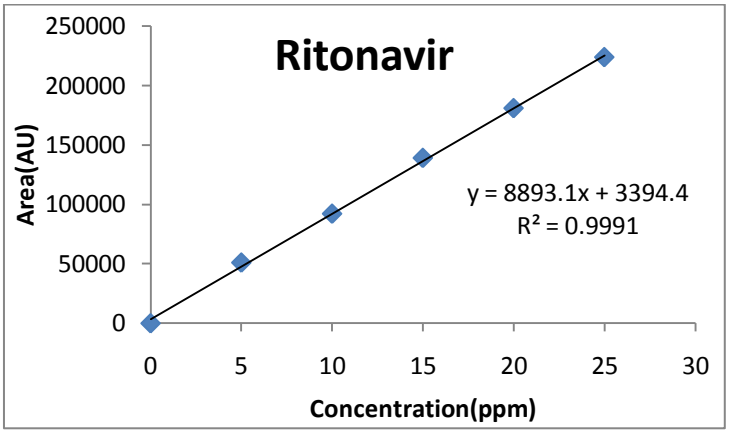

Figure. *Calibration graph for Ritonavir*

Atazanavir

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33	10	224573
66	20	441895
100	30	635379
133	40	842226
166	50	1041381

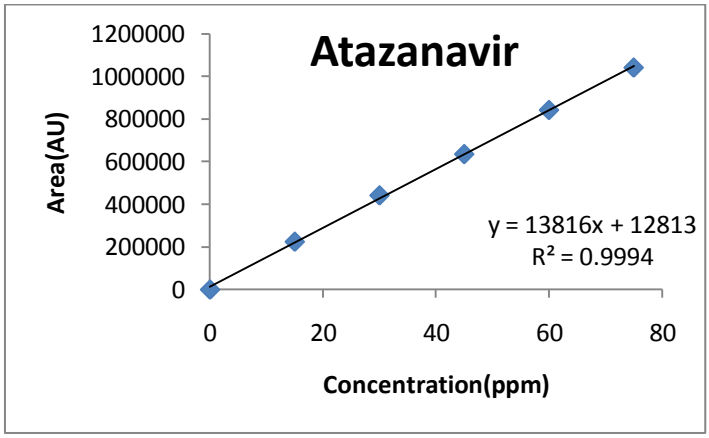

Figure. *Calibration graph for Atazanavir*

Precision:

Table. *Results of repeatability for Ritonavir*

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Ritonavir	2.269	135148	13802	7405.7	1.2
2	Ritonavir	2.255	135369	13826	7338.4	1.2
3	Ritonavir	2.252	135451	13797	7474.5	1.19
4	Ritonavir	2.267	135812	13858	7422.2	1.18
5	Ritonavir	2.260	137007	14018	7326.6	1.21
Mean			135757.4			
Std. Dev			738.3402			
% RSD			0.54			

Table. *Results of method precession for Atazanavir*

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Atazanavir	5.274	630076	40629	5075.5	1.1	4.4

2	Atazanavir	5.266	630126	40937	5120.4	1.1	4.6
3	Atazanavir	5.265	632484	41279	5212.4	1.1	4.3
4	Atazanavir	5.278	636524	41454	5883.0	1.1	4.3
5	Atazanavir	5.305	621812	41320	5041.5	1.1	4.3
Avg			630204.4				
Std. Dev			5375.615				
% RSD			0.8				

Intermediate precision:

Table. *Results of Intermediate precision for Ritonavir*

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Ritonavir	2.248	134029	13603	7519.3	1.2
2	Ritonavir	2.245	134202	13520	7372.9	1.2
3	Ritonavir	2.242	134745	13636	7411.8	1.19
4	Ritonavir	2.239	135442	13775	7323.5	1.20
5	Ritonavir	2.243	135535	13768	7433.4	1.23
6	Ritonavir	2.246	135699	13739	7336.9	1.3
Mean			134942			
Std. Dev			720.3716			
% RSD			0.5			

Table. *Results of Intermediate precision for Atazanavir*

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Atazanavir	5.284	636831	40102	5180.2	1.1	4.8
2	Atazanavir	5.293	638856	40464	5155.6	1.1	4.5
3	Atazanavir	5.306	630174	39977	5039.6	1.0	4.5
4	Atazanavir	5.319	630603	40748	5119.3	1.1	4.8
5	Atazanavir	5.346	632578	39772	5183.9	1.1	4.6
6	Atazanavir	5.352	636550	40083	5009.1	1.1	4.9
Mean			634265.3				
Std. Dev			3629.748				
% RSD			0.57				

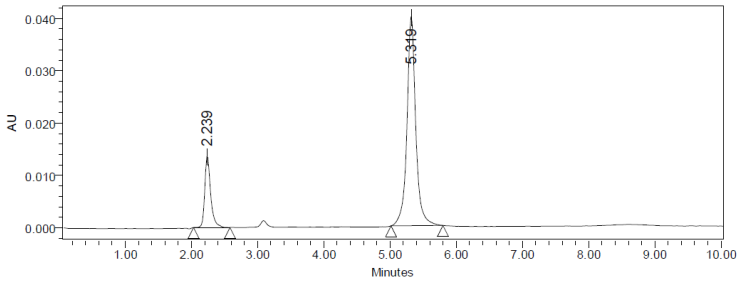

Fig. *Chromatogram showing Day1 injection -4*

Table. *Results of Intermediate precision Day 2 for Ritonavir*

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Ritonavir	2.255	135442	40102	7180.2	1.2
2	Ritonavir	2.260	135535	40464	7155.6	1.20
3	Ritonavir	2.242	135699	39977	7039.6	1.1
4	Ritonavir	2.245	134657	40748	7119.3	1.2
5	Ritonavir	2.260	136754	39772	7183.9	1.24
6	Ritonavir	2.255	135908	40083	7009.1	1.3
Mean			135665.8			
Std. Dev			682.4683			
% RSD			0.5			

Table. *Results of Intermediate precision for Atazanavir*

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Atazanavir	5.266	638856	39977	5039.6	1.0	4.5
2	Atazanavir	5.265	630174	40748	5119.3	1.1	4.8
3	Atazanavir	5.306	630603	39772	5183.9	1.1	4.6
4	Atazanavir	5.293	639542	40083	5009.1	1.1	4.9
5	Atazanavir	5.265	631265	56430	5023.8	1.2	4.1
6	Atazanavir	5.266	638531	47652	9123.1	1.0	4.3
Mean			634828.5				
Std. Dev			4568.678				
% RSD			0.7				

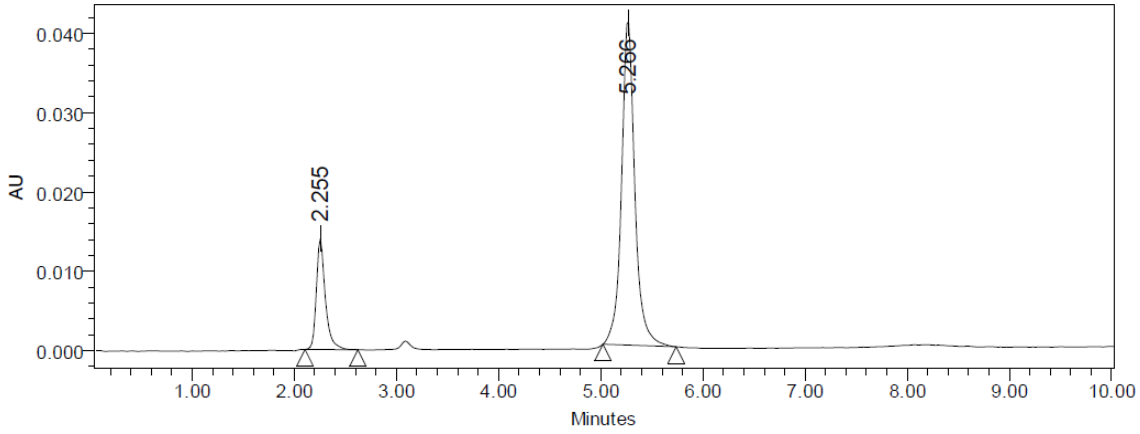

Fig. Chromatogram showing Day 2 injection -6

Accuracy:

Table. *Results of Accuracy for concentration-50%*

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Ritonavir	2.251	69955	7522		1.30	7018	1
2	Atazanavir	5.466	329816	20373	4.24	1.02	5647	1
3	Ritonavir	2.251	69648	7490		1.30	7303	2
4	Atazanavir	5.447	319997	20741	4.0	1.05	5001	2
5	Ritonavir	2.252	69984	7553		1.33	7000	3
6	Atazanavir	5.425	319049	20762	4.92	1.02	5678	3

Table. *Results of Accuracy for concentration-100%*

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Ritonavir	2.261	135064	13901		1.34	7531	1
2	Atazanavir	5.416	638215	40860	4.84	1.06	5978	1
3	Ritonavir	2.261	136160	14268		1.34	7489	2
4	Atazanavir	5.395	624542	40973	4.8	1.06	5029	2
5	Ritonavir	2.267	135179	13716		1.35	7366	3
6	Atazanavir	5.382	633708	40591	4.87	1.07	5934	3

Table. *Results of Accuracy for concentration-150%*

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Ritonavir	2.271	199987	18953		1.40	7833	1
2	Atazanavir	5.368	581475	59177	4.69	1.10	5151	1

3	Ritonavir	2.272	199992	19231		1.39	7907	2
4	Atazanavir	5.354	582305	59272	4.62	1.11	5206	2
5	Ritonavir	2.273	199949	19127		1.39	7901	3
6	Atazanavir	5.339	579831	59532	4.48	1.10	5143	3

The accuracy results for Ritonavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	69862.33	7.5	7.47	99.6	98.8%
100%	135467.7	15	14.8	98.6	
150%	199976	22.5	22.1	98.2	

The accuracy results for Ritonavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	69862.33	7.5	7.47	99.6	98.8%
100%	135467.7	15	14.8	98.6	
150%	199976	22.5	22.1	98.2	

The accuracy results for Atazanavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	322954	22.5	22.47	99.3	99.5%
100%	632155	45	44.8	99.3	
150%	945870.3	67.5	67.49	100	

Limit of Detection

Ritonavir:

=3.3 × 1476.577/8893

=0.54µg/ml

Atazanavir:

=3.3 × 6116.702/13816

=1.4µg/ml

Limit of Quantitation

Ritonavir:

=10×1476.577/8893

= 1.6µg/ml

Atazanavir:

=10 × 6116.702/13816

= 4.4µg/ml

Robustness:

Table. *Results for Robustness*

Ritonavir:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	134994	2.256	7535	1.32
Less Flow rate of 0.9 mL/min	159987	2.505	7891	1.27
More Flow rate of 1.1 mL/min	120653	2.046	7085	1.20
Less organic phase	149987	2.505	7098	1.20
More organic phase	120654	2.046	7123	1.27

Atazanavir:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	637906	5.427	5101	1.01
Less Flow rate of 0.9 mL/min	657680	5.599	5407	1.03
More Flow rate of 1.1 mL/min	607899	4.576	5584	0.98
Less organic phase	646750	5.599	5407	1.02
More organic phase	609025	4.576	5584	0.99

5. CONCLUSION

- In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Atazanavir and Ritonavir in bulk drug and pharmaceutical dosage forms.
- This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps.
- Atazanavir and Ritonavir was freely soluble in ethanol, methanol and sparingly soluble in water.
- Methanol: Water (90:10% v/v) was chosen as the mobile phase. The solvent system used in this method was economical.
- The %RSD values were within 2 and the method was found to be precise.
- The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.

This method can be used for the routine determination of Atazanavir and Ritonavir in bulk drug and in Pharmaceutical dosage forms.

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