Development, Validation and Statistical Correlation of RP–LC Methods for Determination of Atazanavir Sulfate in Capsule Dosage Form

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Abstract: To study the effective therapeutic bioavailability of Atazanavir Sulfate (ATV), administered singly or in combination with Ritonavir, a cost effective and rapid method is required. In order to assess an in-depth study, it is primarily thought prudent to develop an effective analytical method for estimation of ATV in marketed dosage forms. The present work is to develop a simple and precise analytical method for in depth evaluation of therapeutic efficacy of ATV. The novelty of the method shows linearity in the concentration range of 10-100µg/mL at two wavelengths, i.e. 254nm and 284nm respectively. The chromatographic system consists of HiQSil C18HS column; an isocratic mobile phase consisted of methanol and tetrahydrofuran (95:5 v/v). The developed method is validated according to ICH guidelines in capsule dosage form. Validation of the developed method shows good result in range, linearity, accuracy and precision. Student's *t*-test was used to correlate the two methods and applied to raw materials and capsule dosage form.

Key words: Atazanavir Sulfate; Capsule dosage form; Dual wavelength; Method Validation; RP – LC method; Statistical analysis.

Introduction

Atazanavir (ATV) is the 7th Protease Inhibitor used in the treatment of HIV Type II infection (Fig 1)¹. It is an azapeptide having chemical formula (3S,8S,9S,12S) – 3,12 – Bis (1,1 – dimethylethyl) – 8 – hydroxyl – 4,11 – dioxo – 9 – (phenylmethyl) – 6 – [[4 – (2 – pyridinyl)phenyl]methyl] – 2,5,6,10,13 pentaazatetra decanedioic acid dimethyl ester, sulphate. In some literature ATV is reported as poorly water soluble and is known substrate of both hepatic metabolizing enzyme Cytochrome 450 (CYP3A) and intestinal drug efflux pump, P- glycoprotein (Pgp), so having low oral bioavailability². So to avoid this problem low dose of Ritonavir (RTV) is administered as booster. So clinically low dose of RTV has been used to increase the bioavailability of other PI³⁻⁵. It is also reported RTV is not only a potent inhibitor, but also a potent inducer of CYP3A and Pgp with chronic use^{6, 7}.

The contradictory reports regarding ATV inspired to develop an analytical method for an in – depth study of therapeutic bioavailability of ATV administered singly or in combination in in-vitro condition. ATV has been determined in blood serum⁸, blood plasma⁹⁻¹¹, CSF¹² and biological cells^{12,13}. No method is reported for pharmaceutical dosage form. So a simple, rapid, cost effective and extremely precise analytical method is developed to estimate much costlier 2nd line drug (ATV) for treatment of HIV Type II infection. The developed method follows the linearity at two wavelengths, i.e. 254nm and 284nm. The method is validated according to ICH guidelines¹⁴.

Fig 1: Chemical structure of Atazanavir Sulfate (ATV)

Experimental

Chemicals and Reagents

ATV (Atazanavir Sulfate) was obtained from Cipla Pvt. Ltd. (Mumbai, India). Methanol and Tetrahydrofuran for HPLC were procured from Merck (Mumbai, India). Commercially available capsule formulations are collected, Atavir 300 (Cipla) and Atazor 200 (Emcure).

Chromatographic System and Conditions

The chromatographic system consisted of a Jasco (Japan) chromatograph equipped with an LC – Net II/ADC, an MU – 2010 Plus PDA Detector, a PU – 2089 Plus quaternary pump, an online degasser and a Rheodyne model 7725 injector valve with 20µl sample loop. The chromatograph is coupled with “Chrompass” software. Separation of ATV was done on a HiQ Sil C18HS (250mm x 4.6mm, Particle size 5µm) under reverse phase partition chromatographic conditions. The isocratic mobile phase consisted of a mixture of Methanol and Tetrahydrofuran (95:5, v/v) and was filtered through 0.45µm nylon filter membrane before use. The flow rate was 1mL/min and the assay run time was 10mins. Absorbance was measured at 254nm and 284nm.

Preparation of Stock Solution, Working Solution and Standard Calibration Curve

A stock solution of 400µg/ml of ATV was prepared in Methanol. The stock solution was diluted further with methanol to obtain six working solutions with concentrations of 100-10µg/mL. The prepared samples were also filtered through 0.45µm nylon filter membrane before injection. The standard calibration curve was plotted by AUC Vs Concentration at 254nm and 284nm.

Sample Preparation

Commercially marketed sample of Atavir 300 (Cipla) and Atazor 200 (Emcure) were purchased. Twenty capsules were weighed carefully, equivalent amount of solid content was weighed accurately. Weighed amount was dissolved in methanol to prepare the sample solution of concentration 400 µg/mL. The sample was filtered through Whatman filter paper No 41, then through 0.45 µm nylon filter membrane before injection.

Method Validation

The developed methods were validated according to ICH guidelines¹⁴. The validation parameters were linearity, specificity, accuracy, precision, Limit of detection (LOD), Limit of Quantification (LOQ) and Robustness at both the wavelengths (Method A and B). Intra-day and Inter-day precision values were estimated by assaying the pharmaceutical dosage form containing three different concentrations of ATV six times on the same day and on three different days. Accuracy was determined by recovery study by standard addition method. The standard was added to a predetermined concentration at 25%, 50% and 100% level. The LOD and LOQ was determined by using equation (1) and (2) respectively

$$\text{LOD} = 3.3 \sigma / S \quad (1)$$

$$\text{LOQ} = 10 \sigma / S \quad (2)$$

where 'σ' is the standard deviation of y-intercept and 'S' is the slope of calibration curve.

Statistical Analysis

To correlate the difference between the two developed methods of HPLC, six different samples were taken from two different brands and quantification was done simultaneously. To test difference between the proposed HPLC methods statistical tests were performed for the level of confidence 95% ($P = 0.05$). Two way ANOVA and Student's *t* – test were applied to test the significant difference between both the methods.

Result and Discussion*Method Development*

To develop an efficient and reproducible method for assay of ATV in pharmaceutical dosage form many solvent system combinations were tried, like methanol, water, acetonitrile and tetrahydrofuran. Generally the retention time of PIs is dependent on pH of the solvent system¹⁵. To avoid the tedious preparation and maintenance of buffers, the pure organic solvents were chosen. So the solvent, methanol and tetrahydrofuran in the ratio 95:5 were selected, which is easy and cost-effective. Isocratic mode was preferred to gradient elution as it requires long re-equilibrium time, perfect mixing. The analysis was done at normal room temperature. Another major advantage of this method is that the analysis can be done at two different wavelengths with excellent accuracy and precision. The two wavelengths were 254nm and 284nm. At these both the wavelengths the chromatographic separation was very good, following very good linearity. The method was found to be accurate, precise and specific at both the wavelengths (Figure 2 and 3). Using these chromatographic conditions, the retention time of ATV at both the wavelengths was found to be 3.227 ± 0.014 minutes.

Fig 2: Representative Chromatogram of Standard ATV at 254nm

Fig 3: Representative Chromatogram of Standard ATV at 284nm

Calibration Curve and Linearity

ATV showed good correlation coefficient in concentration range of 10-100 μ g/mL in method A and B respectively. A six point calibration standard curve of ATV was plotted ranging from 10 μ g/mL-100 μ g/mL standard working solutions containing ATV in triplicate for method A and B (Table 1). For both the methods linearity of calibration graphs was validated by high value of correlation of coefficient and the S.D. for intercept value was less than 2%. No significant difference was observed in the slopes of standard curves.

Table 1. Linear regression data for calibration curves.

Parameters	Method A	Method B
Linearity range (μ g/mL)	10 -100	10-100
$r \pm$ S.D.	0.9997 ± 0.0003	0.9999 ± 0.0002
Slope $\pm$ S.D.	615.23 ± 1.18	726.45 ± 1.25
Intercept $\pm$ S.D.	425.05 ± 1.62	499.2 ± 1.78

Accuracy

Both the proposed methods when used for extraction and subsequent quantification of ATV in preanalyzed sample by standard addition method at the level of 25, 50 and 100%. In both the methods assay of each concentration were repeated for three times. The mean recoveries for ATV from the marketed formulation are listed in Table 2.

Table 2. Accuracy of the method.

Name of the formulation	Amount of sample taken (µg/mL)	Amount of standard added (µg/mL)	Amount found* (%)		Mean* ± S.D	
			Method A**	Method B [§]	Method A**	Method B [§]
Atavir 300	40	10	103.34	100.37	101.38± 1.75	99.63
	40	20	100.84	100.26		±
	40	40	99.97	98.269		1.18
	40	10	100.51	100.24	100.53± 0.22	100.22±
Atazor 200	40	20	100.32	100.20		0.023
	40	40	100.76	100.20		

*Mean of three determinations ** Method A at 254nm [§] Method B at 284nm.

Precision

The intra-day and inter-day precision were determined by assaying the tablets in triplicate for three different concentrations in a day and for consecutive six days and expressed as relative standard deviation. The relative standard deviations were below 2%, which signifies the precision of both the methods (Table 3).

Table 3. Precision of the method.

Name of the formulation	Intra-day precision (n =6)		Inter-day precision (n = 3)	
	Mean*(%) ± S.D		Mean*(%) ± S.D	
	Method A**	Method B [§]	Method A**	Method B [§]
Atavir	100.32±0.61	100.5±0.55	99.78±0.63	99.36±0.15
Atazor	100.22±0.72	100.50±0.55	102.79±0.63	101.09±0.84

*Mean of six determinations ** Method A at 254nm [§] Method B at 284nm.

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

The LOD and LOQ values were found to be 1.9µg/mL, 0.2475µg/mL and 5.7µg/mL, 0.75µg/mL for method A and B at 254nm and 284nm respectively.

Robustness

To evaluate method robustness few parameters were varied. The variation was done in composition of solvent system (± 2% of organic phase), flow rate (± 0.2 mL/min), wavelength (± 2 nm). Robustness was done in triplicate at a concentration level of

100µg/mL and 200µg/mL for ATV in method A and B respectively and the S.D of retention time, capacity factor and tailing factor were calculated (Table 4).

Specificity

To evaluate the specificity of the methods (A and B), two brands of ATV tablet were selected, injected and the effect of excipients were studied in respect to retention time, capacity factor, tailing factor and no. of theoretical plates. The method is very much specific as there is no interference of excipients (Figure 4, 5, 6, 7).

Fig 4: Representative Chromatogram of Atavir 300 at 254nm

Table 4. Robustness of method A and B.

Chromatographic changes		Method A			Method B		
Factors	levels	R_t^* (min)	K^*	T^*	R_t^* (min)	K^*	T^*
Methanol:	97:3	3.02	2.35	1.09	3.05	2.38	1.04
Tetrahydrofuran	95:5	3.227	2.58	1.01	3.227	2.58	1.05
(v/v)	93:7	3.78	3.2	0.97	3.82	3.24	0.98
Mean $\pm$ S.D. (n = 3)		3.34 $\pm$ 0.4	2.71 $\pm$ 0.44	1.02 $\pm$ 0.06	2.48 $\pm$ 0.4	2.73 $\pm$ 0.45	1.02 $\pm$ 0.04
Change in the	0.8	3.75	3.16	1.11	3.73	3.14	1.13
flow rate	1.0	3.227	2.58	1.01	3.227	2.58	1.05
(ml/min)	1.2	3.16	2.51	0.95	3.12	2.46	0.99
Mean $\pm$ S.D. (n = 3)		3.50 $\pm$ 0.32	2.75 $\pm$ 0.36	1.02 $\pm$ 0.08	3.36 $\pm$ 0.32	2.72 $\pm$ 0.36	1.05 $\pm$ 0.07
Wavelength (nm)	252	3.131	2.48	1.00	3.165	2.51	1.03
	254	3.227	2.58	1.01	3.227	2.58	1.05
	256	3.358	2.73	1.03	3.287	2.65	1.09
Mean $\pm$ S.D. (n = 3)		3.23 $\pm$ 0.11	2.59 $\pm$ 0.125	1.01 $\pm$ 0.02	3.22 $\pm$ 0.06	2.58 $\pm$ 0.07	1.05 $\pm$ 0.03

* R_t , K , T are retention time, capacity factor and tailing factor respectively.

Fig 5: Representative Chromatogram of Atavir 300 at 284nm

Fig 6: Representative Chromatogram of Atazor 200 at 254nm

Fig 7: Representative Chromatogram of Atazor 200 at 284nm

Statistical Correlation

To correlate the difference between the two developed methods of HPLC, six different samples were taken from two different brands and quantification was done simultaneously. To test difference between the proposed HPLC methods statistical tests were performed for the level of confidence 95% ($P = 0.05$). Two way ANOVA was applied to test both method – sample interaction and differences in method precision. In both the cases ***F stat* is less than *F crit***, signifying the method – sample interaction and the differences between the methods are not significant (Table 5).

Table 5. Two-way ANOVA test of ATV by determination in six independent samples in two different brands by Method A & B.

Sample	Method A* (%)		Method B* (%)	
	Atavir	Atazor	Atavir	Atazor
1	99.72	99.87	99.86	99.96
2	99.89	98.92	99.89	98.99
3	98.85	100.05	99.95	99.58
4	99.59	99.77	100.05	99.69
5	98.87	99.82	99.66	100.04
6	99.79	99.85	98.87	99.95

ANOVA - two way with replication

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0.0937	1	0.0937	0.521	0.4788	4.35124
Columns	0.0937	1	0.0937	0.521	0.4788	4.35124
Interaction	0.112	1	0.112	0.622	0.4393	4.35124
Within	3.599	20	0.179			
Total	3.8986	23				

* The results are presented as % of label claim of ATV in tablet.

To test means a paired student's t – test was applied. The test removes any variation between samples. From the student's t – test, $t_{\text{Stat}} < t_{\text{critical}}$ was found in both the cases signifying there is no significant difference between the means (Table 6).

Table 6. Average results of Atavir (a) and Atazor (b) determination by Method A and B and their correlation by paired t –test.

Sample	Method A (%)	Method B (%)
a) Atavir		
1	99.98	99.96
2	98.82	98.89
3	100.05	98.58
4	99.37	99.49
5	99.87	100.04
6	99.68	99.75
Average	99.63	99.45
t-Test: Paired Two Sample for Means		
	Variable 1	Variable 2
Mean	99.6283	99.4516
Variance	0.2165	0.354
Observations	6	6
Pearson Correlation	0.2982	
Hypothesized Mean Difference	0	
df	5	

t Stat	0.6796	
P(T<=t) one-tail	0.2634	
t Critical one-tail	2.015	
P(T<=t) two-tail	0.5269	
t Critical two-tail	2.5705	
Sample	Method A (%)	Method B (%)
b) Atazor		
1	99.67	99.82
2	99.78	100.12
3	100.04	99.43
4	99.89	99.45
5	99.56	99.86
6	100.09	100.06
Average	99.83	99.79
t-Test: Paired Two Sample for Means		
	<i>Variable 1</i>	<i>Variable 2</i>
Mean	99.8383	99.79
Variance	0.04317	0.0865
Observations	6	6
Pearson Correlation	-0.2159	
Hypothesized Mean Difference	0	
df	5	
t Stat	0.2996	
P(T<=t) one-tail	0.3882	
t Critical one-tail	2.015	
P(T<=t) two-tail	0.7765	
t Critical two-tail	2.5705	

t Stat < t critical

* The results are presented as % of label claim of ATV in tablet

Conclusion

A simple, precise, selective and sensitive isocratic HPLC assay method with UV detection for ATV in pharmaceutical dosage form has been developed and validated. The advantage of the method is that it can be applicable for quantification of ATV at two wavelengths which indicates the specificity of the method. Statistical analysis signifies that there is no difference between the two methods, so gives a universal applicability of the methods for quality control of Atazanavir Sulfate in raw materials and dosage form.

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