

**Research Article**

# Development And Validation Of Liquid Chromatography Method For The Analysis Of Atazanavir In Pharmaceutical Dosage Forms

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**Abstract**

Atazanavir is an important antiretroviral drug. The objective of the current study was to develop a simple accurate, precise and rapid RP-HPLC method development and validation for determination of Atazanavir in tablet dosage form. Chromatographic separation was achieved on C<sub>18</sub> Column with mobile phase Methanol: (0.1%) OPA (80:20) v/v with flow rate 0.7ml/min and UV detection was carried out on 250nm. Retention time of Atazanavir was found to be 4.302 & Linearity of proposed method was found to be in the range of 10-50 µg/ml (r<sup>2</sup>=0.999). Method was statistically validated for its linearity, accuracy and precision. Both interday and intraday variation was found to be showing less %RSD (Relative Standard Deviation) value indicating high grade of precision of method.

**Keywords:** RP-HPLC Method, Atazanavir, Validation, Recovery.

**Introduction**

Analysis is important in every product but it is

vital in medicines as it involves life. The assurance of quality is achieved through analysis of drug product.<sup>1</sup> Now days the drug are very much useful as a antiretroviral, it is mainly used in single therapies, rather than drug used in multiple formulation because of multiple action side effects.<sup>2</sup> Quantification of drug molecule is important task for routine analysis of API in its finished product analysis.<sup>3</sup> Literature survey exposed very few analytical procedures for routine analysis of Atazanavir.<sup>4</sup>

Atazanavir binds to the protease active site and inhibit the activity of enzyme. It selectively inhibits the virus – specific processing of viral Gag and Gag-polyproteins in HIV -1 infected cells by binding to the active site of HIV -1 protease, thus preventing the formation of mature virions<sup>5</sup>.

The analysis of Atazanavir by UV and HPLC techniques in various biological fluids was reported earlier. In addition the reported methods are complex time consuming and high costly therefore in present research an attempt was made to develop and validate simple, precise, accurate and economic method for routine analysis of Atazanavir in bulk and tablet dosage form.

We have developed the method and validated the method as per ICH Guidelines.

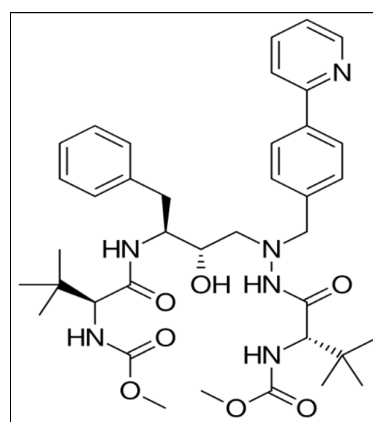


Figure 1. Chemical structure of Atazanavir

**Materials And Methods**

The Atazanavir were gifted from Mylan Pharmaceutical Ltd. Tablet formulation of Atazanavir (Label claim: Atazanavir10 mg) (Mylan pharmaceuticals) was purchased from local market. Methanol:OPA (HPLC grade) were obtained from E. Merck Ltd, Mumbai, India.

Agilent 1100 series HPLC unit accomplished with UV Detector, enable C<sub>18</sub> (250\*4.6\*5) Column, Quantitative HPLC was performed on isocratic mode with 20ul injection of sample loop. Output signal was monitored & integrated using Autochrome-3000 Software.

## RESULTS AND DISCUSSIONS

### Identification test for drug and solubility:

Melting point of Atazanavir was determined using digital melting point apparatus. The melting point was found to be 214° which matches with reference<sup>5, 6</sup>. For solubility determination 100 mg of Atazanavir bulk drug is taken and slowly added into 100 ml water and 100 ml Methanol separately. The drug was found to be soluble in water and methanol.

### Wavelength determination:

An accurately weight quantity 10 mg of Atazanavir were transferred to 100 ml volumetric flasks containing a diluent methanol, and volume was made up to mark with same solvent to obtain concentration 100 µg/ml. The absorbance of the latter was recorded using UV visible spectrophotometer in range 200-400nm.<sup>6, 7</sup>

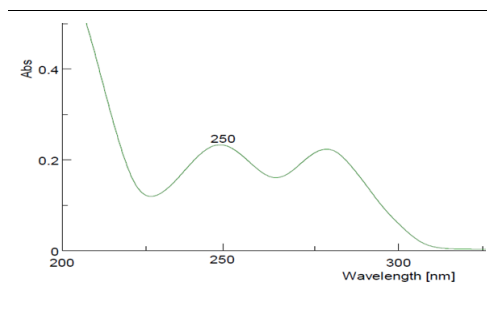


Figure 2. UV Spectrum of Atazanavir

The wavelength of 250nm was selected for quantitative determination of Atazanavir as given in further sections.

### Mobile phase selection:-

Initially the composition and the flow rate of mobile phase were selected according to separation condition using the standard solution and sample solution. After number of trial it was established that isocratic flow suitable for high eluting power compare to acid, base and other phosphate buffer, and still found suitable for closely eluting peaks.

### Preparation of Mobile Phase

HPLC grade acetonitrile was filtered through 0.4 µm membrane filter paper. Mobile phase was prepared by mixing 40 ml of Methanol with 60 ml (0.05%) of Phosphate buffer and was sonicated for 15 mins.

### Preparation of Standard Stock Solution:-

An accurately weighed quantity of Atazanavir (10 mg) was transferred in a 10 ml Methanol. To obtain standard solution having concentration of Atazanavir (1000 µg/ml). And then take 0.1ml stock solution and make with mobile phase methanol: 0.1 % OPA water (10µg/ml.). Six replicates of this solution were injected and results were recorded for RT, Area, tailing factor and theoretical plates. Mean, SD, %RSD were calculated and other parameters were also verified for acceptability level.<sup>8, 9</sup>

### Sample Solution

Twenty tablets of each brand each contain 10 mg of Atazanavir weighed and finely powdered; a quantity of powder equivalent to 10mg of Atazanavir transferred in 10mL flask and made the volume up to the mark with Water. Further the stock solution was diluted with mobile phase to get 100 µg/ml final concentration of sample solution.

### Chromatographic Conditions:-

Mobile Phase containing both Methanol : (0.1%) OPA (80:20) v/v0 was selected as the optimum composition of mobile phase because it was found that this solvent system resolved both components ideally. Flow rate was set to 0.7 ml/min. and UV detection was carried out at 250nm. Mobile phase and sample were degassed by sonication for 15 min and filtered through 0.4µm membrane filter. All the determination was performed at constant column temperature (25°C).

### System Suitability test:

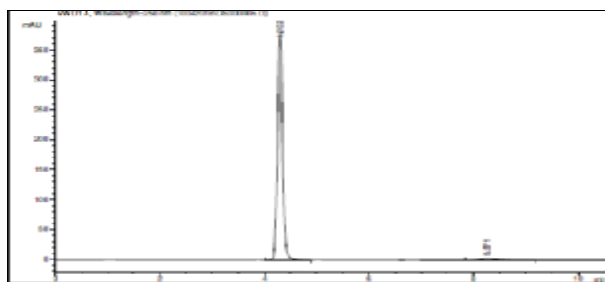
System suitability is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis is to be done.<sup>10</sup> The test was performed by six replicates of standard solution of Atazanavir. The result obtained at each replicate were assessed for parameters like retention time, theoretical plates and tailing factors and evaluated against standard limits. The results were in agreement with the

limit as per ICH guidelines. Therefore from system suitability experiment it was concluded that the system was found to be suitable for quantitative estimation of Atazanavir with Methanol and

0.1 % OPA (80:20) as a mobile phase at 250nm.

**Table no.1 Optimized chromatographic conditions for proposed methods**

Sr. No	Instrumentation	Optimized Conditions
1	Column	C <sub>18</sub> (primesil)
2	Column Temperature	25°C
3	Flow Rate	<b>0.7 ml/min</b>
4	Injection Volume	20ul
5	Wavelength	250 nm
6	Run Time	10mins
7	Mobile Phase Composition	Methanol :( 0.1%) OPA (80:20) v/v

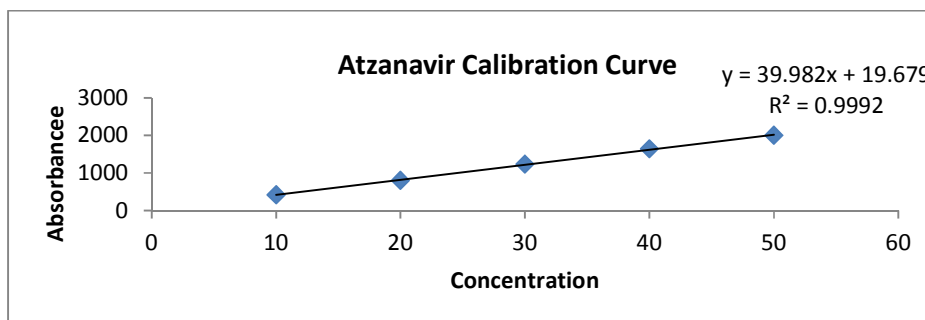


No.	RT[min]	Area[mV*s]	TP	TF
1	4.302	2571.13013	11609.4	1.1004
Sum		2571.13013		

**Figure.3 HPLC Chromatogram of Atazanavir**

**Table no. 02. Linearity of Atazanavir**

Conc. (µg/ml)	Peak area
10	419.41
20	802.77
30	1232.99
40	1641.3246
50	1999.256
<b>Regression equation</b>	<b>Y=39.9823x+19.679</b>
<b>R<sup>2</sup></b>	<b>0.999</b>



**Figure.4 linearity graph of Atazanavir**

**Table no.03 Accuracy study**

Level of recovery	Amount of drug present (µg/ml)	Amount of drug added	Area	Amount of drug found(µg/ml)	Amount received	% Recovery	%RSD
80%	8	8	730.10	17.76	7.76	97.12	0.28
	8	8	731.32	17.80	7.80	97.50	
100%	10	10	812.63	19.83	9.83	98.30	0.72
	10	10	816.72	19.93	9.93	99.30	
120%	12	12	893.96	21.86	11.86	98.90	0.10
	12	12	894.65	21.88	11.88	99.04	

**Table 04: Interday Precision of Atazanavir**

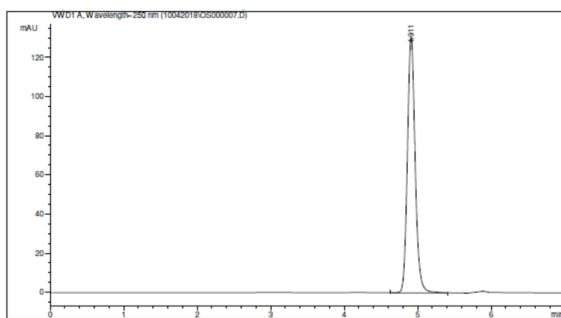
Sr No.	Conc	Area	II	Mean	Amt Found	% AmtFnd	SD	%RSD
1	20	797.3605	799.27	798.32	19.47	97.37	100.45	0.89
2	30	1210.85	1214.54	1212.69	29.84	99.46	2.61	0.22
3	40	1631.15	1623.24	1627.20	40.20	100.50	5.59	0.34

**Table 05: Intraday Precision of Atazanavir**

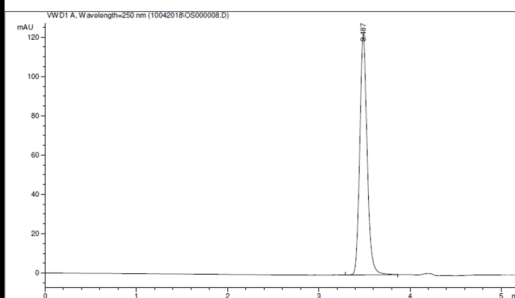
Sr No.	Conc	Area	II	Mean	Amt Found	% AmtFnd	SD	%RSD
1	20	795.32	793.23	794.28	19.37	101.75	0.96	0.12
2	30	1209.32	1212.56	1210.94	29.79	99.30	2.29	0.19
3	40	1628.36	1630.11	1629.24	40.25	100.63	1.24	0.08

**Table 06: Robustness study of Atazanavir (Change of flow rate)**

Flow rate-0.6			Flow rate-0.8		
Sr No.	µgm/ml	Area	Sr No.	µgm/ml	Area
1	20	956.84	1	20	687.92
2	20	960.21	2	20	680.36
	Mean	958.53		Mean	684.14
	SD	2.38		SD	5.35
	%RSD	0.25		%RSD	0.78



Flow rate: 0.6 ml/min.



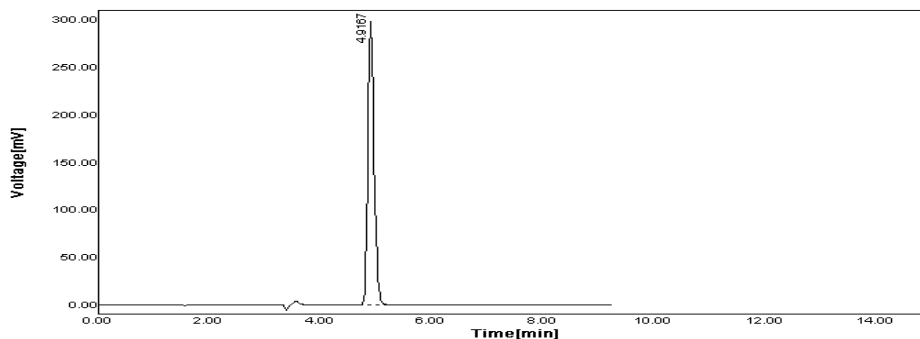
Flow rate: 0.8ml/min

**Table 07: Robustness study of Atazanavir (Change of Mobile phase composition)**

Mobile phase (81+ 19)			Mobile phase (79+21)		
Sr No.	Conc(µg/ml)	Area	Sr No.	Conc(µg/ml)	Area
1	8	894.2	1	8	644.39
2	8	901.25	2	8	651.31
	Mean	897.7		Mean	647.85
	SD	4.99		SD	4.89
	%RSD	0.56		%RSD	0.76

**Table 08: Robustness study of Atazanavir (wavelength Change)**

229 nm			231nm		
Sr No.	Conc(µg/ml)	Area	Sr No.	Conc(µg/ml)	Area
1	8	896.31	1	8	896.21
2	8	899.56	2	8	895.24
	Mean	897.9		Mean	895.73
	SD	2.30		SD	0.69
	%RSD	0.26		%RSD	0.08



**Figure 5 graph of 80% recovery for marketed formulation**

**Table no 09: % Recovery of assay**

Conc (µg/ml)	Area I	Amt Found	% Label Claim	SD	%RSD
40.00	1626.64	40.19	100.48	5.53	0.34
40.00	1618.82	39.99	99.98	0.14	0.36
			100.23	0.04	0.04

**Table no 10: Validation parameters**

Method characteristics	Atazanavir
Linearity	10-50 µg/mL
Regressions equation	y = 39.98x+19.67
Correlation coefficient	0.999
Retention time (mins)	4.302
LOD(µg/mL)	0.1733
LOQ(µg/mL)	0.5252
Precision (RSD, %); Intraday (n=3) Interday (n=3)	1.4
	0.33

**Method Validation**

Developed analytical method was subjected to validation with respect to various parameters such as Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), Accuracy, Precision, Recovery studies and Reproducibility as per the ICH guidelines<sup>10, 11, 12</sup>.

**A) Linearity****Preparation of Calibration curve:-**

A set of five solutions of Atazanavir concentrations ranging from 10 to 50 µg/ml were prepared. Each sample was analysed in triplicate. Calibration curve was constructed by plotting the peak area against concentration using linear regression analysis.<sup>14</sup>

**B) Accuracy**

The accuracy was determined by standard addition method. Three different levels (80%, 100% and 120%) of standards were spiked to commercial tablets in triplicate. The mean of percentage recoveries and the %RSD was calculated<sup>15</sup>. The result of accuracy study are shown in Table 03.

**C) Precision**

The intra-day precision of the developed HPLC method was obtained as %R.S.D. The inter-day precision was also determined by assaying the tablets in triplicate per day for consecutive 3 days, which was found to be 1.4% and 0.33% Atazanavir<sup>10, 11, 12</sup>

**B) Limit of Detection**

Limit of detection is the lowest analytical concentration at which analyte could be detected qualitatively.<sup>10, 11, 12</sup> The value of LOD was found to be 0.1733 mcg/ml.

**C) Limit of Quantification**

It is the lowest analyte concentrations that can be quantified with acceptable precision requiring peak heights 10 to 20 times higher than baseline noise level. Signal to noise ratio is good rule of thumb<sup>10, 11, and 12</sup>. The value of LOQ was 0.5252 mcg/ml.

**F) Robustness**

Robustness of an analytical procedure is its ability to remain unaffected by small variation in the analytical parameters. The robustness is evaluated by varying the analytical parameters such as buffer pH, flow rate, column temperature, injection volume, detection wavelength or mobile

phase composition within a realistic range<sup>10, 11, 12</sup>. There was no significant variation due to the variation of Flow rate, mobile phase composition or wavelength change. The results are tabulated in table 06, 07 and Table 08.

**E) % Recovery****Preparation of stock from API**

Accurately weighed 10mg of Atazanavir was added in volumetric flask containing some amount of mobile phase and volume was made up to the mark using mobile phase. The resulting solution was filtered through 0.45 µ membrane filter and sonicated for three cycles each of 10 min. from the stock solution 1.0ml of stock was pipetted out in triplicate and kept in three different volumetric flask, cleaned previously and diluted up to 10ml by using mobile phase to obtain resultant solution 10 µg/mL. This solution was injected for given chromatographic system in triplicate and mean area was determined.<sup>14, 15</sup>

**Preparation of stock from Dosage form**

Twenty tablets (label claim 10mg of Atazanavir, Mylan Ltd) were weighed; average weight was determined and powdered. Powder equivalent to 10 mg (15) mg was transfer to 10ml of mobile phase. The resulting solution was filtered through 0.45 µ membrane filter and sonicated for three cycles each of 10 min. from the stock solution 0.8, 1.0, 1.2ml solution were pipetted out and diluted with mobile phase to obtain resultant solution of 8, 10, 12 µg/m

**Preparation of test solution for %recovery**

10 µg/mL solution of Atazanavir was spiked into each of above dilutions of 8, 10, 12 µg/mL to obtain solution at 80%, 100%, 120% respectively. Each of these three levels was injected in triplicate and mean area for each level was determined. The mean area obtained on API injection was subtracted from the mean area of each of these three levels to obtain area corresponding to test solution. % recovery was determined from the test and standard area using following formula.

$$\% \text{ recovery} = \frac{\text{sample area} / \text{std. area} * \text{std. dilution}}{\text{factor} / \text{sample dil. factor} * \text{avg. wt} / \text{label claim}} * 100$$

**Discussion**

The RP-HPLC analysis was performed on the idC<sub>18</sub> (100\*4.6) mm, 5 µm particle size in the isocratic mode, at 40°C column oven temperature using Methanol and 0.1 % OPA water as mobile phase; flow rate was adjusted to 0.7ml/min. The

detection was carried out at 250nm. The average retention time for candidate drug was found to be 4.302 min. Linearity was observed in the concentration range of 50-100 µg/ml ( $r^2= 0.999$ ). The method has been successively applied for the determination of candidate drug in tablet formulation. There was no interference from the excipients present in the tablet. The drug content was found to be 101.6% for candidate drug. Accuracy of the method was studied by the recovery studies at three different levels 80%, 100%, 120% the % recovery was found to be within the limit of the acceptance criteria with average recovery of 98-119. The data obtained from proposed RP-HPLC method and validation was summarized in table no. 10

### Conclusion

Proposed study describes a new RP-HPLC method for the estimation Atazanavir by using simple mobile phase. The method gives good resolution between the compounds with a short analysis time. The method was validated and found to be simple, sensitive, accurate and precise. So the developed method can be used conveniently for analysis of Atazanavir in pharmaceutical dosage form.

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