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A new analytical method development and validation for the quantitative estimation of atazanavir in bulk form and marketed pharmaceutical dosage form by using RP-HPLC

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ABSTRACT

Atazanavir is indicated in combination with other antiretroviral agents for the treatment of HIV-1 infection in adults and pediatric patients 3 months of age and older weighing at least 5kg. Here an accurate, valid, elementary, and error-free reverse-phase liquid chromatography strategy was developed for the quantitation of Atazanavir in its bulk form as well as in marketed pharmaceutical dosage form. Effective chromatographic separation of Atazanavir was achieved by using waters, C-18, (250mm*4.6mmØ) column using Phosphate buffer (pH 3.0) and Acetonitrile in the proportion of 20:80 v/v. The Mobile phase was siphoned at a flow rate of 1.0 mL min-1 with a column temperature of ambient, and detection wavelength was carried out at 227 nm. The retention time was found to be 3.51 min for Atazanavir. The dimensionality of Atazanavir was in linear range with a parametric static of 0.9935. Method Validation was carried out in terms of Specificity, Linearity, Precision, Accuracy, LOD, and LOQ as per ICH Guidelines. Results obtained from the validation studies show that the developed method can be useful in the quality control analysis of bulk and marketed pharmaceutical formulations of Atazanavir.

Keywords: Atazanavir, RP-HPLC, Method Development, Validation, Accuracy.

INTRODUCTION

Atazanavir (formerly known as BMS-232632) is an antiretroviral drug of the protease inhibitor (PI) class. Like other antiretrovirals, it is used to treat infection of human immunodeficiency virus (HIV). Atazanavir is distinguished from other PIs in that it can be given once-daily (rather than requiring multiple doses per day) and has lesser effects on the patient's lipid profile (the amounts of cholesterol and other fatty substances in the blood). Like other protease inhibitors, it is used only in combination with other HIV medications. The

U.S. Food and Drug Administration (FDA) approved Atazanavir on June 20, 2003. Atazanavir[1] is an Aza-dipeptide analogue with a bis-aryl substituent on the (hydroxethyl) hydrazine moiety with activity against both wild type and mutant forms of HIV protease. Atazanavir does not elevate serum lipids, a common problem with other protease inhibitors. Atazanavir (ATV) is an azapeptide HIV-1 protease inhibitor (PI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Atazanavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs. Atazanavir is pharmacologically related but structurally different from other protease inhibitors and other currently available antiretrovirals. The IUPAC Name of Atazanavir is methyl N-[(2S)-1-[2-[(2S, 3S)-2-hydroxy-3-[[(2S)-2-(methoxy carbonylamino)-3, 3-dimethyl butanoyl] amino]-4-phenyl butyl]-2-[(4-pyridin-2-yl phenyl) methyl] hydra zinyl]-3, 3-dimethyl-1-oxobutan-2-yl] carbamate. The Chemical Structure of Atazanavir is as follows.

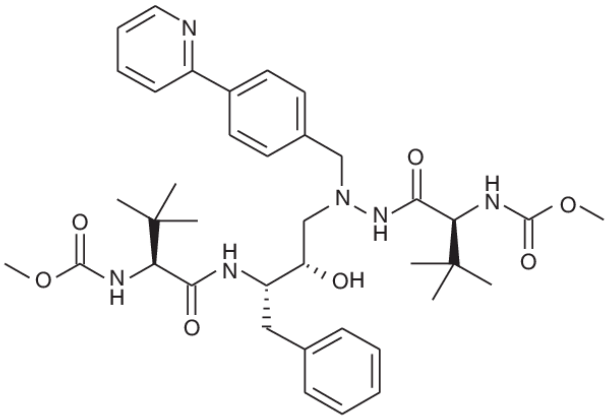

Fig 1: Chemical Structure of Atazanavir

The present strategy focused on isocratic high-performance liquid chromatography[2] method for the estimation of Atazanavir in bulk form and marketed pharmaceutical dosage form. After performing an extensive literature review[20-21], an attempt was made to develop a smooth plain sailing, unambiguous, valid, speedy, and decisive strategy for estimating Atazanavir in bulk form and marketed pharmaceutical dosage form.

MATERIALS AND METHODS

Table 1: List of Instruments Used

S.No.	Instruments/Equipments/Apparatus
1.	HITACHI L2130 with D Elite 2000 Software with Isocratic with UV-Visible Detector (L-2400)
2	ELICO SL-159 UV – Vis spectrophotometer
2	Electronic Balance (SHIMADZU ATY224)
3.	Ultra Sonicator (Wensar wuc-2L)
4.	Thermal Oven
5.	Waters ODS (C_8) RP Column, 250 mm x 4.6 mm.
6.	P^H Analyzer (ELICO)
7.	Triple Quartz Distillation Unit (BOROSIL)

Table 2: List of Chemicals, Reagents and Standards Used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	----	----	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	A.R.	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9	L.R.	Sd fine-Chem ltd; Mumbai
6.	Triethyl amine	99.9	L.R.	Sd fine-Chem ltd; Mumbai
7.	Glacial acetic acid	99.99	L.R	Sd fine-Chem ltd; Mumbai

Method Development

HPLC Instrumentation & Conditions

The HPLC system employed was HITACHI L2130 with D Elite 2000 Software with Isocratic with UV-Visible Detector (L-2400).

Standard & sample preparation for UV-spectrophotometer analysis

25 mg of Atazanavir standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Sample Preparations

The standard API & Test sample solutions were prepared separately in different volumetric flask by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm.

This has been performed to know the maxima of Atazanavir, so that the same wave number can be utilized in HPLC UV detector for estimating the Atazanavir. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450.

Standard Solution Preparation

25mg of Atazanavir Working standard was accurately weighed and transferred into a 25 mL volumetric flask and about 20 ml of diluent was added to it and sonicated to dissolve drug completely and volume was made up to the mark with the same solvent which gave Stock solution of 1000 ppm. 1 ml of the above stock solution was pippetted into a 10ml volumetric flask and was diluted up to the mark with diluents to prepare 100ppm solution. Further 1 ml of prepared 100 ppm solution was pippetted into a 10ml volumetric flask and was diluted up to the mark with diluents which gave 10ppm Atazanavir working standard solution. After mixing the solution was filtered through 0.45µm filter.

Sample Solution Preparation

20 tablets of marketed drug were weighed and the average weight was calculated. The sample equivalent to 25 mg of Atazanavir was accurately Weighed and transferred into a 25 ml volumetric flask. About 20 ml of diluent was added and sonicated to dissolve drug completely and the volume was made up to the mark with diluent which gave stock solution of 1000ppm. The above solution was prepared with proper mixture and filtered through 0.45µm filter. 1 ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent to prepare 100ppm solution. Further 1 ml of prepared 100ppm solution was pippetted into a 10ml volumetric flask and diluted up to the mark with diluent which gave 10 ppm Atazanavir working standard solution. It was mixed well and filtered through 0.45µm filter.

Phosphate Buffer Preparation

Near about 6.8 grams of 'Potassium dihydrogen orthophosphate' was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water. The pH - 3.0 maintained was with Orthophosphoric acid.

Preparation of Mobile Phase

400mL (40%) of above buffer and 600 mL of Acetonitrile HPLC (60%) were mixed well and degassed in ultrasonic water bath for 15 minutes. The

solution was filtered through 0.45 µm filter under vacuum filtration.

Diluent Preparation

Mobile phase as diluent.

Initialization of the Instrument

The HPLC instrument was switched on. The column was washed with HPLC water for 45 minutes. The column was then saturated with mobile phase for 45 minutes. The mobile phase was run to find the peaks. 20 minutes after the standard drug solution was loaded in HPLC.

Method Validation

Accuracy: *Recovery Study*

To determine the accuracy[3] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Atazanavir were taken and added to the pre-analyzed formulation of concentration 10µg/ml. From that percentage recovery[4] values were calculated.

Precision

As per USP, it is the degree of agreement[5] among individual test results obtained upon repeated application of analytical methods to multiple samplings of a homogenous sample or measure of extent to which the data values are close to each other for many measurements (under similar conditions).

Preparation of working standard of 10ppm of Atazanavir

25mg of Atazanavir Working standard was accurately weighed and transferred into a 25 mL volumetric flask and about 20 ml of diluent was added to it and sonicated to dissolve drug completely and volume was made up to the mark with the same solvent[6] which gave Stock solution of 1000 ppm. 1 ml of the above stock solution was pippetted into a 10ml volumetric flask and was diluted up to the mark with diluents to prepare 100ppm solution. Further 1 ml of prepared 100 ppm solution was pippetted into a 10ml volumetric flask and was diluted up to the mark with diluents which gave 10ppm Atazanavir working standard solution. The above solution was filtered through 0.45µm filter after well mixed.

Procedure

The 'standard API solution' was loaded at injection port for five times and measured the area for all five injections in HPLC[7]. The '%RSD' for the peak area of five replicate samples injected was found to be within the specified limits.

Acceptance Criteria

The % RSD[8] for the peak area of five standard samples injected yield not more than 2%.

Linearity & Range

Linearity[9] indicates the ability of analytical procedures to produce results that are directly proportional to the concentration of analyte in the given sample.

Preparation of Stock solution (100ppm)

25mg of Atazanavir was dissolved in 25ml of mobile phase which gave a solution of the strength of 1000ppm. 1ml of this solution was pippetted into a 10ml volumetric flask and the volume was made up to mark with diluents (mobile phase) which finally gave the stock solution of strength of 100ppm. The stock solution[10] was degassed in ultrasonic water bath for 5mins and filtered through 0.45 µm filter under vacuum filtration.

Preparation of Level – I (10µg/ml): 1.0 ml of stock solution was taken into 10ml of volumetric flask and diluted up to the mark with diluent.
Preparation of Level – II (20µg/ml): 2.0 ml of stock solution was taken into 10ml of volumetric flask and diluted up to the mark with diluent.
Preparation of Level – III (30µg/ml): 3.0 ml of stock solution was taken into 10ml of volumetric flask and diluted up to the mark with diluent.
Preparation of Level – IV (40µg/ml): 4.0 ml of stock solution was taken into 10 ml of volumetric flask and diluted up to the mark with diluent.
Preparation of Level – V (50µg/ml): 5.0 ml of stock solution was taken into 10 ml of volumetric flask and diluted up to the mark with diluent.
Procedure: Each solution was injected into the chromatographic system[11] and the peak areas were measured. A graph plotted against the peak area vs concentration on X-axis and Y-axis respectively and the correlation coefficient[12] was calculated.
Acceptance Criteria: Correlation coefficient should be not less than 0.999.

Method Robustness: It influence of small variations in chromatographic conditions[13] such as change in "flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%)". It was studied to determine the robustness of the method of developed RP-HPLC assay[14] of Atazanavir.

LOD & LOQ: The Minimum concentration level at which the analyte can be reliable detected (LOD[15]) & quantified (LOQ[16]) were found to be 0.03 & 0.09 µg/ml respectively.

Assay of Atazanavir in Marketed Pharmaceutical Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed capsules were finally powdered and triturated well. A quantity of powder equivalent[17] to 100 mg of drugs was transferred to 100 ml volumetric flask, and 70 ml of diluents was added and solution was sonicated for 15 minutes then the volume was made up to 100 ml with same solvent. 10 ml of the above solution was diluted to 100 ml by diluents. The solution was filtered[18] through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.

The solution prepared was injected in five replicates into the HPLC system and the observations were recorded. A duplicate standard solution also be introduced into the HPLC and the peak areas were recorded.

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where,

AT = Peak Area of test,
AS = Peak Area of standard,
WS = Weight of Standard API taken in mg,
WT = Weight of sample taken in mg,
DS = Dilution of Standard solution,
DT = Dilution of sample solution,
P = Percentage purity of working standard.

RESULTS AND DISCUSSION

UV Analysis

While scanning[19] the Atazanavir solution we observed the maxima at 227 nm.

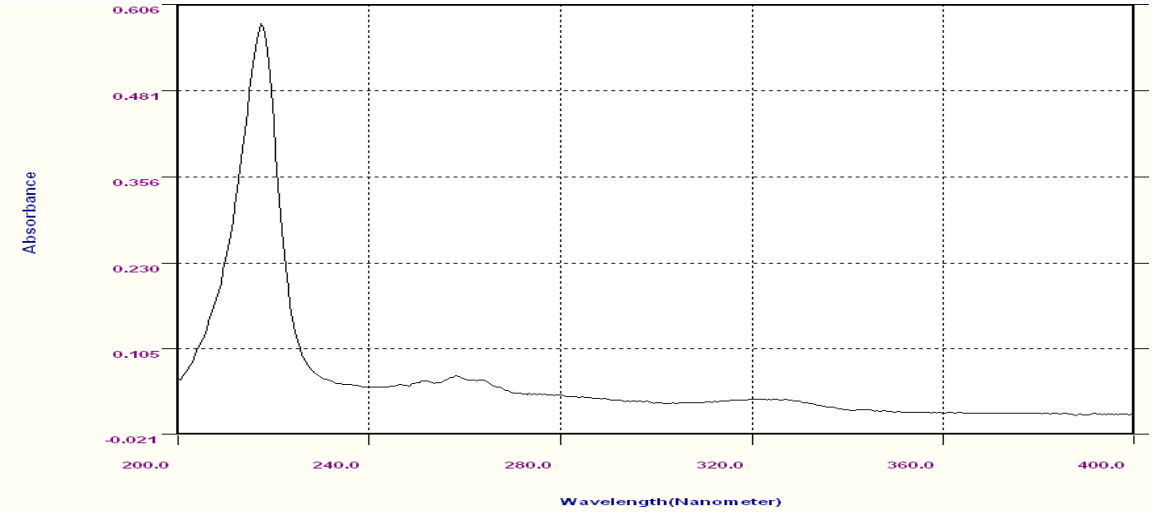

Fig 2: UV Spectrum of Atazanavir

Table 3: Optimized Chromatographic Conditions

Mobile phase	Acetonitrile (ACN): Phosphate Buffer = 20:80% v/v
Wavelength	227nm
Flow rate	1.0 ml/ min
Run time	10 min
Column	Waters, C-18, (250mm*4.6mmØ)

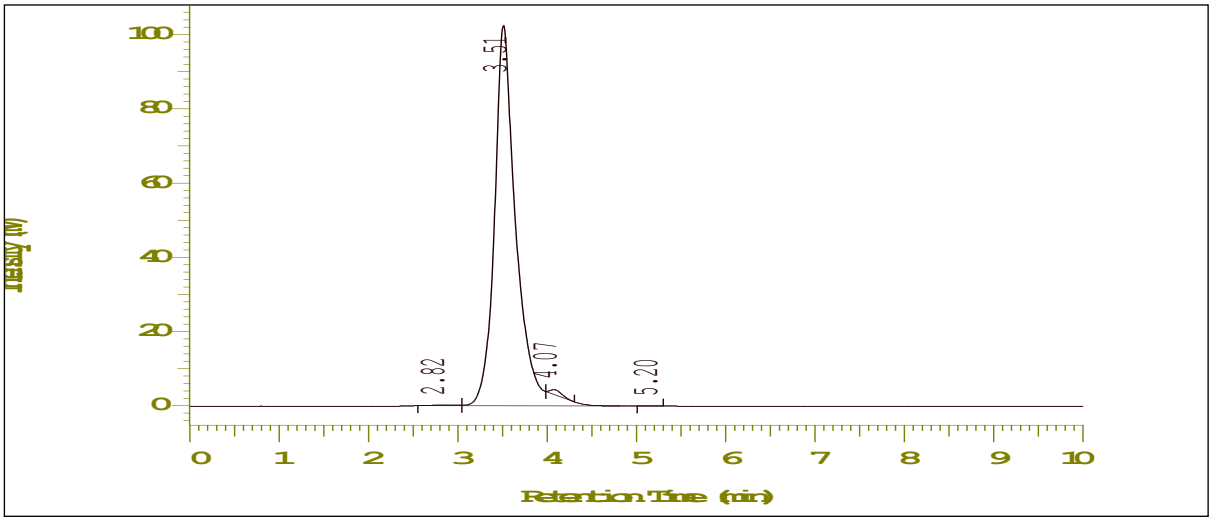

Fig 3: Optimized Chromatographic Condition

Table 4: Accuracy Readings

Sample ID	Concentration (µg/ml)		%Recovery of Pure drug	Statistical Analysis
	Pure drug	Formulation		
S_1 : 80 %	16	20	99.18	Mean= 98.97667%
S_2 : 80 %	16	20	98.58	S.D. = 0.200083
S_3 : 80 %	16	20	99.20	% R.S.D.= 0.202152
S_4 : 100 %	20	20	99.67	Mean= 99.54%
S_5 : 100 %	20	20	99.74	S.D. = 0.33
S_6 : 100 %	20	20	99.21	% R.S.D.= 0.331525
S_7 : 120 %	24	20	99.12	Mean= 99.567%
S_8 : 120 %	24	20	99.85	S.D. = 0.33
S_9 : 120 %	24	20	99.98	% R.S.D. = 0.331159

Table 5: Repeatability Data for Atazanavir

HPLC-Injection for Atazanavir	Area	Retention Time
Replicate – 1	452867	3.57
Replicate – 2	452667	3.59
Replicate – 3	452567	3.55
Replicate – 4	452867	3.55
Replicate – 5	452633	3.56
Average	452720.2	3.55
Standard Deviation	138.74869	0.00
% RSD	0.0306478	0.00

Table 6: Results of Intermediate Precision

Conc. of valsartan (API) (µg/ml)	Observed Conc. of Atazanavir (µg/ml) by the Proposed Method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
10	10.08	0.96	10.03	0.97
20	20.04	0.40	30.03	0.42S
40	39.97	0.33	39.95	0.14

Table 7: Linearity Data of Atazanavir

Conc. in µg/ml	AUC n=6
0	0
10	2124588
20	3124586
30	4258963
40	5258639
50	6541239
60	7586931
70	8521364
80	9874561
90	10923785
100	11548793

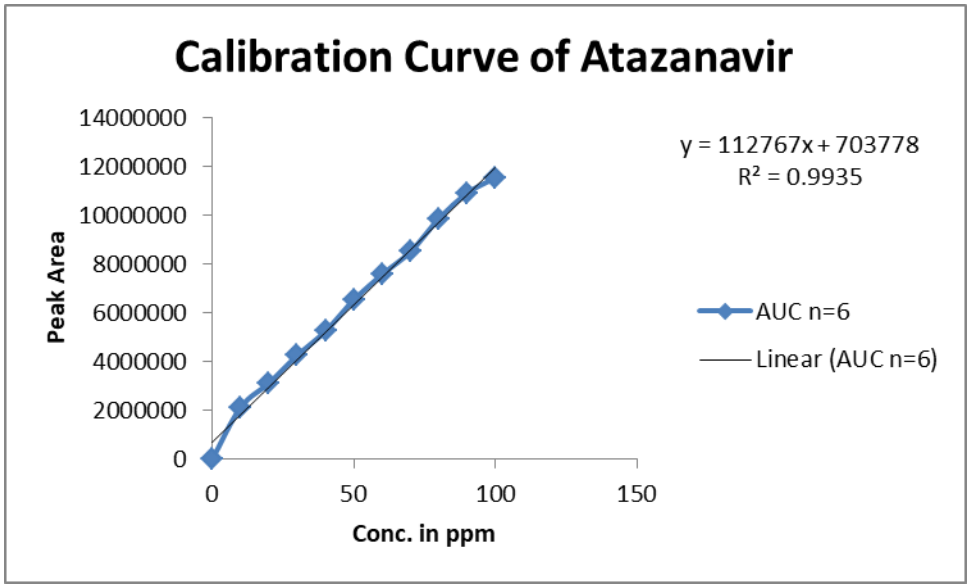

Fig 4: Calibration Curve of Atazanavir

LOD & LOQ

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.05 & 0.15 µg/ml respectively.

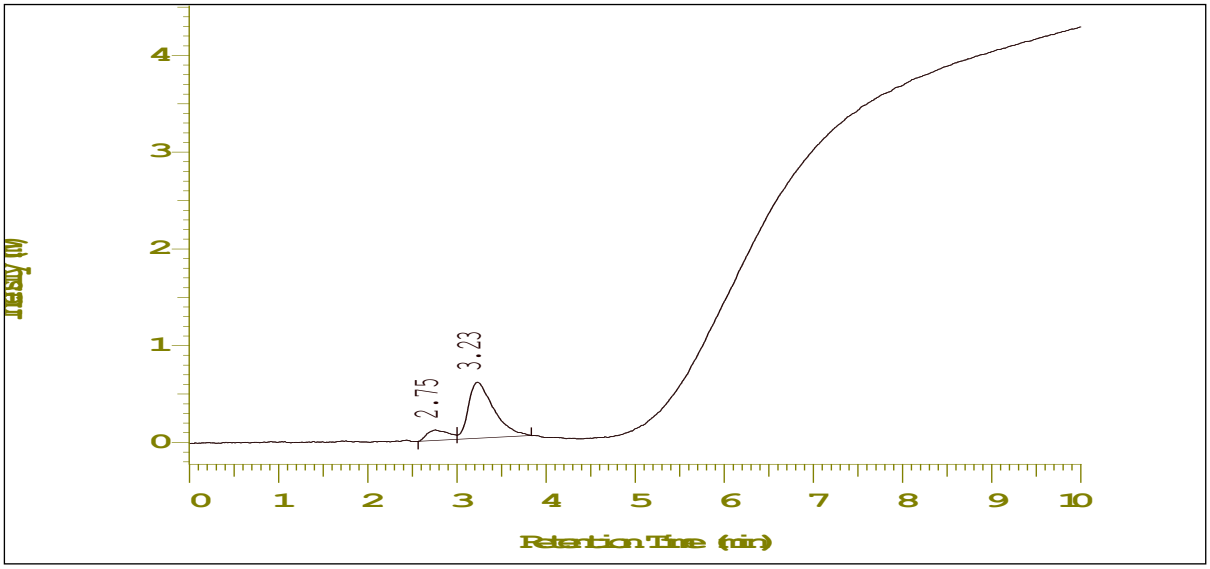

Fig 5: Chromatogram for LOD & LOQ

Table 8: Robustness Data for Atazanavir

Change in parameter	% RSD
Flow (1.1 ml/min)	0.12
Flow (0.9 ml/min)	0.11
Temperature (27^0C)	1.03
Temperature (23^0C)	1.02
Wavelength of Detection (276 nm)	0.95
Wavelength of detection (280 nm)	0.92

Table 9: Assay Results of Atazanavir

Commercial Brand Name of Tablets	Clamed Amount of Drug (mg)	Mean (±SD) amount (mg) found by the Proposed Method (n=6)	Mean (± SD) Assay (n = 6)
Atazor (Emcure Pharmaceuticals Ltd.)	100	100.34 (±0.06)	100.34 (±0.49)

Table 10: Summary of Validation Parameters by RP-HPLC Method

Validation Parameters		Results
Specificity		% interference<0.6 %
Range (µg/ml)	Linear range	0-100µg/ml
	Working range	0.03-60µg/ml
	Target range	20,30,40µg/ml
	Target concentration	30µg/ml
Accuracy (% Recovery)	**80, 100, 120**	98.97, 99.54, 99.56%
Precision (% RSD)	Repeatability	0.0306478
	Intraday (10,30,100 µg/ml)	0.96,0.40,0.33
	Inter day (10,30,100 µg/ml)	0.97,0.42,0.14
LOD (µg/ml)		0.05
LOQ (µg/ml)		0.15

SUMMARY

It is found that the 'Atazanavir' is soluble in Methanol and Acetonitrile but less soluble in water. The uv-visible absorbance spectrum for Atazanavir was detected under 227 nm. The HPLC development was performed for the assay of Atazanavir and detected under the range of 200-280 nm conveniently while the flow rate of 1 ml/min & an injection volume of 20 µl were maintained for the best analysis. The present work develops a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of 'Atazanavir' in different chromatographic conditions. In this work we used waters $C_{18,}$ 'ODS HG-5'- RP 150mm x 4.6mm 5µm particle size with column peak shape, peak resolution and good absorbance. For preparation of various samples the Solvent & diluent were confirmed after examining the solubility, pH and Dissociation of API in different solvents of our disposal (methanol, ethanol, acetonitrile, buffer, water, 0.1N NaOH, 0.1NHCl). The drug was found to be soluble in acetonitrile and buffer but the drug was less soluble in water. When using these solvents with proper composition new methods can be developed and validated for authentication. The calibration curve showed good linearity in the range of 0-50 µg/ml, for Atazanavir (API) with correlation coefficient (r^2) of 0.993. A typical calibration curve has the regression equation of y = 112767x + 703778 for Atazanavir. The accuracy, Precision, LOD &LOQ results were found within limit.Nearly 100% purity for the commercial drug was confirmed during assay procedure.

CONCLUSION

A sensitive & Selective RP-HPLC method has been developed & validated for the analysis of Atazanavir API. The present developed RP-HPLC method has excellent sensitivity, precision and reproducibility. The result shows the developed method is yet another suitable method for assay, purity & stability which can help in the analysis of Atazanavir in different formulations.

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