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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC FOR THE DETERMINATION OF AZELNIDIPINE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

The new RP-HPLC method for the estimation of Azelnidipine was found out by using different chromatographic parameters. Chromatography was performed by gradient reverse phase separation using a Water's XBridge C18 column of particle size 5µ 250×4.6mm. The separations were achieved at the UV detection at 255nm using the mobile phase of Potassium dihydrogen phosphate and orthophosphoric acid (buffer): Methanol (60:40v/v). Flow rate was 1ml/min and the injection volume was set at 20 µl with 10 mins of runtime. The retention time was observed at 4.49 mins. The method was validated by using various validation parameters like accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ). These results showed the method could find practical application as a quality control tool for analysis of the drug in its pharmaceutical dosage forms in quality control laboratories. The standard curve was linear over a working range of 5-30 µg/ml and gave an average correlation factor of 0.9984 for Azelnidipine. The limit of detection and the limit of quantitation were found to be 1.38µg/ml µg/ml and 4.17µg/ml respectively. The method showed good recoveries and relative standard deviations ofintra and inter day assay less than 2. This method can be easily and conveniently used for routine analysis of Azelnidipine in bulk and tablet dosage forms.

KEYWORDS: Azelnidipine, RP-HPLC, Accuracy, Precision, Linearity, LOD, LOQ.

1. INTRODUCTION[1-4]

Azelnidipine is a new dihydropyridine calcium channel antagonist with selectivity for L-type calcium channels that has recently been approved in Japan for the treatment of patients with hypertension.

Clinical investigations found that long-term treatment with azelnidipine efficiently regulates blood pressure in 95 people with mild-to-moderate hypertension (BP). After one year of treatment, the mean reduction in sitting systolic/diastolic BP from baseline was 27.8/16.6 mm Hg.

In randomised, double-blind studies, the antihypertensive efficacy of azelnidipine was found to be similar to that of amlodipine and nitrendipine in patients with mild to moderate hypertension.

Amlodipine and azelnidipine had similar effects on 24-hour blood pressure. The most common causes of azelnidipine are vasodilator effects as headache and hot face flushes. It does not cause reflex tachycardia when used.

DRUG PROFILE

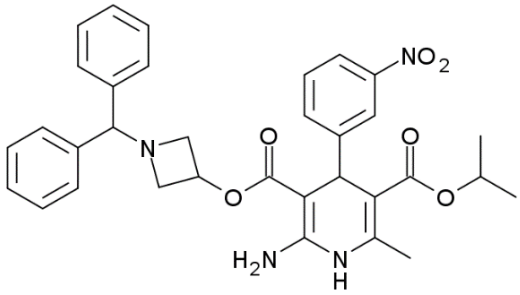

Fig. 1: Structure of Azelnidipine.

Structure : AZELNIDIPINE

IUPAC name : 3-(1-Benzhydrylazetidin-3-yl) 5-isopropyl 2-amino-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate

Molecular Formula : $C_{33}H_{34}N_4O_6$

Molar Mass : 582.6 $g.mol^{-1}$

Melting point : 193-195 ^0C

Solubility : Insoluble in water

Half - life : 16-28 hours

pKa	:	19.88 (Strongest Acidic), 6 (Strongest Base)
Refractivity	:	172.06 $m^3.mol^{-1}$
Drug Category	:	It is a dihydropyridine calcium channel blocker.

Mechanism of Action:[3]

Azelnidipine inhibits trans-membrane Ca^{2+} influx through the voltage-dependent channels of smooth muscles in vascular walls. Ca^{2+} channels are classified into various categories, including L-type, T-type, N-type, P/Q-type, and R-type Ca^{2+} channels. The L-type Ca^{2+} channels 6. Normally, calcium induces smooth muscle contraction, contributing to hypertension. When calcium channels are blocked, the vascular smooth muscle does not contract, resulting in relaxation of vascular smooth muscle walls and decreased blood pressure.

Uses of AZELNIDIPINE

Hypertension (High blood pressure).

Directions for Use

Swallow AZELNIDIPINE as a whole with water. Do not crush or chew the tablet. The dose and duration will be decided by your doctor based on your medical condition. Follow your doctor's instructions carefully while taking this medicine.

Side Effects of AZELNIDIPINE

- Headache
- Fast heart rate
- The feeling of fast or uneven heartbeat (palpitations)
- Sudden reddening of your face
- Neck or upper chest (flushing)
- Ankle swelling

2. HPLC METHODOLOGY[5-10]

2.1. Collection of reagents and solvents

Azelnidipine API procured from Ajanta Pharma Limited, Kunjhal, Tehsil Nalagarh, Solan, Himachal Pradesh of these working standard drugs. HPLC grade Acetonitrile (Merck), Analytical grade Potassium dihydrogen phosphate and orthophosphoric acid was used as the solvents throughout the experiment. Pharmaceutical formulation tablet Azusa (label claim

containining 16mg) was used in HPLC analysis. HPLC grade water obtained in-house by using Direct-Q water purification system (Millipore, Milford,USA) was used in HPLC study.

2.2. Apparatus and software

The Agilent 1120 Compact LC HPLC system consisting of gradient pump (LC-10AT vp pump) (4MPa or 40barr), rheodyne injector, UV variable wavelength detector, Standard cell and agilent syringe was used. The separations were achieved on a waters X- Bridge C18 column 5µm 4.6x250mm with UV detection at 255nm. Analytical weighing balance (Shimadzu AUX 220) was used for weighing, sonicator (EQUITRON-230VAC, 50Hz), vaccum pump (SUPER FIT), filtration kit (TARSONS) and Nylon membrane filter (Merck Millipore) for solvents and sample filtration were used throughout the experiment. Double beam UV Visible spectrophotometer (SHIMADZU-UV 1700) was used for wavelength detection. The EZ Chrome Elite software- single channel was used for acquisition, evaluation and storage of chromatographic data.

2.3. Chromatography

After several trials with the different combination and ratio of solvents, the mobile phase Potassium dihydrogen phosphate and orthophosphoric acid (buffer): Methanol (60:40 v/v) was selected, because it was found that it ideals with retention time (Rt) 5.49 min. Wavelength was selected by scanning the standard drug over a wide range of wavelength 200 nm to 400 nm. The component show reasonably good response at 255nm.

Absorption maxima of Azelnidipine

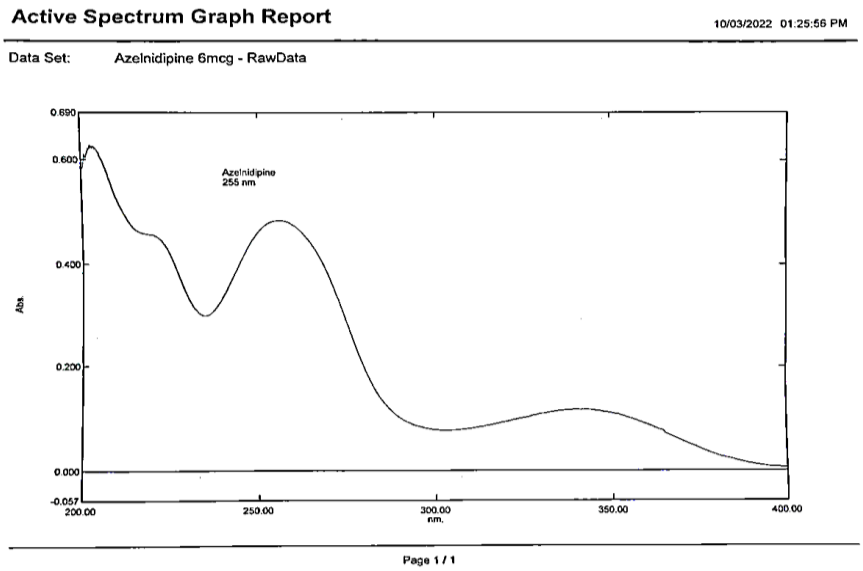

Fig. 2: Absorption maxima for Azelnidipine.

2.4. Preparation of mobile phase

Potassium dihydrogen phosphate and orthophosphoric acid buffer solution was prepared by dissolving 0.5ml of each in 500 ml of HPLC grade water. HPLC experiments were carried out using binary pump A containing Methanol and pump B containing Potassium dihydrogen phosphate and orthophosphoric acid.

2.5. Standard solutions for HPLC estimation of Azelnidipine

Accurately weighed 50 mg of Azelnidipine is transferred into 50 ml of volumetric flask and was dissolved in acetonitrile and the volume were made up to the mark with the same solvent. This gave the concentration of 1000 µg ml^{-1} of Azelnidipine. From the above 5ml of Azelnidipine solution was pipetted out into a separate 50ml volumetric flask and the volume was made upto the mark with acetonitrile. This gave the concentration of 100µgml^{-1} of Azelnidipine. From the above, six dilutions in between 5-30µgml^{-1} of working concentration made up by using mobile phase as a solvent.

2.6. Sample preparation for HPLC estimation of Azelnidipine

Ten tablets of Azelnidipine containing 16mg of Azelnidipine were weighed and powdered for further study. The powder equivalent to 10mg of Azelnidipine was accurately weighed and transferred to 10 ml volumetric flask. After that drug mixture is dissolved in Methanol. The volume is maintained with Methanol and is sonicated for 10 min. The above solution was carefully filtered through nylon membrane filter paper (0.45µ).

From this solution, required dilutions for HPLC method were prepared by using Methanol as a solvent.

2.7. Calibration

The calibration curve was plotted with seven concentrations of the standard drug solution 5-30 µg/ml and chromatography was repeated six times for each dilution. The linearity was evaluated by linear regression analysis. Before injecting solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. Seven determinations were carried out for each solution, peak areas were recorded for all the solutions. All stock and working solutions were sonicated for 10 min then filtered through the nylon membrane filter (0.45µ) prior to use. 10µL injections were made for each concentration and chromatographed six times under specified condition at ambient temperature (25^{oc}). The correlation graph was constructed by plotting the peak areas obtained

at the optimum wavelength of detection v/s the injected amounts of the respective concentrations.

2.8. Specificity and selectivity

Specificity is a procedure to detect quantitatively the analyte in presence of the components that may be expected to be present in the sample matrix. While selectivity is a procedure to detect the analyte qualitatively in presence of components that may be expected to be presented in the sample matrix. The excipients in tablet formulation were spiked in pre weighted quantity of drugs and then absorbance was measured and calculations were done to determine the quantity of the drugs.

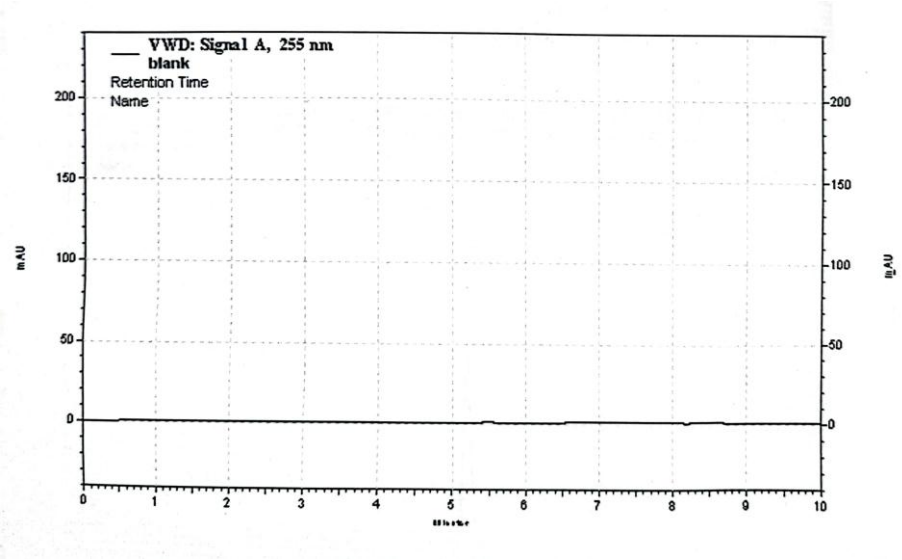

Fig. 3: Blank Chromatogram of Azelnidipine.

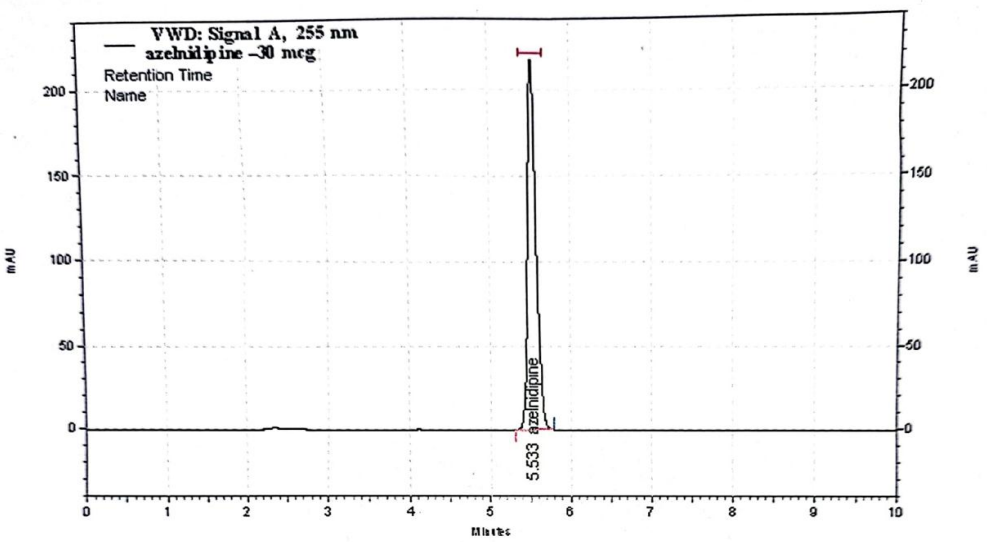

Fig. 4: Chromatogram of standard Azelnidipine.

2.9. Linearity and range

By using the working standard, aliquots of 5, 10, 15, 20, 25, 30µg/ml were prepared with Methanol. Six dilutions of each of the above-mentioned concentrations were prepared separately and from these six dilutions, 20µl of each concentration were injected into the HPLC system. Then their chromatogram was recorded. Peak areas were recorded for all the peaks and a standard calibration curve of peak area against concentration was plotted.

2.10. Accuracy

The procedure for the preparation of the solutions for Accuracy determination at 80%, 100% and 120% level were prepared in the acetonitrile.

For 80% Accuracy for Azelnidipine: standard concentration is 10 µg/ml.

8 µg/ml Concentration Taken.

For 100% Accuracy for Azelnidipine: standard concentration is 10 µg/ml.

10 µg/ml Concentration Taken.

For 120%Accuracy for Azelnidipine: standard concentration is 10 µg/ml.

12 µg/ml Concentration Taken.

Then they were mixed, filtered and diluted to get the concentration of 10µg/ml of the solution. The following mixer is subjected to analysis by RP-HPLC method under the same chromatographic conditions as described above. The consecutive 3 determinations were performed. The results obtained were compared with expected results and were statistically validated and is shown in table.

2.11. Precision

The precision of the assay was determined in terms of intra and inter day variation in the peak area for a set of drug solution 15µg/ml, assayed six times on the same day and on different 2 days.

The intra and inter day variation in the peak ratio of the drug solution was calculated in terms of co-efficient of variation (CV) and obtained by multiplying the ratio of the standard deviation to the mean with 100(CV=SD/MEAN × 100) shown in the graph.

2.12. Robustness

As defined by the ICH, the robustness of an analytical procedures describes to its capability to remain unaffected by small and deliberate variation in the chromatographic conditions and

found to be unaffected by small variation ±0.2ml/min in flow rate of mobile phase, wavelength ±2nm and pH ±1 results are shown.

2.13. Limit of detection and limit of quantification

LOD and LOQ were calculated according to ICH recommendations where the approach is based on the signal –to-noise ratio. Chromatogram signals obtained with known low concentrations of analytes was compared with the signals of the blank samples. A signal – to –noise ratio 3:1 and 10:1 was considered for calculating LOD and LOQ respectively. The values of LOD and LOQ were given in the table 5.

3. Method Validation[11-15]

Validation is the process of establishing a documented evidence, which provides a high degree of assurance that a specific activity will contistently produce desire result or product meeting its predetermined specification and quality characteristic. The method was validated as per ICH Guidelines.

3.1. Linearity

The linearity of estimation of Azelnidipine was determined by the analysis of analyte concentration across 5µg/ml to 30µg/ml of Azelnidipine. They were prepared and then tested at 255 nm. Absorbance is plotted graphically as a function of analyte concentration.

3.2. Linearity of estimation of Azelnidipine

10mg of Azelnidipine were taken into 10ml volumetric flask. It was dissolved in acetonitrile and is sonicated at room temperature for about 5minutes and finally the volume was made up with Methanol. From the above stock solution, 50µl, 100µl, 150µl, 200µl, 250µl and 300µl solutions were pipetted out and are made volume up to mark (10ml) with Methanol in 10ml volumetric flasks which gave 5µg/ml- 30µg/ml solutions. Absorbance was plotted graphically.

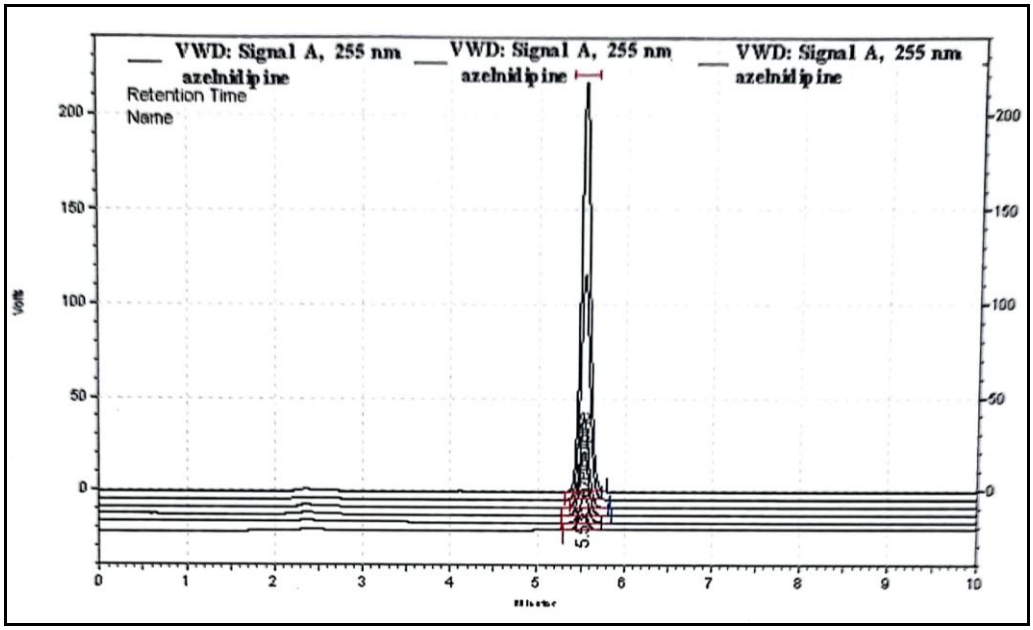

Fig. 5: Chromatogram of Azelnidipine at concentration 5-30µg/ml.

Table 1: Chromatogram of Azelnidipine at concentration 5-30µg/ml.

S. No.	Concentration	Peak Area at 255nm
1.	5µg/ml	970331
2.	10µg/ml	1812523
3.	15µg/ml	3033219
4.	20µg/ml	4069963
5.	25µg/ml	5135718
6.	30µg/ml	6081826

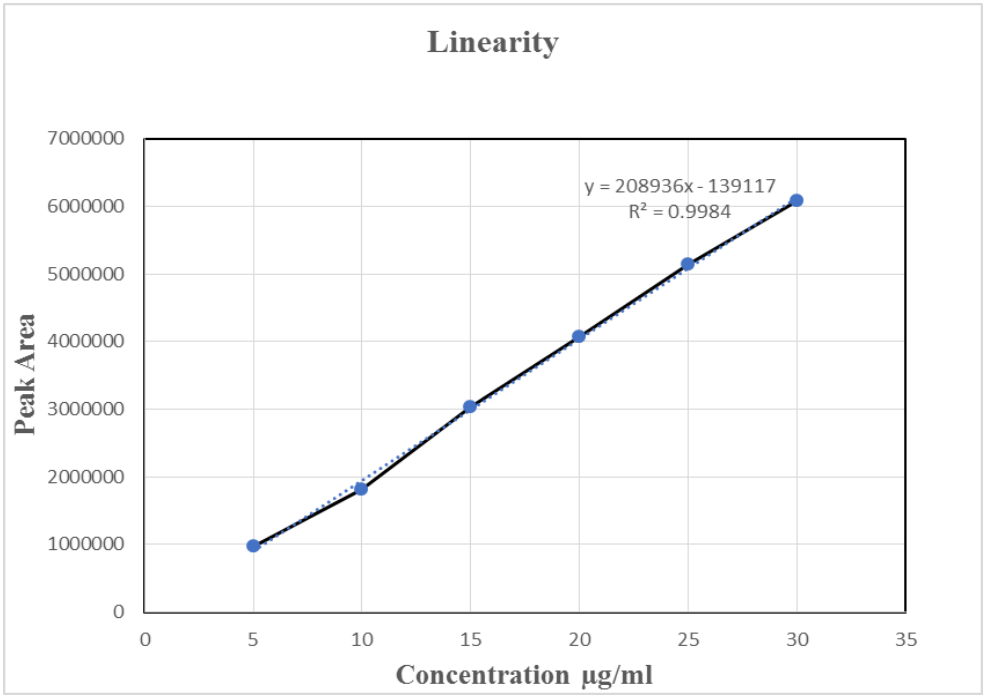

Fig. 6: Chromatogram of Azelnidipine at concentration 5-30µg/ml.

3.3. Precision

Precision of the analytical method was studied by analysis of multiple sampling of homogenous sample. Interday (between 2 days) and intraday (at the same days: morning and evening) precision were carried out. The variation of results were calculated and %RSD was determined.

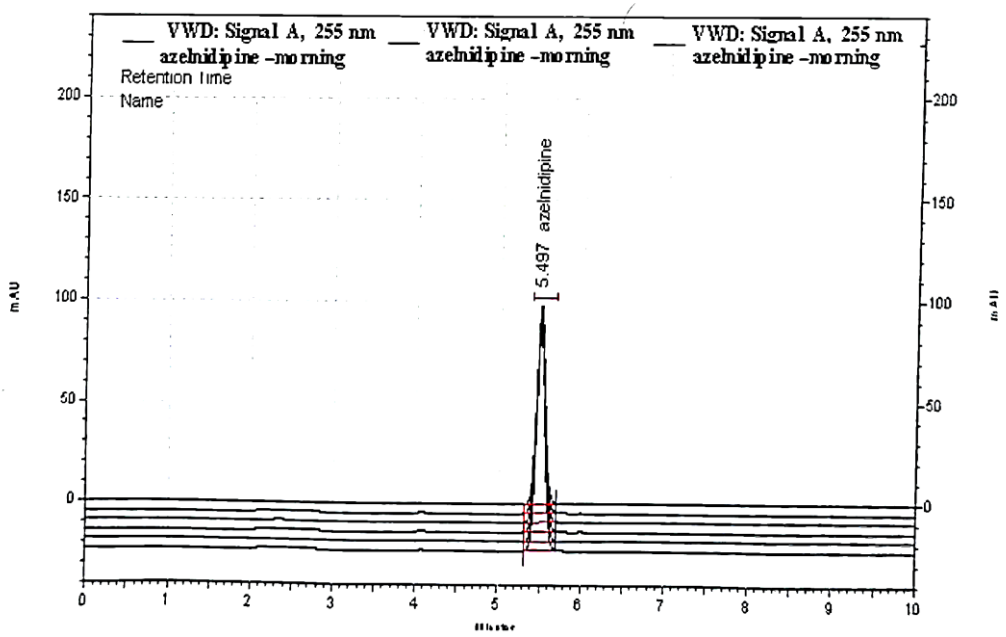

Fig. 7: Chromatogram showing Intraday precision (At morning).

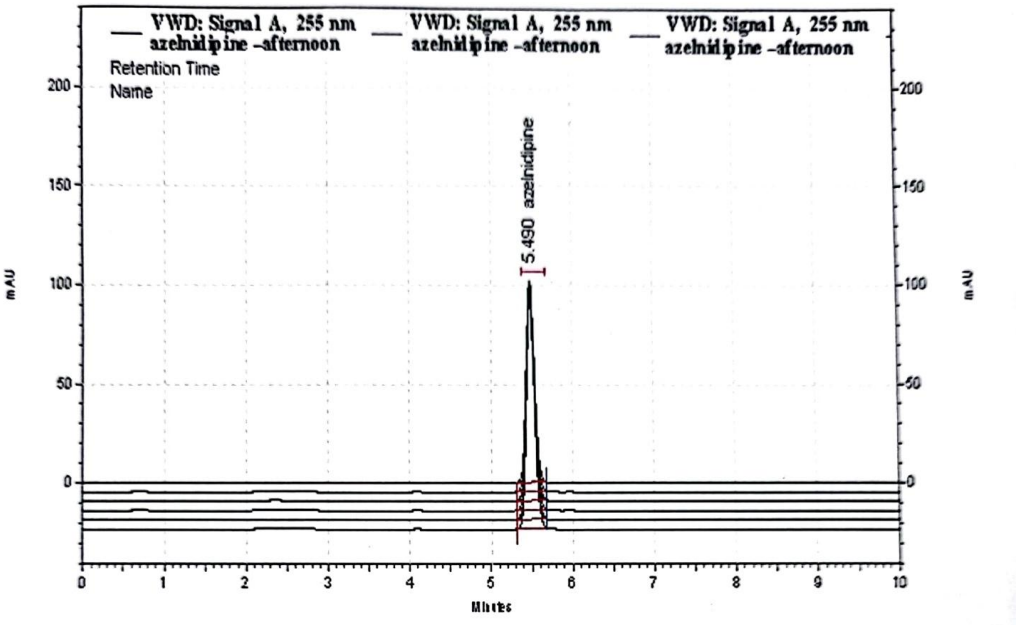

Fig. 8: Chromatogram showing Intraday precision (Afternoon).

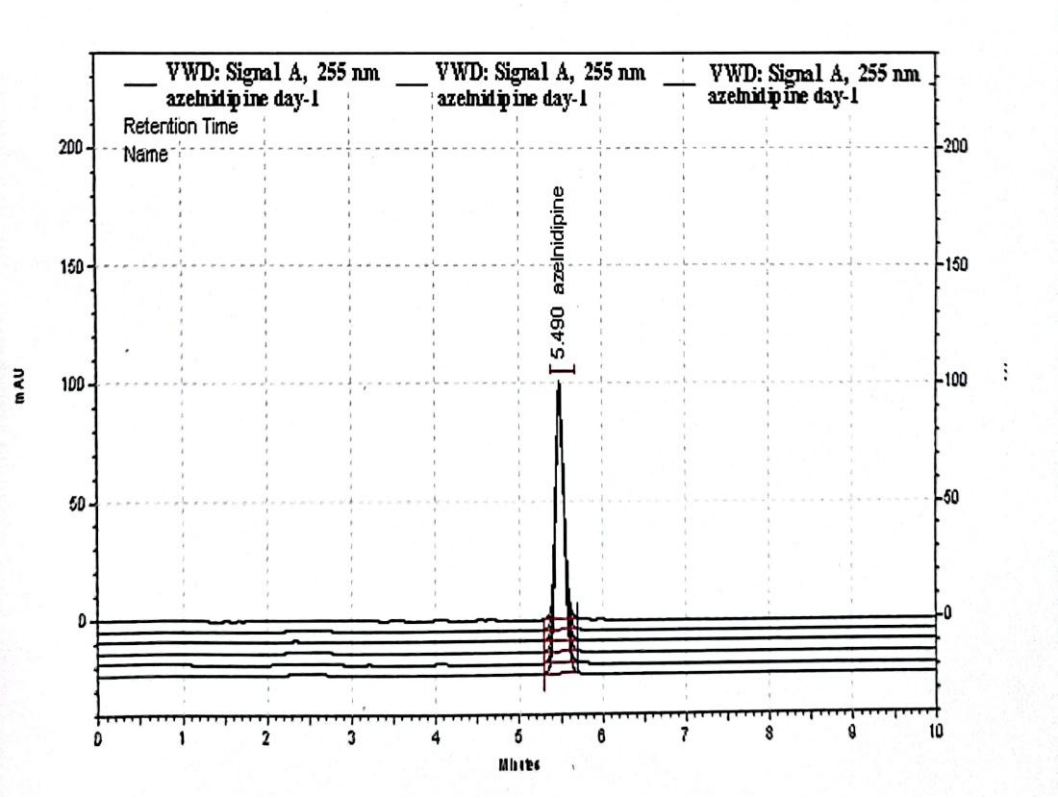

Fig. 9: Chromatogram showing interday precision (Day -1).

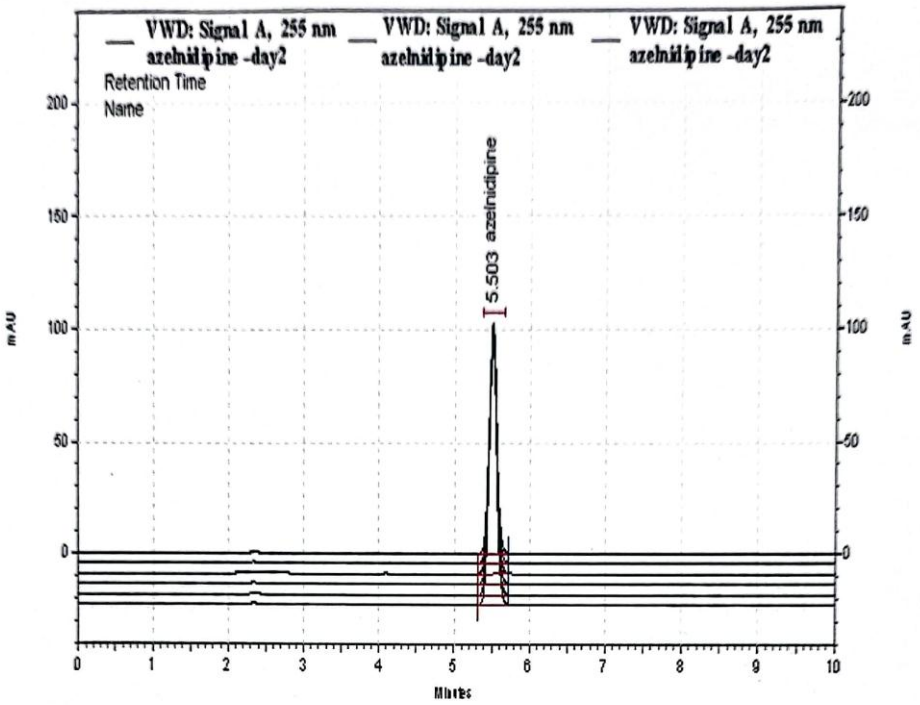

Fig. 10: Chromatogram showing interday precision (Day – 2).

Table 1: Intraday precision of estimation of Azelnidipine.

Morning				
Concentration(15µg/ml)	**Absorbance**	**AVG**	**SD**	**%RSD**
1	3033267	3039825.7	6193.37235	0.20%
2	3034978			
3	3037178			
4	3039578			
5	3044154			
6	3049799			
Afternoon				
Concentration(15µg/ml)	**Absorbance**	**AVG**	**SD**	**%RSD**
1	3036578	3050000.3	12786.56904	0.42%
2	3037865			
3	3044621			
4	3052477			
5	3059231			
6	3069230			

Table 2: Interday precision of estimation of Azelnidipine.

Day - 1				
Concentration(15µg/ml)	**Absorbance**	**AVG**	**SD**	**%RSD**
1	3033219	3038596.5	4356.90274	0.14%
2	3034932			
3	3037132			
4	3039632			
5	3041832			
6	3044832			
Day - 2				
Concentration(15µg/ml)	**Absorbance**	**AVG**	**SD**	**%RSD**
1	3033200	3038573.83	4354.93414	0.14%
2	3034913			
3	3037107			
4	3039613			
5	3041797			
6	3044813			

3.4. Accuracy

The accuracy for estimation of Azelnidipine using Acetonitrile was determined by adding known amount of the analyte. The accuracy was calculated from the test results as the percentage of the analyte recovered by the assay.

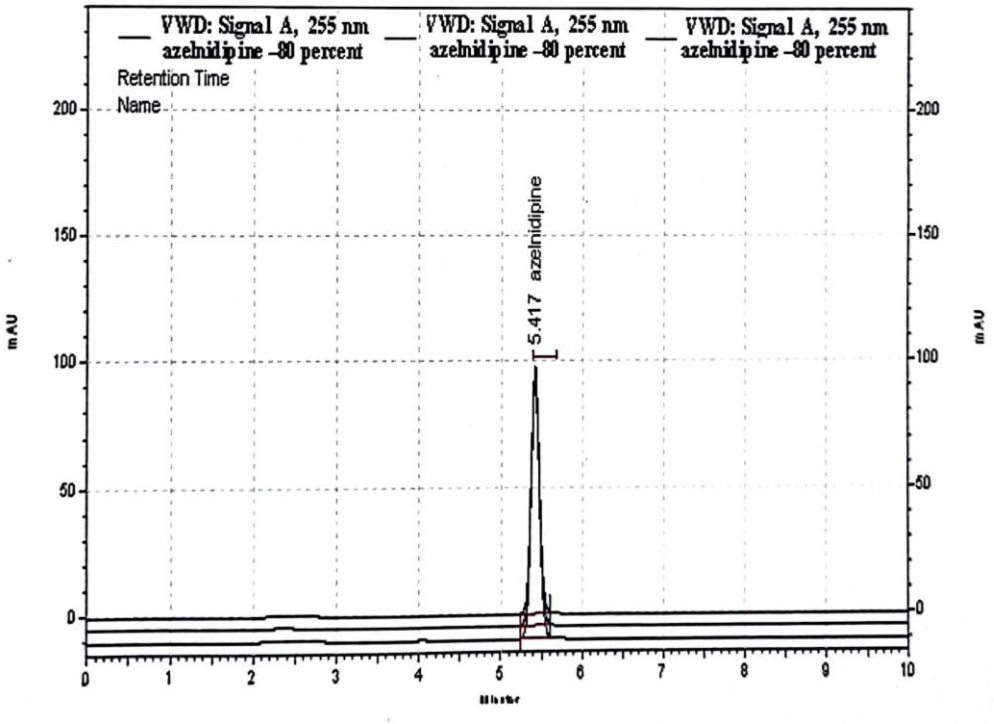

Fig. 11: Chromatogram for 80% accuracy.

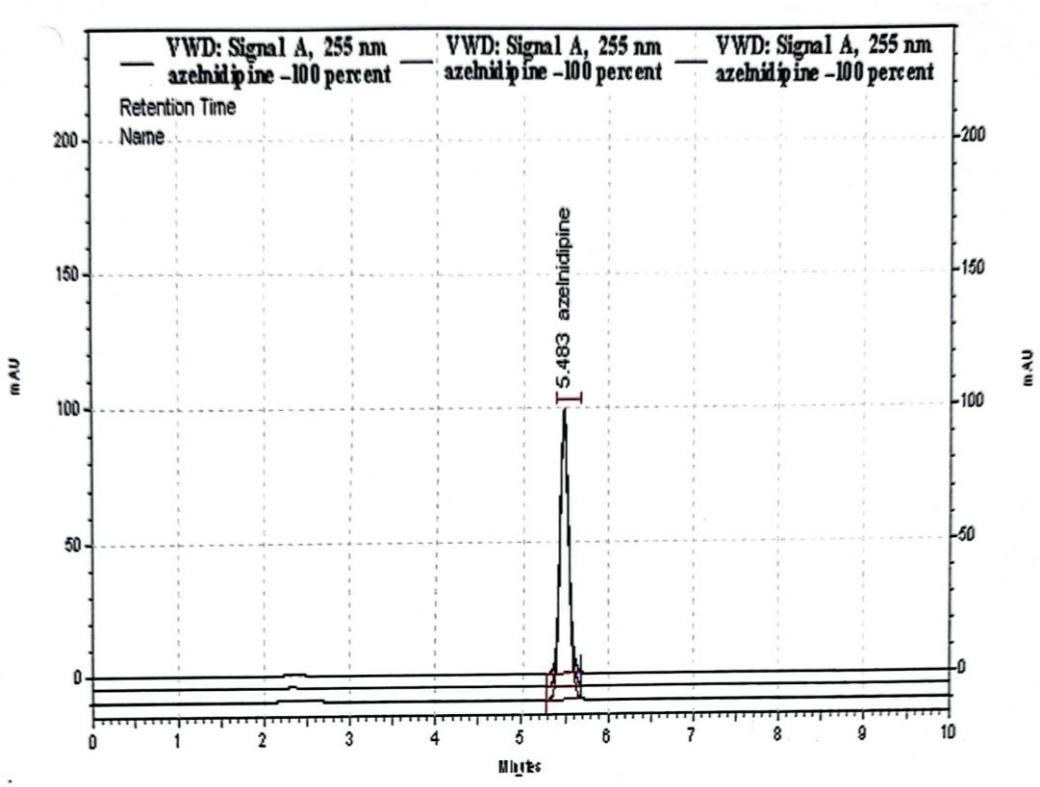

Fig. 12: Chromatogram for 100% accuracy.

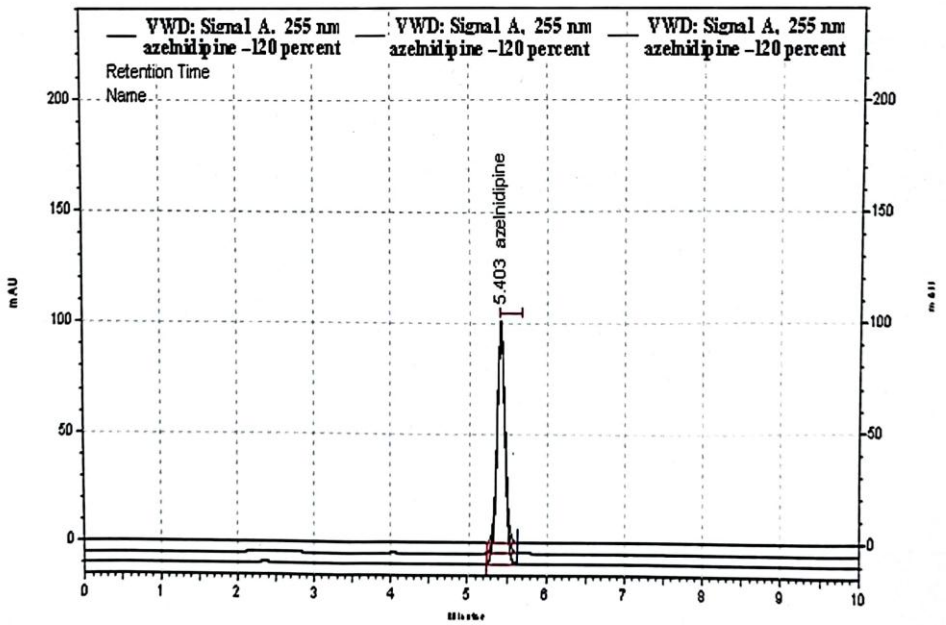

Fig. 13: Chromatogram for 120% accuracy.

Table 3: Accuracy for Azelnidipine.

Accuracy	Peak Area	Average	Standard Deviation	RSD	Recovery	Average
80%	181252	182062.33	861.9630696	0.47%	99.67	100
	181967				99.59	
	182968				100.73	
100%	1812523	1816012.3	3186.805506	0.18%	100.23	100
	1818769				100.63	
	1816745				99.14	
120%	2175027	2180543	7243.460292	0.33%	99.28	99.54
	2177856				99.91	
	2188746				99.44	

3.5. 4. Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were calculated according to ICH recommendations where the approach is based on the signal-to-noise ratio. Chromatogram signals obtained with known low concentrations of analytes was compared with the signals of blank samples. A signal to noise ratio 3:1 and 10:1 was considered for calculating LOD and LOQ respectively. The values of LOD and LOQ were given in Table No 5.

Table 4: LOD and LOQ for estimation of Azelnidipine.

S.N	Name of Validation parameter	Signal/noiseRatio (S/N)	LOD(µg/ml)	LOQ (µg/ml)
1.	Azelnidipine	194.817	1.38µg/ml	4.17µg/ml

Table 5: Optimum conditions, optical characteristics and Statistical data of the regression equation in RP-HPLC method for estimation of Azelnidipine.

Parameter	Method	Acceptance Criteria
λ max	255nm	-
Linear Range(µg/ml)	5-30	-
Correlation Coefficient(r2)	0.9984 for Azelnidipine	NLT .995
Limit of Detection (µg/ml)	1.38µg/ml	-
Limit of Quantification(µg/ml)	4.17µg/ml	-
Number of Theoretical Plates per meter	5064	NLT 2000
Tailing Factor	1.27842	NMT 2
Capacity Factor	0.03381	-

CONCLUSION

In addition to positive requirements for analytical methods, the striking advantage of all the developed method is that they are economical, cheap, precise. The proposed RP-HPLC method were suitable technique for the determination of Azelnidipine. All the parameters analyzing Azelnidipine met the criteria of ICH guidelines for Method Validation. In the present investigation, we have developed a simple, sensitive, precise and accurate RP-HPLC method for the quantitative estimation of Azelnidipine in bulk and pharmaceutical formulations. The recoveries achieved were found good by the method. The HPLC method is more sensitive, precise and accurate compared to the spectrophotometric methods. The HPLC method developed may be recommended for the routine determination of Azelnidipine in bulk drug and pharmaceutical formulations.

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