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ANALYTICAL METHOD DEVELOPMENT, VALIDATION AND FORCE DEGRADATION STUDY FOR ESTIMATION OF AZELNIDIPINE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

A novel simple, rapid, sensitive and stability indicating LC-MS/MS method was developed and validated for the determination and identification of a degradation product for Azelnidipine. Shimadzu LC-20 AT equipped with a mass spectrometer ABScix API 200 was used for LC-MS/MS. The chromatographic analysis was carried out using Agilent, Zorbax, C18 (150mm x 4.6mm, 5µm) column, using a 20:80 mixture of 10mM ammonium formate buffer in water having pH 4.0 and acetonitrile as a mobile phase. The flow rate was kept at 1 mL/min with a run time of 10 minutes and the sample injection volume was 20 µL. MRM scans for m/z products 583.400 (daughter ion 496.200) have been performed for Azelnidipine. A force degradation study has been performed using LC-MS for Azelnidipine at different stress levels (acidic, alkaline, oxidative, photolytic and thermal). Azelnidipine was unstable in acidic, basic as well as oxidation conditions, whereas it was stable in thermal and photo-degradation conditions. Validation parameters such as linearity, range, LOD and LOQ, accuracy, precision and robustness were evaluated as per ICH guidelines. The proposed LC-MS/MS method and its validation according to the ICH guidelines show that the developed method is sensitive, precise, accurate and simple for the determination of Azelnidipine.

KEYWORD:- Azelnidipine, Force degradation, Liquid Chromatography-Mass Spectrometry, Method development, Method validation.

1. INTRODUCTION

Azelnidipine is a novel long acting dihydropyridine calcium channel blocker having chemical name is 3-(1-diphenylmethylazetidin-3-yl)-5-isopropyl-2-amino-1,4- dihydro-6-methyl-4-(3-nitrophenyl)-3,5 pyridine dicarboxylate.[1] Azelnidipine works primarily by inhibiting L and T calcium channel blockers. The drug has the potential to lower blood pressure in the same way that other anti-hypertensive dihydropyridines, such as amlodipine, do, but without increasing pulse rate. The calcium channel blockers are shown that the retard atherogenesis in animal models and to prevent the development of early lesions in human coronary arteries. They are used to treat angina pectoris and high blood pressure.[1,2]

A review of previously published literature reveals that very few Analytical methods for the determination of Azelnidipine are reported. Which includes UV-Spectroscopy,[2,3] HPLC,[4-8] LC-MS,[9-11] LC-ESI-MS,[1,12] and HPTLC,[13] methods. The purpose of this study was to develop a single, simple, fast and suitable stability-indicating LC-MS/MS method for detection and quantification of Azelnidipine. The method was extensively validated and subsequently applied for the force degradation studies for using LC-MS/MS. The developed method was validated as per International Conference on Harmonization (ICH) guidelines.[14]

Morever, Azelnidipine degradation behaviour under different degradation conditions has been reported with limited shreds. The study was set out to explore the degradation behavior of Azelnidipine under different degradation conditions. The force degradation study was performed to assess the stability of Azelnidipine in drugs and their products.

2. MATERIALS AND METHODS

2.1. Materials

Azelnidipine was purchased from par Drugs Pvt Ltd. The LC-MS grade solvents used in the mobile phase were Water (Aquarch), Acetonitrile (J.T. Baker), and Methanol (J.T. Baker). Ammonium formate, hydrogen peroxide, and hydrochloric acid were the chemicals used in the analysis.

2.2. Instrumentation

A Shimadzu LC-20 AT instrument equipped with an ABScix API 200 mass spectrometer was used in the study. The LC-MS/MS instrument included an auto sampler, auto-injector, column oven, and an ESI (electron spray ioniser) ion source with Q1 and collision energy. Agilent, Zorbax, C18column, (150mm x 4.6mm), 5µm size was used for the analysis. The

experiments were carried out using a Shimadzu ATX-224 Weighing Balance, a Frontline 1870 Ultra Sonicator, an Analab Scientific Private Ltd pH metre, a Kesar Control System Hot Air Oven, and a Photo stability Chamber.

2.3. Standard and Sample preparation

Water: Acetonitrile (50:50) was used as the diluent.

Azelnidipine Standard Stock Solution: Weigh and transfer approximately 16.0mg of Azelnidipine into a 100ml volumetric flask, then make up the volume with diluent (160µg/ml). Working Standard Solution was made by pouring 1ml of Azelnidipine stock solution into a 100ml volumetric flask and filling the remaining volume with diluent. (AZEL-1.6µg/ml).

2.4. LC-MS/MS analysis

The LC-MS/MS analysis was carried out as part of the method development, validation, and degradation. As the isocratic mobile phase, a 20:80 mixture of 10mM ammonium formate buffer in water having pH 4.0 and acetonitrile was used. The mobile phase flow rate was set at 1.0 mL/min for the entire study, with a total run time of 10 minutes. The injection volume was 20 µL, and the column oven temperature was kept at 35 °C. A MRM (multiple reaction monitoring) scan study was performed using positive ESI mode for mass spectrometry. Whereas the Curtain gas 30psi, the Ion spray voltage 5000Kv, the Source temperature 400°C, and the Ion source gases 1 and 2 were 50psi and 50psi, respectively, Declustering potential 20 volts, Entrance potential 10 volts, and Focusing potential 400 volts. MRM scan for m/z product 583.400 (daughter ion 496.200) (centroid) has been carried out. To identify and characterise the degradation products, all experimental conditions were optimised to increase sensitivity at very low concentrations. The method was validated by evaluating LOD, LOQ, linearity, ruggedness, accuracy, and precision.

2.5. Method validation

2.5.1. Linearity and Range

Linearity refers to the ability to obtain test results that are directly proportional to the concentration (amount) of the chemical substance being analyzed in the sample within a given range. The standard preparation range was from 50% to 150%. The standard solutions for Linearity are as shown in Table.1.

Table 1: Standard solutions for linearity.

Linearity Level (%)	Std stock soln of Azelnidipine (ml)	Make up with diluent (ml)	Conc of Azelnidipine (µg/ml)
50	0.50	100	0.8
75	0.75	100	1.2
100	1.00	100	1.6
125	1.25	100	2.0
150	1.50	100	2.4

2.5.2. Precision

For intraday precision, three concentrations in the given range (n=3) were measured on the same day. For interday precision, three concentrations within the range (n=3) were measured on three consecutive days. Concentrations of Azelnidipine considered were: Lower concentration: 50% (0.8 µg/ml), Middle concentration: 100% (1.6 µg/ml) and Higher concentration: 150 %(2.4 µg/ml). At each level, %RSD (relative standard deviation) should not exceed 2.0%.

2.5.3.Repeatability

Repeatability of the method (n=6) was assessed for one concentration, 100% (1.6 µg/ml).

2.5.4. Recovery

Standard Stock Solution of Azelnidipine was prepared by Weighing and transferring approximately 16.0mg of Azelnidipine into a 100ml volumetric flask, then make up the volume with diluent (160 µg/ml). Different Recovery level of solutions were prepared as shown in the Table.2.

Table 2: Recovery level of solutions.

Recovery Level (%)	Solution 1(ml)	Std stock soln of Azelnidipine (ml)	Diluted with diluents (ml)
80	0.5	0.4	10
100	0.5	0.5	10
120	0.5	0.6	10

2.5.5. Robustness

In order to test robustness, different flow rates and different mobile phase compositions of working standard solution were injected. (Flow rate: ±0.2ml/mint, Solvent % in mobile phase: ±2% solvent).

2.5.6. Assay for marketed formulation

Azelnidipine standard stock solution was prepared by weighing approximately 16.0mg Azelnidipine into a 100ml volumetric flask and diluting the volume with diluent (160µg/ml). To make the working standard solution, 1ml of the Azelnidipine stock solution was diluted with diluent in a 100ml volumetric flask (AZEL-1.6µg/ml). The sample stock solution for the assay was prepared by weighing 16mg of Azelnidipine tablets. Afterwards, the crushed tablet was transferred into a 100ml volumetric flask and Adding 60 ml of diluent to the flask and sonicating for 15 minutes in a sonicator. The final volume was makeup to 100ml with diluent. Finally this solution was filtered using Whatman filter paper no-1. Sample Working Solution for the assay was made up of 1ml from sample stock solution diluted with diluent into a 100ml volumetric flask (AZEL -1.6µg/ml).

2.6. Force degradation studies

Force degradation experiments were carried out to determine the stability of Azelnidipine under various experimental conditions, including acidic, basic, oxidative, thermal, and photolytic.

2.6.1.Forced degradation solution preparation to find out degrading compound from standard (By infusing sample into mass spectrometer)

Azelnidipine degradation procedure

1) **Acid degradation:** 1.0 ml of standard stock solution was transferred into a 100 ml volumetric flask, followed by 1.0 ml 1N HCL, and put the volumetric for 4 hours on water bath at 60^0C. After 4 hours, it was diluted to make up the volume with diluent (1.6 µg/ml).
2) **Base degradation:** 1.0 ml of standard stock solution was transferred into a 100 ml volumetric flask, followed by 1.0 ml 1N NaOH, and put the volumetric for 3 hours on water bath at 60^0C. After 3 hours, it was diluted to make up the volume with diluent (1.6 µg/ml).
3) **Oxidation degradation:** 1.0 ml of standard stock solution was transferred into a 100 ml volumetric flask, followed by 1.0 ml 30.0% Hydrogen Peroxide, and put the volumetric for 5 hours on water bath at 60^0C. After 5 hours, it was diluted to make up the volume with diluent (1.6 µg/ml).
4) **Thermal degradation:** 40.0 mg of Azelnidipine standard was taken into a petridish and placed the petridish into hot air oven at 105^0C for 5 days. After 5 days, weighed and

transferred about 16.0 mg of above powder into a 100 ml volumetric flask and diluted to make up the volume with diluent. Further, transferred 1.0 ml solution into a 100 ml volumetric flask and diluted to make up the volume with diluent (1.6 µg/ml)

5) **Photo degradation:** Put about 40.0 mg of Azelnidipine standard into petridish and place the petridish into a photo stability chamber for 5 days. After 5 days, weighed and transferred about 16.0 mg of above powder into a 100 ml volumetric flask and diluted to make up the volume with diluent. Further, Transferred 1.0 ml above solution into a 100 ml volumetric flask and diluted to make up the volume with diluent (1.6 µg/ml).

Note: The forced degradation preparation solution of Azelnidipine is infused into the mass spectrometer to identify the degradation product of Azelnidipine.

2.6.2.Forced degradation solution preparation for LC-MS/MS

Sample Stock Solution for forced degradation: 16.0 mg of AZEL was accurately weighed into a 100 ml volumetric flask and added 60 ml of diluent and Shake for 15 minutes in sonicator. Make up volume with diluent upto the mark. Filtered this solution with whatman filter paper no-1. 1.0 ml of this solution was transferred into 100 ml volumetric flask and make up volume up to the mark with diluent.

(AZEL-1.6µg/ml).

Azelnidipine degradation procedure

1) **Acid degradation:** Standard Preparation: 1.0 ml of standard stock solution was transferred into a 100 ml volumetric flask, followed by 1.0 ml 1N HCL, and put the volumetric for 4 hours on water bath at 60^0C. After 4 hours, it was diluted to make up the volume with diluents (1.6 µg/ml). Sample Preparation: 1.0 ml of sample stock solution was transferred into a 100 ml volumetric flask, followed by 1.0ml 1N HCL, and put the volumetric for 4 hours on water bath at 60^0C. After 4 hours, it was diluted to make up the volume with diluent (1.6 µg/ml).
2) **Base degradation:** Standard Preparation: 1.0 ml of standard stock solution was transferred into a 100 ml volumetric flask, followed by 1.0 ml 1N NaOH, and put the volumetric for 3 hours on water bath at 60^0C. After 3 hours, it was diluted to make up the volume with diluent (1.6 µg/ml). Sample Preparation: 1.0 ml of sample stock solution was transferred into a 100 ml volumetric flask, followed by 1.0 ml 1N NaOH, and put the volumetric for 3 hours on water bath at 60^0C. After 3 hours, it was diluted to make up the volume with diluents (1.6 µg/ml).

3) **Oxidation degradation:** Standard Preparation: 1.0 ml of standard stock solution was transferred into a 100 ml volumetric flask, followed by 1.0 ml 30.0% Hydrogen Peroxide, and put the volumetric for 5 hours on water bath at 60^0C. After 5 hours, it was diluted to make up the volume with diluent (1.6 µg/ml). Sample Preparation: 1.0 ml of sample stock solution was transferred into a 100 ml volumetric flask, followed by 1.0 ml 30.0% Hydrogen Peroxide, and put the volumetric for 5 hours on water bath at 60^0C. After 5 hours, it was diluted to make up the volume with diluents (1.6 µg/ml).
4) **Thermal degradation:** Standard Preparation: 40.0 mg of Azelnidipine standard was taken into petridish and place the petridish into hot air oven at 105^0C for 5 days. After 5 days, weighed and transferred about 16.0 mg of above powder into a 100 ml volumetric flask and diluted volume with diluent. Transferred 1.0 ml above solution into a 100 ml volumetric flask and diluted volume with diluents (1.6 µg/ml). Sample Preparation: 40.0 mg of Azelnidipine sample was taken into petridish and place the petridish into hot air oven at 105^0C for 5 days. After 5 days, weighed and transferred about 16.0 mg of above powder into a 100 ml volumetric flask and diluted volume with diluent. Transferred 1.0 ml above solution into a 100 ml volumetric flask and diluted volume with diluent (1.6 µg/ml).
5) **Photo degradation:** Standard Preparation: 40.0 mg of Azelnidipine standard was taken into petridish and place the petridish into a photo stability chamber for 5 days. After 5 days, weighed and transferred about 16.0 mg of above powder into a 100 ml volumetric flask and diluted volume with diluent. Transferred 1.0 ml above solution into a 100 ml volumetric flask and diluted volume with diluent (1.6 µg/ml). Sample Preparation: 40.0 mg of Azelnidipine sample was taken into petridish and place the petridish into a photo stability chamber for 5 days. After 5 days, weighed and transferred about 16.0 mg of above powder into a 100 ml volumetric flask and diluted volume with diluent. Transferred 1.0ml above solution into a 100 ml volumetric flask and diluted volume with diluent (1.6 µg/ml).

3. RESULTS AND DISCUSSION

3.1. Method development

The developed method enables the quantification of AZEL by liquid chromatography coupled with tandem mass spectrometry. This LC-MS/MS method was designed to be optimized for separation and resolution based on the chemical composition of the mobile phase, pH level, flow rate, column selection, column oven temperature, and analyte

concentration. An optimised mobile phase was a 20:80 mixture of 10mM ammonium formate buffer in water having pH 4.0 and acetonitrile. Agilent, Zorbax, C18column, (150mm x 4.6mm), 5µm size was used for the analysis. The column temperature was set to 35 °C, the injection volume was 20µL, and the run time was set to 10 minutes with a flow rate of 1 mL/min. After injection of Azelnidipine in LC-MS/MS, a Chromatogram with a single peak at retention time 3.29 min and Mass spectrum of Azelnidipine were obtained as shown in Fig.1-A and Fig.1-B respectively.

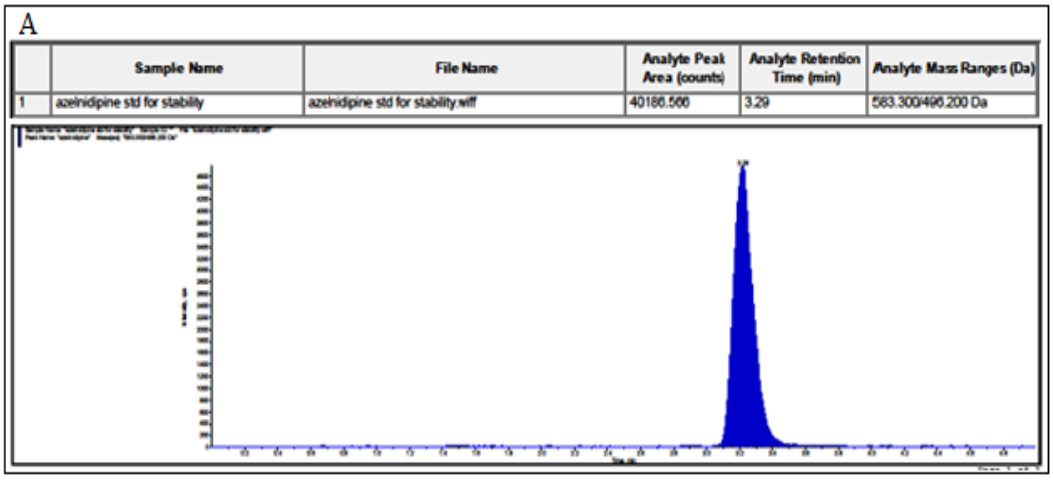

Fig. 1: A Chromatogram of azelnidipine.

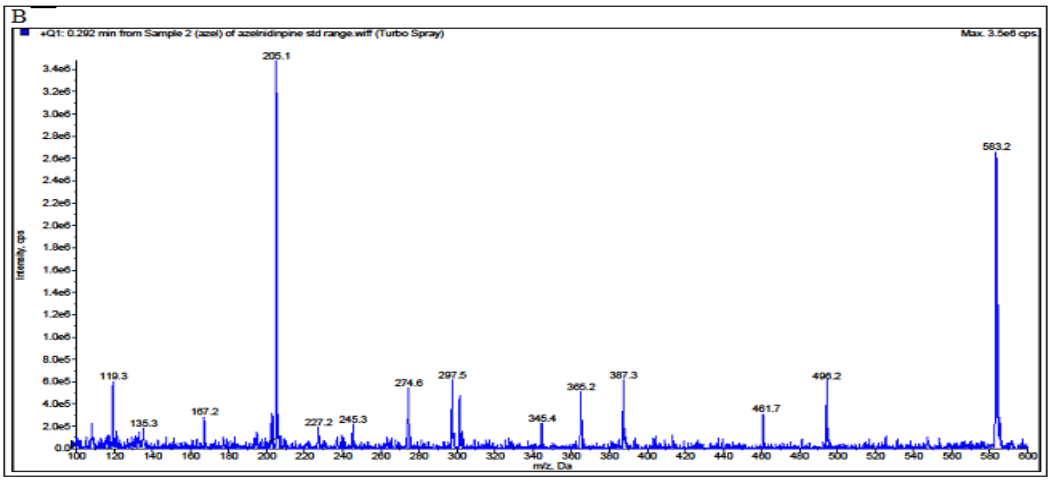

Fig. 1: B Mass spectrum of azelnidipine.

3.2. Method validation

The further test for LOD and LOQ were performed after optimization, which showed that Azelnidipine's LOD and LOQ were 0.173 and 0.526 respectively. Drug response was found to be linear in the range of 50% to 150%. As shown in Figure 2, the calibration curve was linear with a correlation coefficient of 0.997. The method was precise by intraday and

interday precision study. The mean percent recovery achieved at 80%, 100%, and 120% was between 98% and 102% for all three levels. Robustness was studied by changing two conditions. These conditions include change in Flow rate: ±0.2ml/mint and Solvent % in mobile phase: ±2% solvent. Table.3 to Table.7-B shows results for Precision, Repeatability, Recovery, Robustness, and Assay respectively for Azelnidipine. The percentage of RSD for the area at each level should not exceed 2.0%.

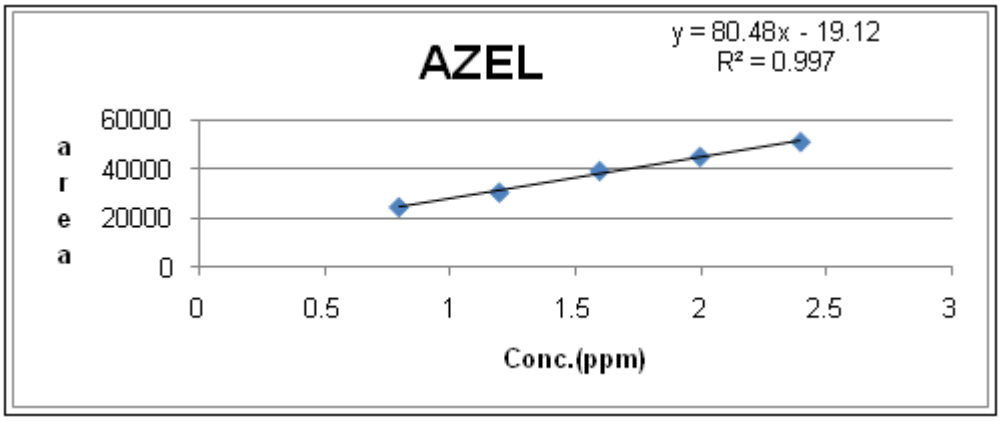

Fig. 2: Calibration curve azelnidipine.

Table 3: Results of precision study for azelnidipine (n=3).

	Concentration µg/ml	Intraday Precision			Interday Precision		
		Concentration measured		%RSD	Concentration measured		%RSD
		AVG	SD		AVG	SD	
50%	0.8	25190.911	449.015	1.782	24892.269	228.435	0.918
100%	1.6	39025.812	349.649	0.896	39613.314	675.742	1.706
150%	2.4	51827.516	785.970	1.517	51328.091	427.463	0.833

Table 4: Results of repeatability study for azelnidipine.

Std	Area
1	38961.542
2	39642.826
3	38426.820
4	39641.595
5	39014.580
6	37983.582
Avg	38945.158
%RSD	1.692

Table 5: Results of accuracy (%Recovery) study for azelnidipine.

Amounted added in concentration level (%)	amount added(µg/ml)	amount recovered(µg/ml)	%recovery	avg	%rsd
80	0.64	0.636 0.644 0.647	99.421 100.578 101.140	100.380	0.873
100	0.80	0.801 0.814 0.790	100.067 101.780 98.780	100.209	1.502
120	0.96	0.975 0.960 0.943	101.524 100.044 98.235	99.934	1.649

Table 6: Results of robustness study for azelnidipine (Average of three replicates).

Factors	Level	Peak Area	RSD (%)
Flow rate	+0.2	27956.672	0.935
	-0.2	41312.162	1.393
Mobile phase	+2	33062.800	1.104
	-2	40938.373	1.216

Table 7: A Results of Assay for Azelnidipine (At 100% of sample solution).

Area of std.	39558.426
Area of samples 1) 38992.641 2)39824.942 3)39543.654	%Assay 98.570 100.674 99.963
Avg. assay %RSD of assay	99.735 1.073

Table 7: B Results of Assay for Azelnidipine (Lable claim).

Label claim(W/W)	Result(W/W)	% Assay	Avg %Assay	SD	%RSD
16 16 16	15.771 16.108 15.994	98.570 100.674 99.963	99.735	1.070	1.073

3.3. Force degradation study

3.3.1. Acidic condition

The mass spectrum changed after Azelnidipine injection, as shown in Figure 3-A, indicating Azelnidipine degradation. As a result, it demonstrates that the drug is unstable in acidic conditions. Major degradation occurred at retention times of 3.30 minutes for the standard and 3.25 minutes for the sample, as shown in Fig.3-B and Fig.3-C respectively.

A

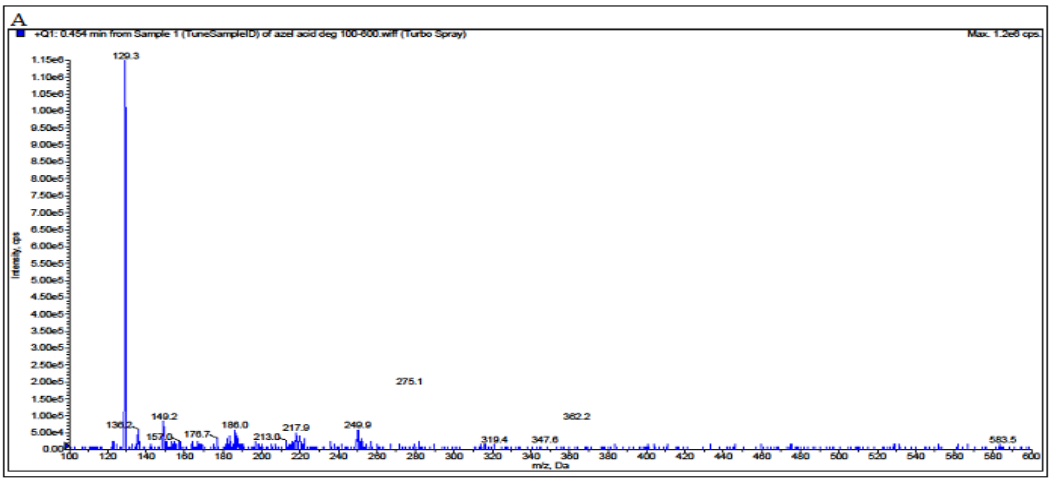

Fig. 3: A Azelnidipine acid degradation mass spectra.

B

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	azel acid deg std DP1	azel acid deg std DP1.wiff	7737.435	3.31	583.300/496.200 Da
2	azel acid deg std DP1	azel acid deg std DP1.wiff	764.453	3.30	362.200/275.100 Da

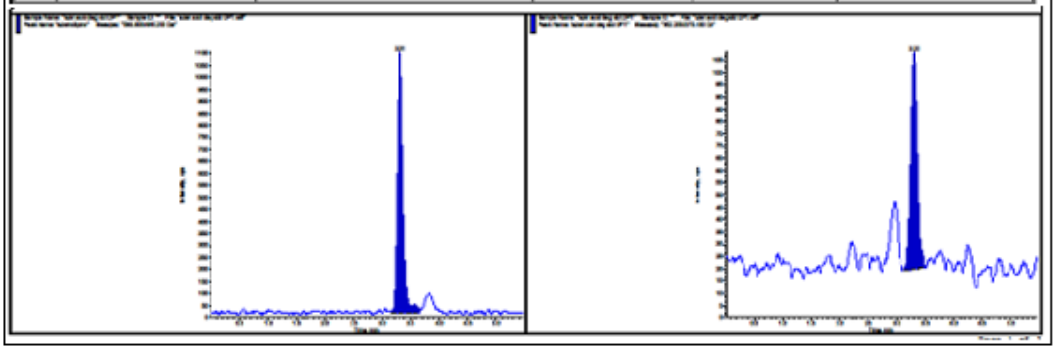

Fig. 3: B Chromatogram of degraded product in acid (standard).

C

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	azel acid deg sample DP1	azel acid deg sample DP1.wiff	7658.596	3.34	583.300/496.200 Da
2	azel acid deg sample DP1	azel acid deg sample DP1.wiff	782.143	3.25	362.200/275.100 Da

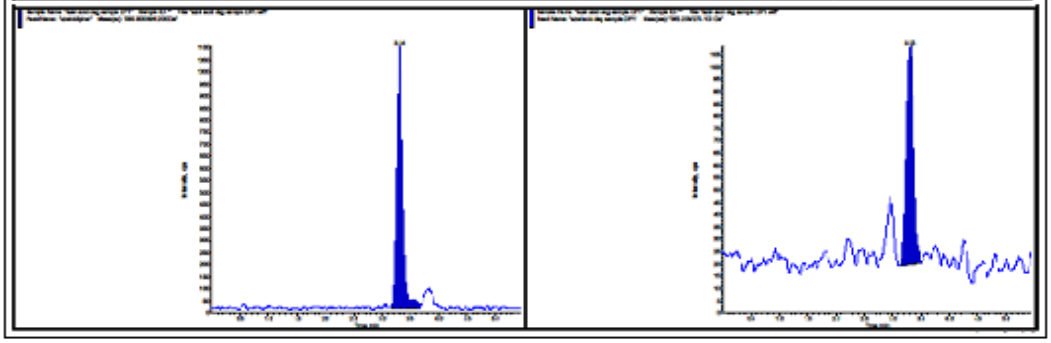

Fig. 3: C Chromatogram of degraded product in acid (sample).

3.3.2. Basic condition

Azelnidipine degrades in alkaline condition. As a result, it confirms that the drug was unstable in its alkaline condition. The mass spectrum for base degradation is shown in Fig.4-A, with degradation occurring at retention times of 1.60 min for standard and 1.62 min for sample, as shown in Fig.4-B and Fig.4-C respectively.

A

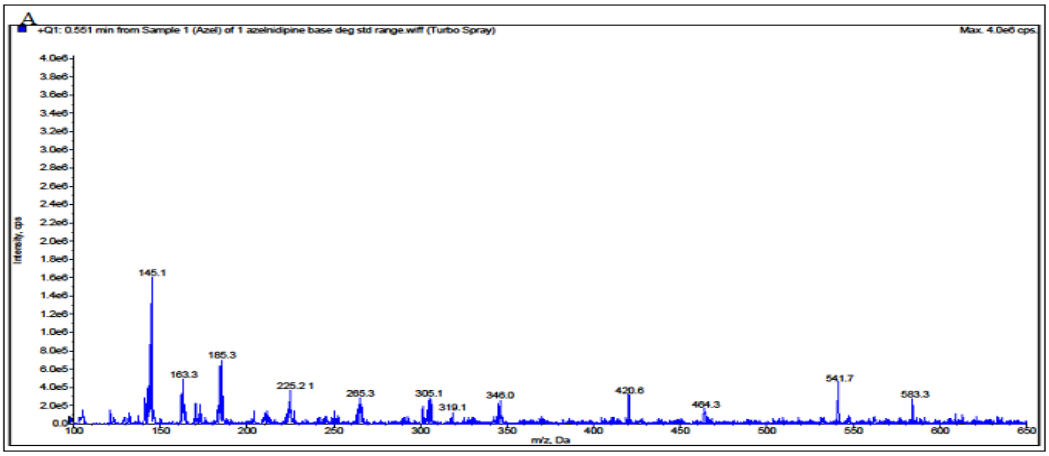

Fig. 4: A Azelnidipine base degradation mass spectra.

B

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	azel base deg std DP2	azel base deg std DP2.wiff	19334.302	3.38	583.300/496.200 Da
2	azel base deg std DP2	azel base deg std DP2.wiff	15638.246	1.60	541.700/420.600 Da

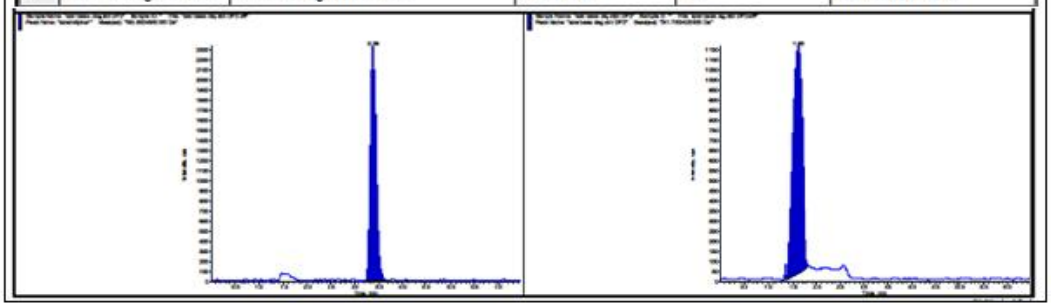

Fig. 4: B Chromatogram of degraded product in base (Standard).

C

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	azel base deg sample DP2	azel base deg sample DP2.wiff	19263.425	3.31	583.300/496.200 Da
2	azel base deg sample DP2	azel base deg sample DP2.wiff	15744.979	1.62	541.700/420.600 Da

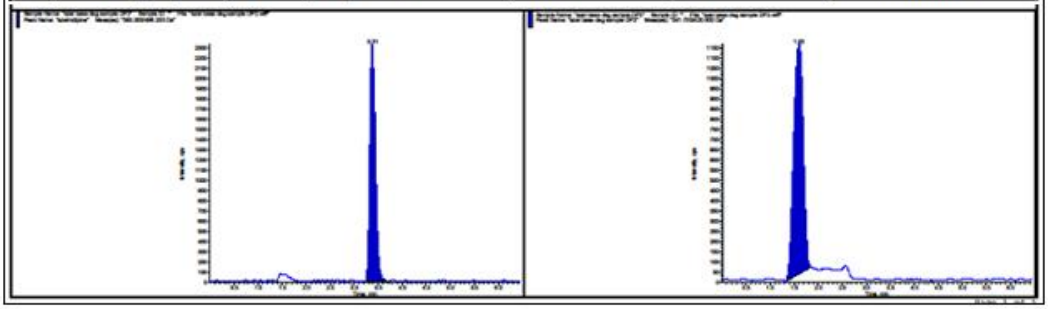

Fig. 4: C Chromatogram of degraded product in base (Sample).

3.3.3. Oxidation condition

In hydrogen peroxide solution, degradation was observed. The mass spectrum for Oxidation degradation is shown in Fig.5-A, indicating degradation at retention times of 2.42 minutes for standard and 2.40 minutes for sample, as shown in Fig.5-B and Fig.5-C respectively. Therefore, it can be concluded that Azelnidipine is unstable in oxidation.

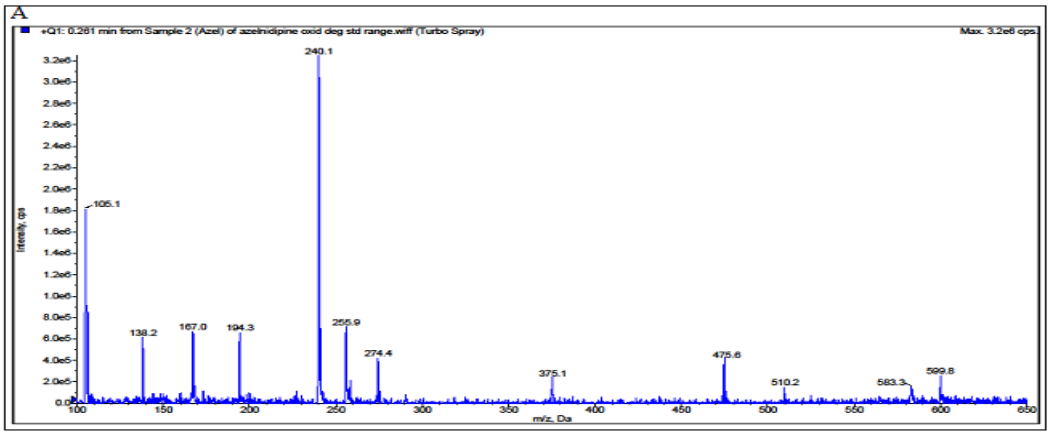

Fig. 5: A Azelnidipine oxidation degradation mass spectra.

B

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	azel oxd deg std DP3	azel oxd deg std DP3.wiff	3743.307	3.30	583.300/496.200 Da
2	azel oxd deg std DP3	azel oxd deg std DP3.wiff	1191.592	2.42	599.800/475.600 Da

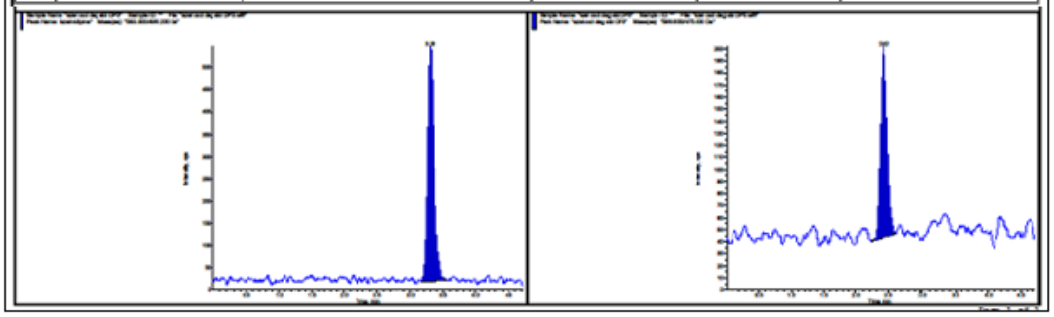

Fig. 5: B Chromatogram of degraded product in oxidation (Standard).

C

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	azel oxd deg sample DP3	azel oxd deg sample DP3.wiff	3621.966	3.36	583.300/496.200 Da
2	azel oxd deg sample DP3	azel oxd deg sample DP3.wiff	1240.133	2.40	599.800/475.600 Da

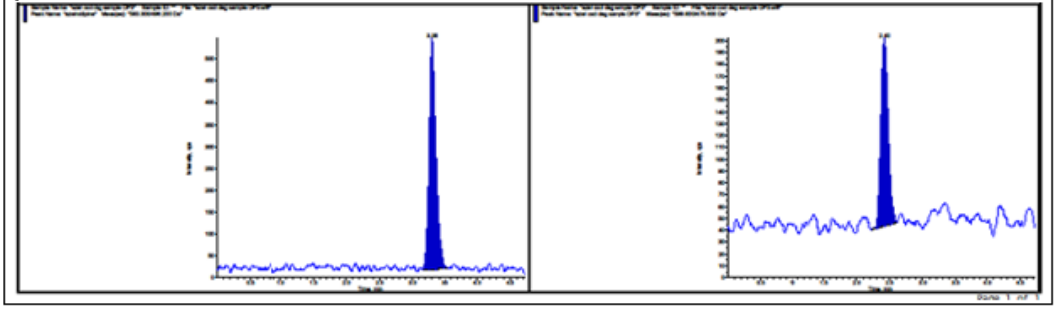

Fig. 5: C Chromatogram of degraded product in oxidation (Sample).

3.3.4. Thermal condition

Azelnidipine did not degrade under thermal condition. Therefore, Azelnidipine was stable in thermal condition. The mass spectrum of thermal degradation is shown in Figure 6.

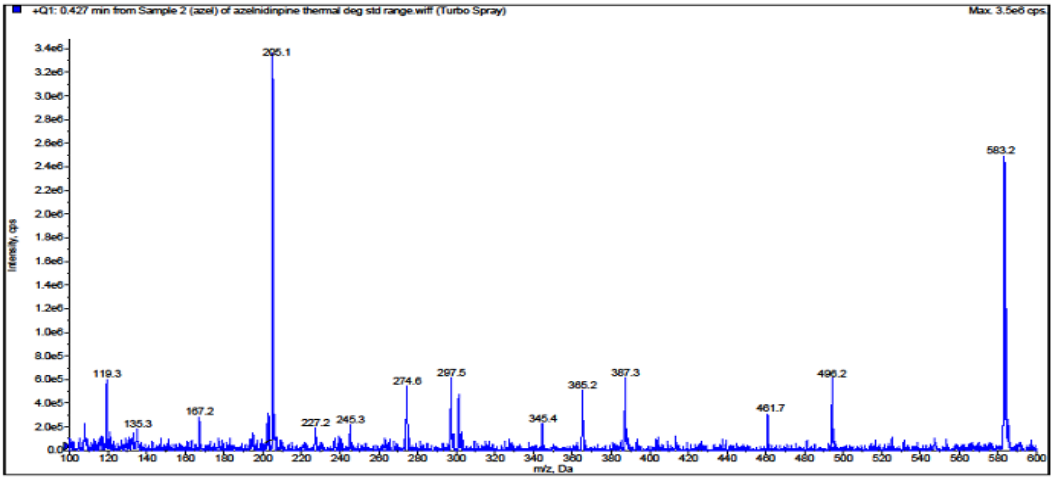

Fig. 6: Azelnidipine thermal degradation mass spectra.

3.3.5. Photo condition

Azelnidipine did not show any degradation in photo condition. Thus, Azelnidipine was stable in photo condition. Mass spectrum for photo degradation is shown in Fig.7.

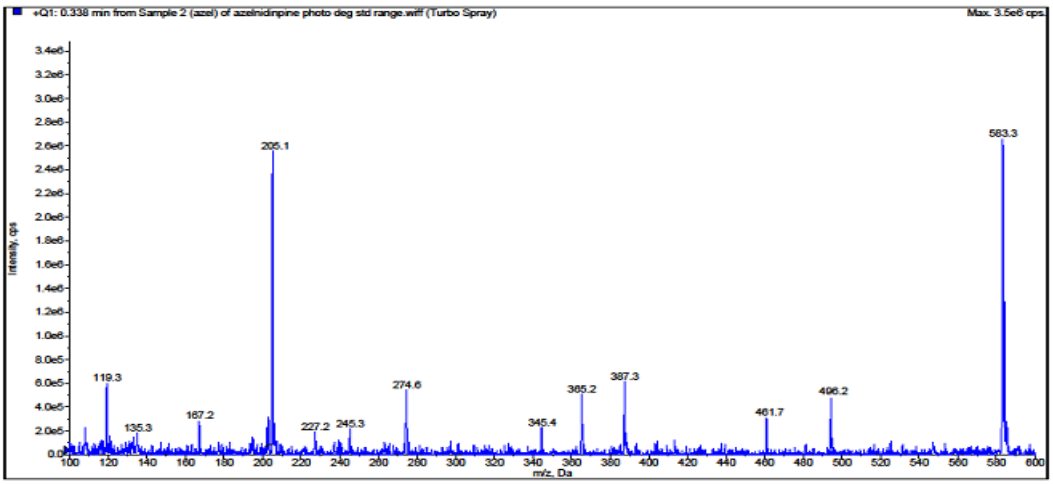

Fig. 7: Azelnidipine photo degradation mass spectra.

3.4. Fragmentation pathway

The degradation products were analysed using LC and LC–MS. The degradation products were subjected to MRM studies in ESI positive mode to determine their molecular ion peaks and to establish their fragment profile. The fragmentation of Azelnidipine [MRM:(Q1)583.400 Da and (Q3) 496.200 Da], Azel DP1 [MRM:(Q1)362.200 Da and (Q3)

275.100 Da], Azel DP2 [MRM:(Q1)541.700 Da and (Q3) 420.600 Da], Azel DP3 [MRM:(Q1)599.800 Da and (Q3) 475.600 Da] are shown in fig. 8-A,B,C,D respectively, where Q1 is for product ion and Q3 is for fragment ion.

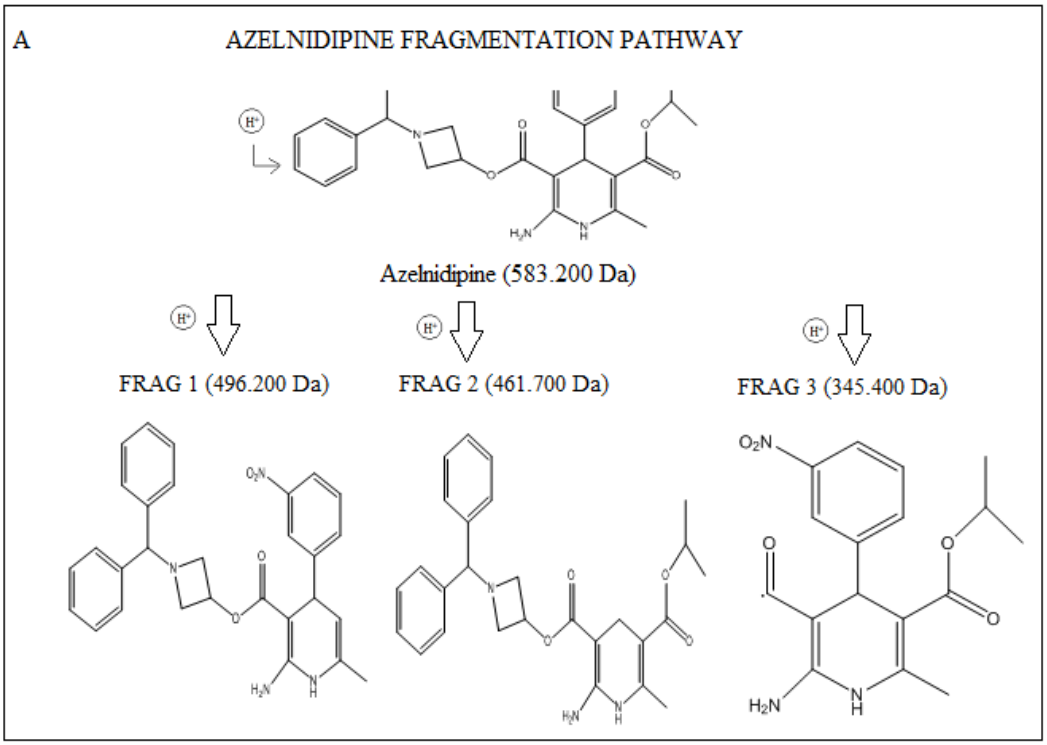

Fig. 8: A azelnidipine fragmentation pathway.

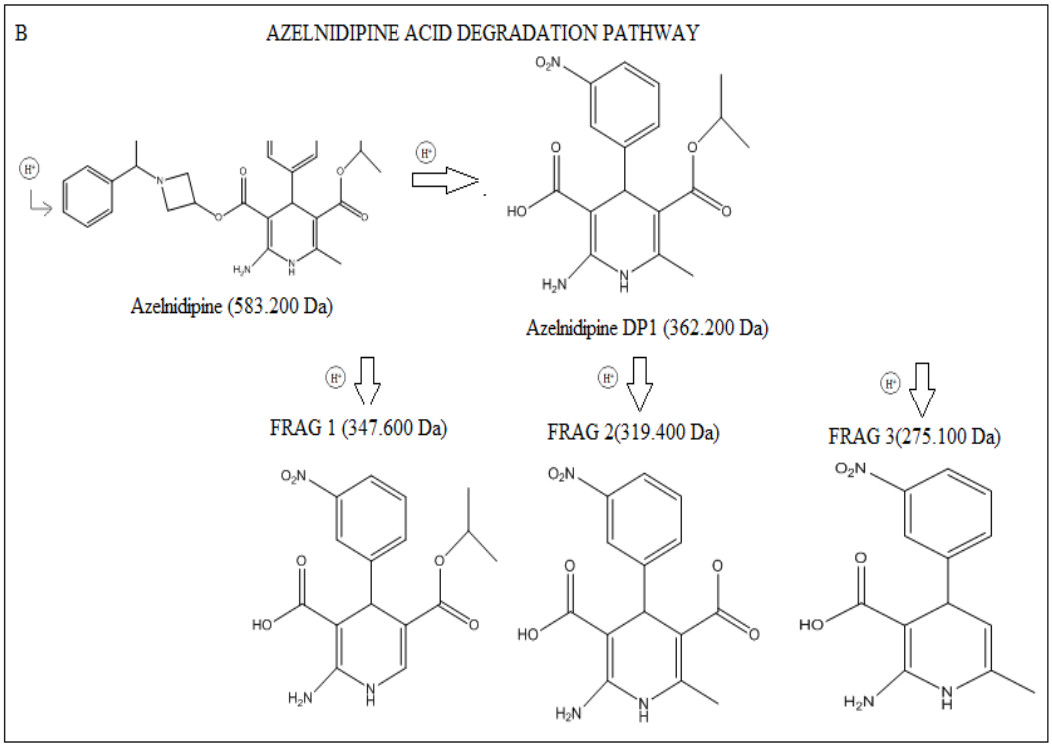

Fig. 8: B azelnidipine acid degradation pathway.

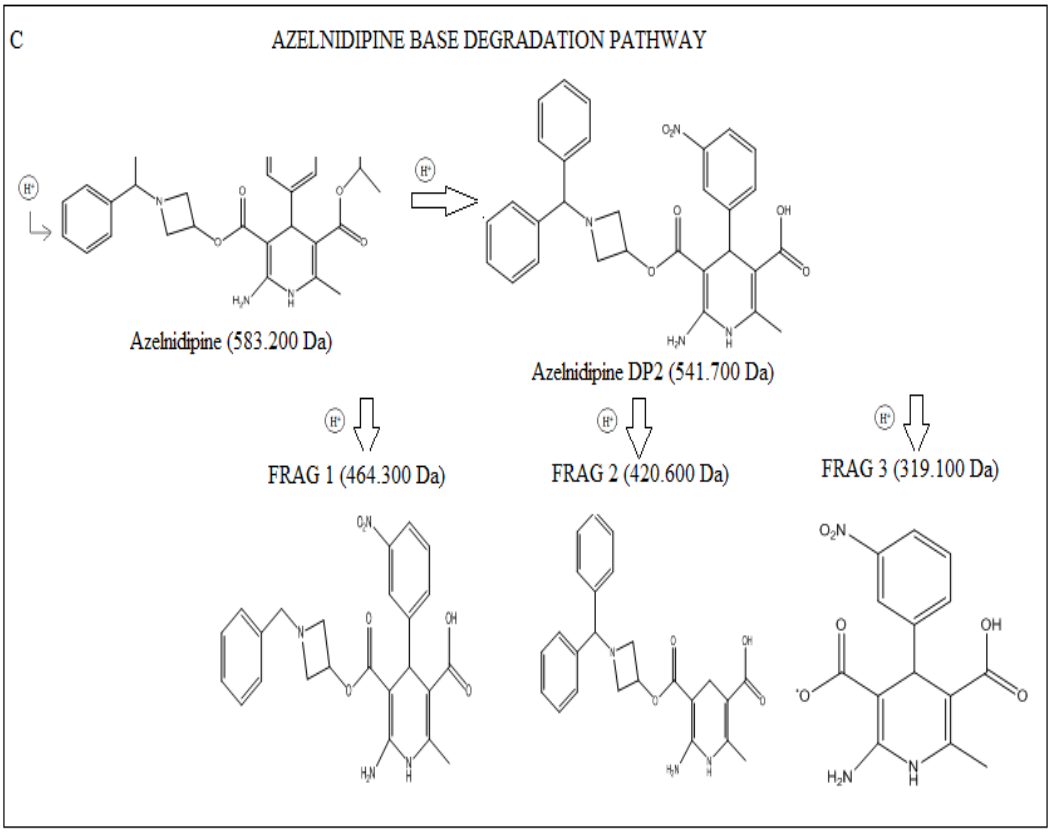

Fig. 8: C Azelnidipine base degradation pathway.

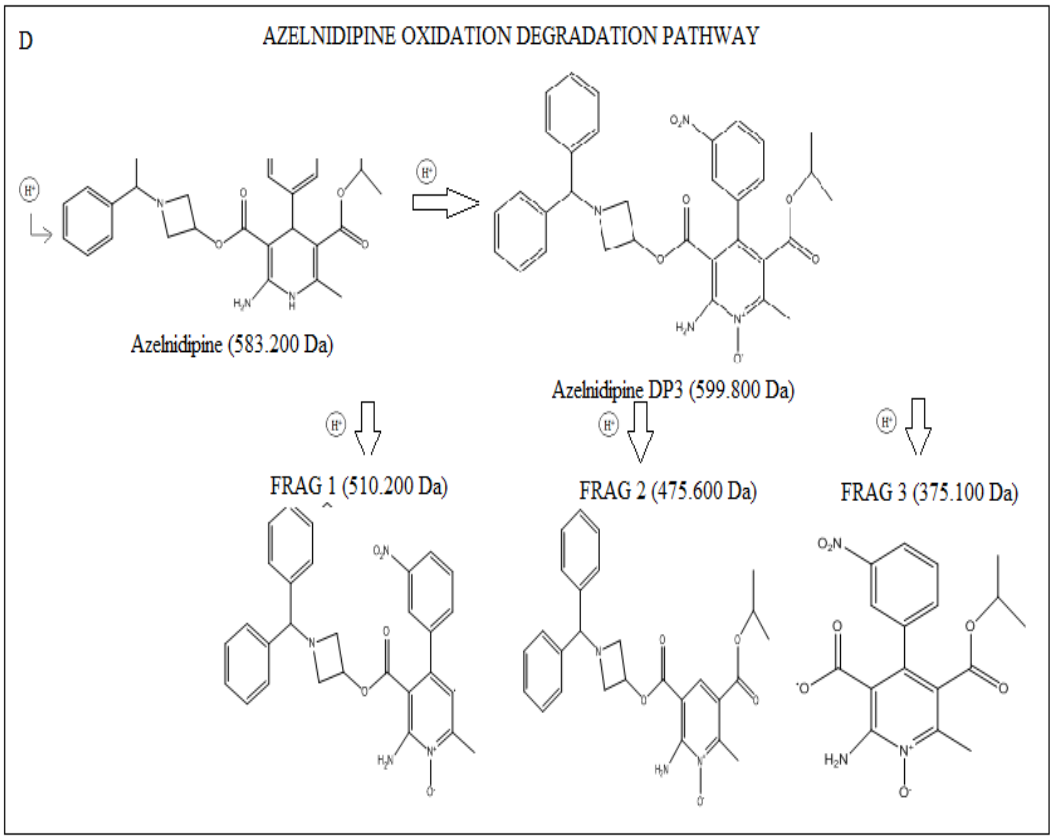

Fig. 8: D Azelnidipine oxidation degradation pathway.

4. CONCLUSION

Here we present research related to the development and validation of LC-MS/MS methods for identifying Azelnidipine's stability in various stress conditions, as well as the possible structures of degradation products. The method proved to be simple and economic as the separation was achieved on C18 coloumn with a mixture of 10mM ammonium formate buffer in water having pH 4.0 and acetonitrile in the proportion of 20:80 v/v and all the formed degradation products along with Azelnidipine were separ ated out within 10 min of run time. Azelnidipine was found to degrade in acidic, basic, and oxidation conditions, but remained stable in thermal and photo degradation conditions. The method proved to be more sensitive and selective toward the determination of degradation products than previously reported methods. According to the ICH Guideline, all validation parameters such as linearity, accuracy, precision, and robustness were found to be less than 2% RSD, indicating that the proposed method is sensitive. The present proposed method is more simple, selective, and sensitive for routine analysis.

5. AKNOWLEDGEMENT

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6. Conflict of interest

The authors have declared no conflict of interest.

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