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RESEARCH ARTICLE

Structural Identification and Characterization of Potential Impurities of Azelnidipine

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ABSTRACT

Azelnidipine (AZL) is a pale yellowish white tablet (16mg) with diameter of 9.2mm and thickness of 3.3mm. A reverse phase performance liquid chromatographic method was developed for the determination of AZL in bulk and pharmaceutical dosage form. During the synthesis of bulk drug of AZL, we observed four impurities. All the impurities were detected by a gradient high performance liquid chromatographic (HPLC) method. LC-MS was performed to identify the mass number of these impurities. A thorough study was carried out to characterize the impurities. These impurities were synthesized, characterized and were co-injected with the sample containing impurities and are found to be matching with the impurities present in the sample. Based on the complete spectral analysis (UV, IR, NMR and MS) these impurities were characterized as 1) Azelnidipine Stage-I para impurity [Impurity 1], whose molecular formula is $C_{14}H_{15}NO_5$ and molecular weight is 277.27, 2) Azelnidipine Intermediate [Impurity 2], whose molecular formula is $C_{14}H_{15}NO_5$ and molecular weight is 277.27, 3) 4-Nitro Azelnidipine [Impurity 3], whose molecular formula is $C_{33}H_{34}N_4O_6$ and molecular weight is 582.65 and, 4) 2-Nitro Azelnidipine [Impurity 4], whose molecular formula is $C_{33}H_{34}N_4O_6$ and molecular weight is 582.65. The proposed method was validated as per International Conference on Harmonization (ICH) guidelines. The method was accurate, precise, specific and rapid found to be suitable for the quantitative analysis of the drug and dosage form.

KEYWORDS

Azelnidipine, Impurity Profiling, Impurities, Identification, IR, UV, NMR, MS, Isolation, Characterization

INTRODUCTION

Azelnidipine (AZL) is a white crystalline powder and is used as Cardiovascular agent. Its brand name is Afine or Beiqi (China) or Calblock (Japan). The therapeutic action of AZL is that it acts as anti-hypertensive agent and also as calcium channel blocker. The present review covers various aspects related to the analytical method development for impurity profiling of an active pharmaceutical ingredient[1]. Impurity profiling procedure was adopted in bulk drugs and in pharmaceutical preparation[2]. Stability indicating HPLC – DAD/UV-ESI/MS impurity profiling of the anti-malarial drug lumefantrine was also studied[3]. The International Conference on Harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use had published guidelines for validation of methods for analyzing impurities in

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new drug substances, products, residual solvents and micro biological impurities[4]. A number of articles have stated guidelines and designed approaches for isolation and identification of process-related impurities and degradation products, using Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography (HPLC), Fourier Transform Ion Cyclotron Resonance Mass Spectroscopy (FTICR – MS), and Tandem Mass Spectroscopy for pharmaceutical substances[5-7].

The presence of impurities in an active pharmaceutical ingredient (API) can have a significant impact on the quality and safety of the drug product. During the analysis of laboratory batches of AZL, four impurities were observed in HPLC method. In order to commercialize an API, it is a mandatory requirement by regulatory authorities to identify and characterize all the unknown impurities that are present in it at a level more than 0.1%[8]. These impurities are required in pure form to check the HPLC method performance such as specificity, linearity, range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, system suitability testing and relative response factor (RRF)[9,10]. The recommended dosing of AZL is 16 mg per day. A literature survey revealed that very few analytical methods have been reported for the determination of AZL, which include HPLC[11,12], an LC-MS method[13], HPLC-MS-MS[14] and LC-ESI-MS[15], in which two methods were for the formulation and the remaining for human plasma. No single method was discussed about the impurity profiling of AZL drug. These related substances are also used to check the accuracy of the analytical method of API.

The structure of possible impurities related to raw materials or degradants is identified by the various characterization techniques such as UV, IR, NMR and Mass Spectrography studies. In our studies of AZL, we have identified following four impurities namely, propan-2-yl-2-(4-nitro benzylidene)-3-oxo butanoate [Impurity 1]; propan-2-yl 2-(3-nitro benzylidene)-3-oxo butanoate [Impurity 2]; 3-[1-(diphenyl methyl)azetidin-3-yl] 5-propan-2-yl 2-amino-6-methyl-4-(4-nitro phenyl)-1,4-dihydro pyridine-3,5-dicarboxylate [Impurity 3]; and 3-[1-diphenyl methyl)azetidin-3 yl] 5-propan-2-yl 2-amino-6-methyl-4-(2-nitro phenyl)-1,4-dihydro pyridine-3,5-dicarboxylate [Impurity 4]. An increasing number of publications on development of analytical methods for AZL bulk drug analysis indicate the significance of impurities of AZL. The structural formulae of these impurities 1, 2, 3 and 4 were mentioned in Figures a, b, c and d respectively.

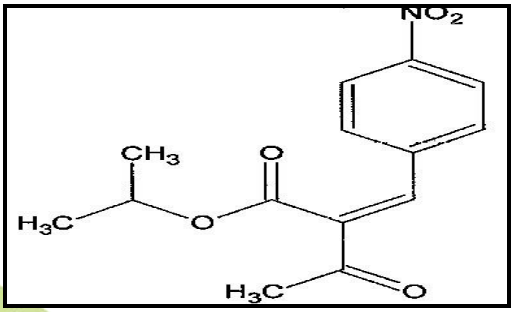


Figure a: Structural formula of para Impurity of AZL

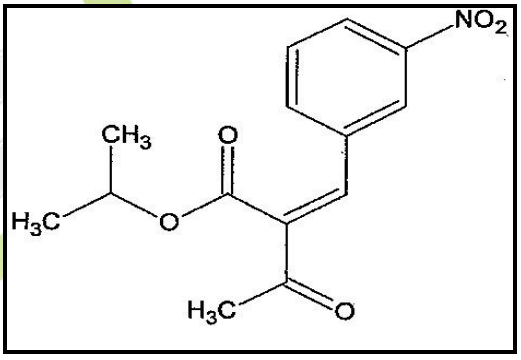


Figure b: Structural formula of Azelnidipine Intermediate

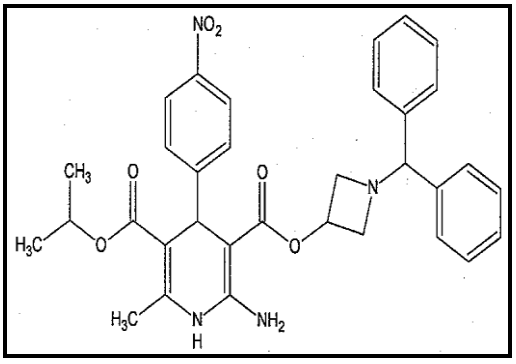


Figure c: Structural formula of 4-Nitro Azelnidipine

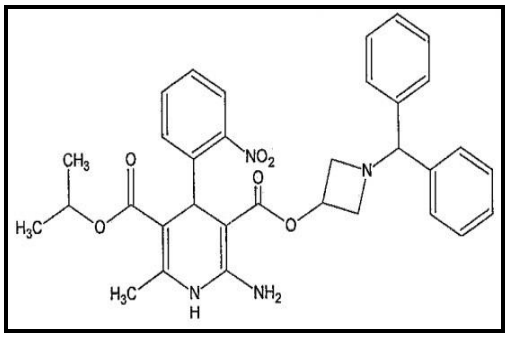


Figure d: Structural formula of 2-Nitro Azelnidipine

EXPERIMENTAL

Samples and Chemicals

Samples of AZL bulk material and its four-related compounds, namely Impurity 1, Impurity 2, Impurity 3 and Impurity 4 were received from analytical research and development department of Hetero Research Foundation, Hyderabad, Telangana State, India. All the four impurities were synthesized in the laboratory after identification by HPLC and determination of mass number by LC-MS. KBr, Methanol AR grade were obtained from SD Fine Chemicals Limited, Mumbai, India. High pure water was prepared by using Millipore Milli "Q" plus purification system.

FT-IR Spectroscopy

The IR Spectra of AZL impurities were recorded in the solid state as 1% KBr dispersion medium using Perkin-Elmer Spectrum One FT-IR Spectrometer.

The IR Spectrum of Para Impurity was presented in Figure 1. The structural assignment of para Impurity has been correlated with the following frequencies shown in Table 1.

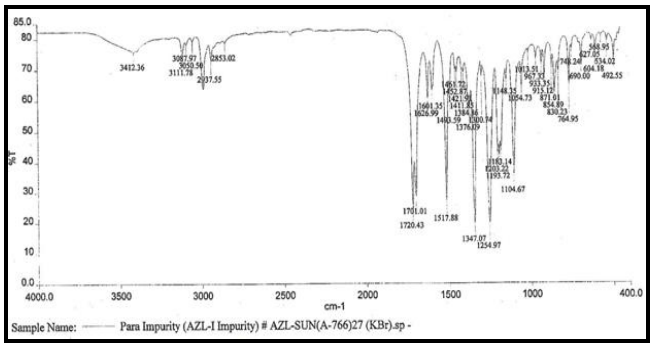


Figure 1: IR Spectrum of Para Impurity of AZL

Table 1: Frequencies of IR spectrum of para Impurity of AZL

Wave number (cm^{-1})	Assignment	Mode of Vibration
3112,3088,3051	Aromatic C-H	Stretching
2986,2938	Aliphatic C-H	Stretching
1720,1701	C=O	Stretching
1623,1601	Aromatic C=C	Stretching
1518	Aromatic $(N=O)_2$	Asymmetric Stretching
1462,1453,1422,1376	Aliphatic C-H	Bending
1347	Aromatic $(N=O)_2$	Symmetric Stretching
1255	C-N	Stretching
1203,1194,1183,1105	C-(C=O)-O	Stretching
855,765	Aromatic C-H	Bending

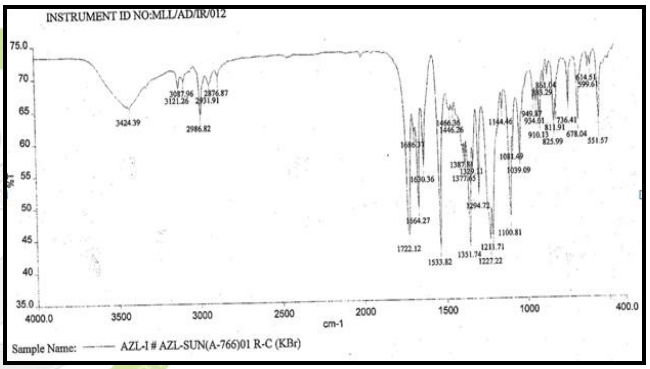


Figure 2: IR Spectrum of AZL-1

Table 2: Frequencies of IR spectrum of AZL – I

Wave number (cm^{-1})	Assignment	Mode of Vibration
3437	N-H	Stretching
3027	Aromatic C-H	Stretching
2978,2927,2850	Aliphatic C-H	Stretching
1677,1646	C=O	Stretching
1516	Aromatic $(N=O)_2$	Asymmetric Stretching
1490	Aromatic C=C	Stretching
1452,1385,1373	Aliphatic C-H	Bending
1345	Aromatic $(N=O)_2$	Symmetric Stretching
1287	C-N	Stretching
1215,1106,1067	C-(C=O)-O	Stretching
828,746,705	Aromatic C-H	Bending

The IR Spectrum of AZL-I was presented in Figure 2. The structural assignment of AZL-I has been correlated with the following frequencies shown in Table 2.

The IR Spectrum of 4-Nitro Azelnidipine was presented in Figure 3. The structural assignment of this impurity has been correlated with the following frequencies shown in Table 3.

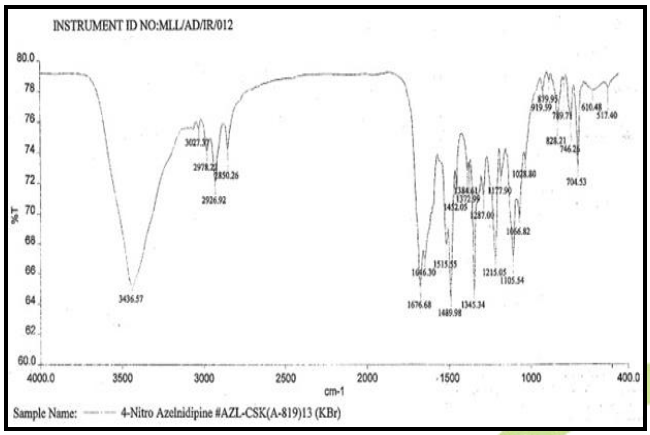


Figure 3: IR Spectrum of 4- Nitro Azelnidipine

Table 3: Frequencies of IR spectrum of 4-Nitro Azelnidipine

Wave number (cm^{-1})	Assignment	Mode of Vibration
3437	N-H	Stretching
3027	Aromatic C-H	Stretching
2978,2927,2850	Aliphatic C-H	Stretching
1677,1646	C=O	Stretching
1516	Aromatic $(N=O)_2$	Asymmetric Stretching
1490	Aromatic C=C	Stretching
1452,1385,1373	Aliphatic C-H	Bending
1345	Aromatic $(N=O)_2$	Symmetric Stretching
1287	C-N	Stretching
1215,1106,1067	C-(C=O)-O	Stretching
828,746,705	Aromatic C-H	Bending

The IR Spectrum of 2-Nitro Azelnidipine was presented in Figure 4. The structural assignment of this impurity has been correlated with the following frequencies shown in Table 4.

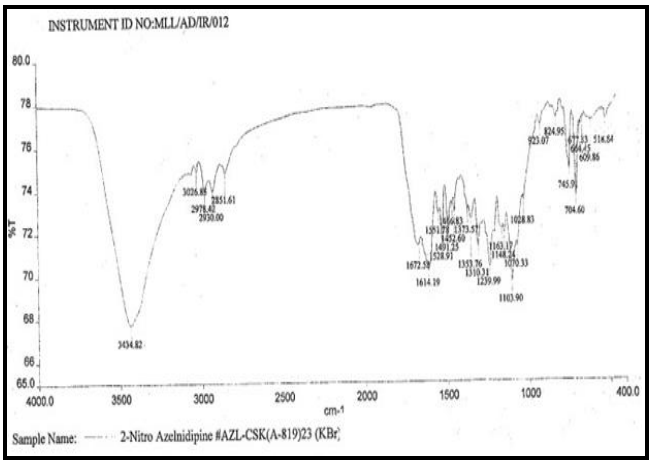


Figure 4: IR Spectrum of 2- Nitro Azelnidipine

Table 4: Frequencies of IR spectrum of 2-Nitro Azelnidipine

Wave number (cm^{-1})	Assignment	Mode of Vibration
3435	N-H	Stretching
3027	Aromatic C-H	Stretching
2978,2930,2852	Aliphatic C-H	Stretching
1673,1614	C=O	Stretching
1552,1491	Aromatic C=C	Stretching
1529	Aromatic $(N=O)_2$	Asymmetric Stretching
1453	Aliphatic C-H	Bending
1354	1347	Aromatic $(N=O)_2$
1310,1240	C-N	Stretching
1104	C-(C=O)-O	Stretching
746,705	Aromatic C-H	Bending

UV Spectroscopy

The UV Spectra of AZL impurities were recorded with a concentration of 0.01 mg/mL solution in methanol using Perkin Elmer Lambda 35 UV-Visible Spectrophotometer.

The UV Spectrum exhibited absorbance of para Impurity is maximum at about 202 nm. The spectrum was presented in Figure 5. The

following absorbance maxima were recorded in Table 5.

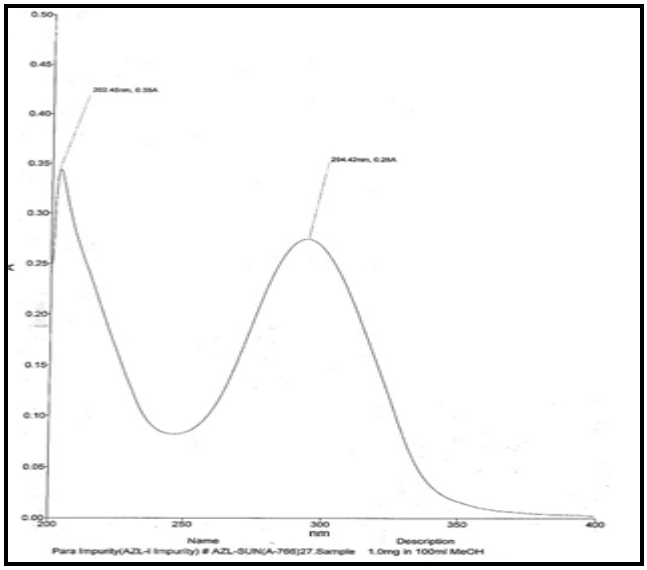


Figure 5: Spectrum of Para Impurity of AZL

Table 5: Absorbance Maxima of UV spectrum of para Impurity of AZL

λ (nm)	Absorbance
~202	~0.35
~294	~0.28

The UV Spectrum exhibited absorbance of AZL-I is maximum at about 267 nm. The spectrum was presented in Figure 6. The following absorbance maxima were recorded in Table 6.

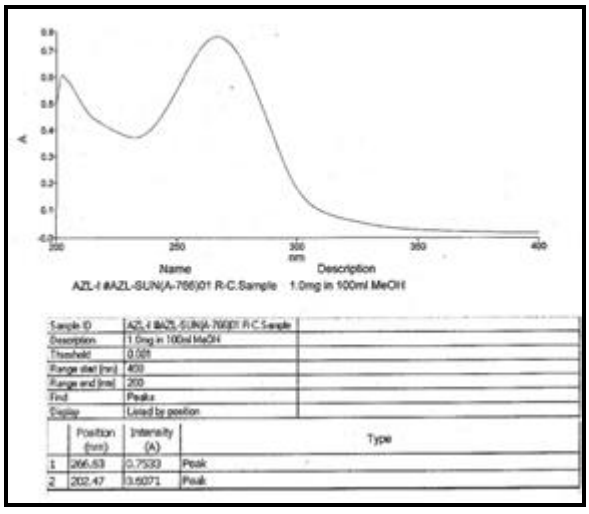


Figure 6: UV Spectrum of AZL-1

Table 6: Absorbance Maxima of UV spectrum of AZL – I

λ (nm)	Absorbance
~267	~0.75
~202	~0.61

The UV Spectrum exhibited absorbance of 4-Nitro Azelnidipine is maximum at about 203 nm. The spectrum was presented in Figure 7. The following absorbance maxima were recorded in Table 7.

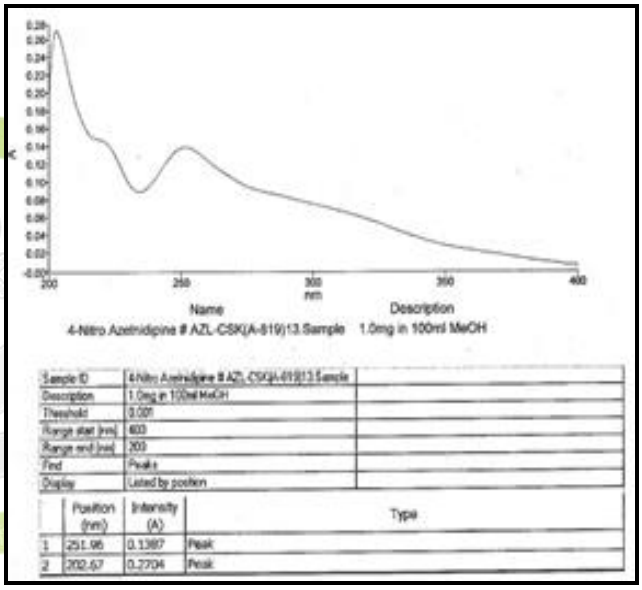
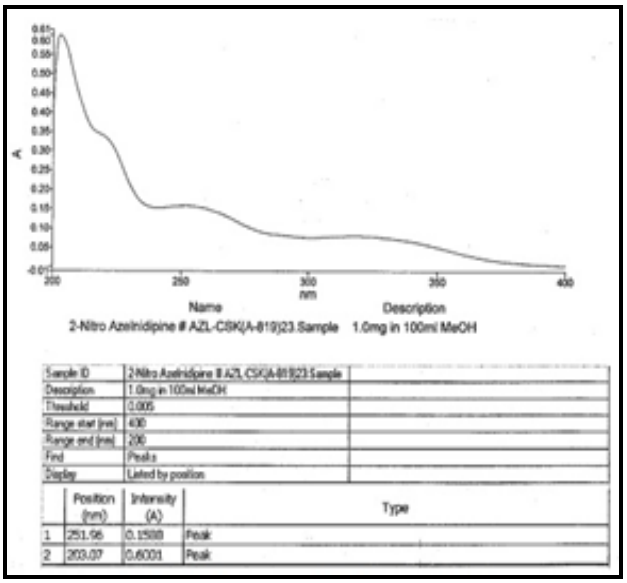


Figure 7: UV Spectrum of 4- Nitro Azelnidipine

Table 7: Absorbance Maxima of UV spectrum of 4 – Nitro Azelnidipine

λ (nm)	Absorbance
~252	~0.14
~203	~0.27

The UV Spectrum exhibited absorbance of 2-Nitro Azelnidipine is maximum at about 203 nm. The spectrum was presented in Figure 8. The following absorbance maxima were recorded in Table 8.



Sample ID	2-Nitro Azelnidipine # AZL-CSK(A-819)23.Sample	
Description	1.0mg in 100ml MeOH	
Threshold	0.005	
Range start [nm]	400	
Range end [nm]	200	
Find	Peaks	
Display	Listed by position	

	Position (nm)	Intensity (A)	Type
1	251.96	0.1588	Peak
2	203.07	0.6001	Peak

Figure 8: UV Spectrum of 2- Nitro Azelnidipine

Table 8: Absorbance Maxima of UV spectrum of 2 – Nitro Azelnidipine

λ (nm)	Absorbance
~252	~0.16
~203	~0.60

NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is an extremely powerful tool for the analysis of drug degradation products. Combining the detailed structural information provided by NMR spectroscopy with a molecular formula and additional structural insight from MN fragmentation experiments can prove extremely valuable in the work flow for drug degradation products. In order to perform NMR-based structure elucidation of drug-degradant products, it is common practice to isolate sufficient material (≥1 mg) for NMR analysis. One-and two-dimensional NMR experiments are then used to piece together correlated fragments of the molecule, allowing the molecular structure to be determined. With improvements in NMR-probe technology, it has become possible to perform these experiments on microgram quantities of isolated sample.

The ^{1}H NMR and ^{13}C NMR experiments for AZL Impurities were performed at 300 MHz and 75 MHz respectively on Bruker 300 MHz Avance NMR Spectrometer. The ^{1}H and ^{13}C chemical shifts were reported on δ scale in ppm, relative to TMS (δ=0.00ppm) and DMSO-d_6 (δ=39.50ppm) as internal standards respectively. The DEPT experiment confirms the methyl and methane carbons. The extra peaks in the data could be due to DMSO-d_6 solvent.

The ^{1}H NMR, ^{13}C NMR and DEPT Spectra of para Impurity were presented in Figures 9, 10 and 11 respectively. The extra peaks in the data could be due to solvents. The list of chemical shift values (in ppm) of para Impurity were tabulated (Table 9).

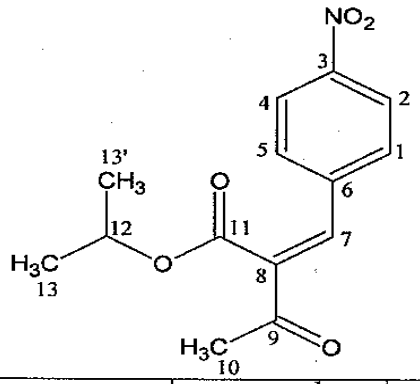
The ^{1}H NMR, ^{13}C NMR and DEPT Spectra of AZL-I were presented in Figures 12, 13 and 14 respectively. The extra peaks in the data could be due to impurities. The list of chemical shift values (in ppm) of AZL-I were tabulated (Table 10).

The ^{1}H NMR, D_2O exchange, ^{13}C NMR and DEPT Spectra of 4-Nitro Azelnidipine were presented in Figures 15, 16, 17 and 18 respectively. The extra peaks in the data could be due to solvents or impurities. The list of chemical shift values (in ppm) of 4-Nitro Azelnidipine were tabulated (Table 11). The ^{1}H NMR, D_2O exchange, ^{13}C NMR and DEPT Spectra of 2-Nitro Azelnidipine were presented in Figures 19, 20, 21 and 22 respectively. The extra peaks in the data could be due to impurities. The list of chemical shift values (in ppm) of 2-Nitro Azelnidipine were tabulated (Table 12).

Mass Spectrometry (MS)

MS is an essential tool in all structure elucidation work flows. This technique provides high sensitivity, high dynamic range, richness of information, and the ability to couple to LC separations directly and provide structural information “on the fly”. MS instrumentation has seen much advancement in the past two decades, which have increased availability of the high resolution instrument.

Table 9: Chemical Shift values of para Impurity of AZL

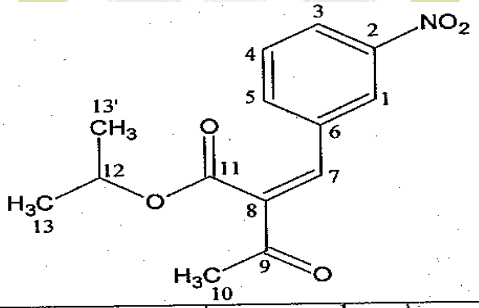


Position	^{1}H	δ(ppm)	J(Hz)^1	^{13}C	DEPT
1,5	2H	8.26	d(8.7)	130.61	CH
2,4	2H	7.69	d(9.0)	123.90	CH
3	-	-	-	147.87	-
6	-	-	-	139.13	-
7	1H	7.72	s	137.51	CH
8	-	-	-	137.43	-
9	-	-	-	202.07	-
10	3H	2.38	s	30.93	CH_3
11	-	-	-	162.89	-
12	1H	5.04-5.12	m	69.42	CH
13,13'	6H	1.28	d(6.0)	21.37	CH_3

s-singlet, d-doublet, m-multiplet.

1.^{1}H-^{1}H Coupling constants.

Table 10: Chemical Shift values of AZL – I

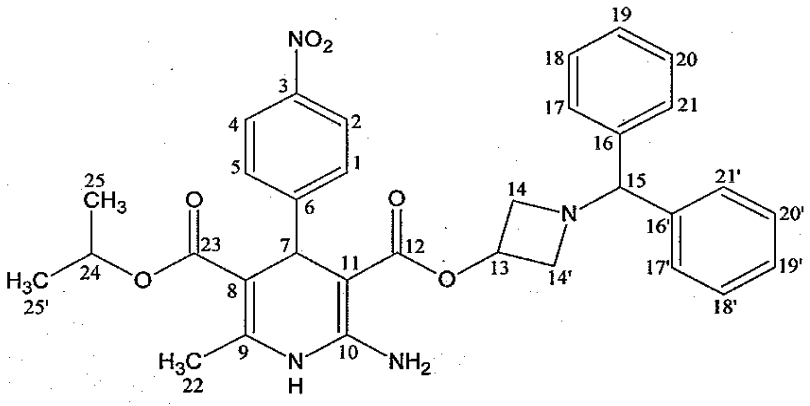


Position	^{1}H	δ(ppm)	J(Hz)^1	^{13}C	DEPT
1	1H	8.31-8.38	m	124.99	CH
2	-	-	-	147.96	-
3	1H	8.31-8.38	m	123.22	CH
4	1H	7.32-7.84	m	130.66	CH
5	1H	7.92-7.97	m	135.50	CH
6	-	-	-	136.64	-
7	1H	7.92-7.97	m	138.68	CH
8	-	-	-	134.56	-
9	-	-	-	195.82	-
10	3H	2.47	s	26.10	CH_3
11	-	-	-	165.95	-
12	1H	5.09-5.17	m	69.33	CH
13,13'	6H	1.21	d(6.3)	21.18	CH_3

s-singlet, d-doublet, m-multiplet.

1.^{1}H-^{1}H Coupling constants.

Table 11: Chemical Shift values of 4 – Nitro Azelnidipine



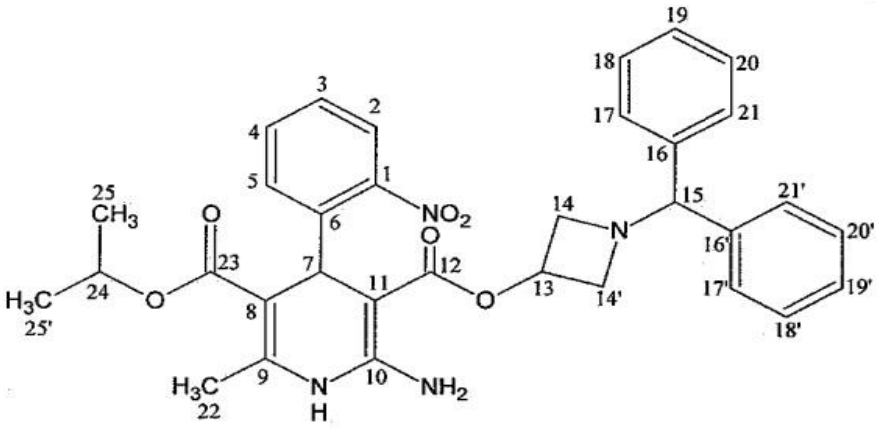
NMR assignments of 4-Nitro Azelnidipine, Batch number AZL-CSK(A-819)13.

Position	^{1}H	δ(ppm)	J(Hz)[1]	^{13}C	DEPT
1,5	2H	8.18	d(8.7)	129.00	CH
2,4	2H	7.45	d(8.7)	123.09	CH
3	-	-	-	145.44	-
6	-	-	-	157.22	-
7	1H	4.75-4.83	m	38.67	CH
8	-	-	-	102.59	-
9	-	-	-	145.23	-
10	-	-	-	151.87	-
11	-	-	-	75.99	-
12	-	-	-	165.92	-
13	1H	4.75-4.83	m	61.68	CH
14Ha	1H	2.91	t(6.6)	59.32	CH_2
14Hb	1H	3.43	t(7.1)		
14'Ha	1H	2.40	br	59.88	CH_2
14'Hb	1H	3.26-3.33	m		
15	1H	4.27	s	76.93	CH
16,16'	-	-	-	142.20,142.30	-
17,21,17',21'	4H	7.14-7.40	m	128.40	CH
18,20,18',20'	4H	7.14-7.40	m	127.01	CH
19,19'	2H	7.14-7.40	m	126.96	CH
22	3H	2.26	s	18.66	CH_3
23	-	-	-	167.23	-
24	1H	4.75-4.83	m	66.41	CH
25,25'	6H	1.03 & 1.18	d(6.3)&d(6.3)	21.53,21.82	CH_3
NH_2	2H	6.78	br	-	-
NH	1H	8.86	s	-	-

s-singlet, d-doublet, t-triplet, m-multiplet, broad.

[1].^{1}H-^{1}H Coupling constants.

Table 12: Chemical Shift values of 2 – Nitro Azelnidipine



NMR assignments of 2-Nitro Azelnidipine, Batch number AZL-CSK(A-819)23.

Position	^{1}H	δ(ppm)	J(Hz)[1]	^{13}C	DEPT
1	-	-	-	147.00	-
2	1H	7.15-7.762	m	123.84	CH
3	1H	7.15-7.762	m	126.78	CH
4	1H	7.15-7.762	m	130.77	CH
5	1H	7.15-7.762	m	133.22	CH
6	-	-	-	144.18	-
7	1H	4.71-4.83	m	32.77	CH
8	-	-	-	103.38	-
9	-	-	-	144.94	-
10	-	-	-	152.20	-
11	-	-	-	77.47	-
12	-	-	-	166.11	-
13	1H	4.71-4.83	m	61.67	CH
14Ha	1H	2.66-2.71	dd(8.3,6.2)	59.32	CH_2
14Hb	1H	3.35-3.40	m		
14'Ha	1H	2.57	d(7.1)	59.51	CH_2
14'Hb	1H	2.83	t(6.8)		
15	1H	4.34	s	76.48	CH
16,16'	-	-	-	142.33,142.75	-
17,21,17',21'	4H	7.15-7.762	m	128.31,128.36	CH
18,20,18',20'	4H	7.15-7.762	m	127.01,127.06	CH
19,19'	2H	7.15-7.762	m	126.95,126.84	CH
22	3H	2.24	s	18.72	CH_3
23	-	-	-	167.50	-
24	1H	4.71-4.83	m	66.22	CH
25,25'	6H	0.90 & 1.13	d(6.3)&d(6.3)	21.18,21.46	CH_3
NH_2	2H	6.77-6.85	br	-	-
NH	1H	8.79	s	-	-

s-singlet, d-doublet, dd-doublet of doublet, t-triplet, m-multiplet, br-broad.

[1].^{1}H-^{1}H Coupling constants.

An ion trap instrument with multiple stage fragmentation has been the standard for structure elucidation. A typical work flow for an unknown identification is first to carry out the fragmentation experiment for the API and then to assign its fragments to understand the fragmentation of the molecule. The same fragmentation experiment can then be carried out for the impurity and fragments for both the API and the impurity can be compared.

The LC-MS analysis has been performed on Agilent 1100 series LC-MSD-TRAP-SL system. The analysis was performed in both ionization modes with Turbo Ion spray interface with the following conditions. Ion source voltage 5500V, declustering potential 80V, Focusing potential 150V, entrance potential 10V, with the nebulizer gas as nitrogen at 60 psi were used for positive ionization mode whereas the negative ionization was performed by switching the polarity of the ion source voltage to -4500V.

The adduct ions at m/z 300 $(M+Na)^+$ and 316 $(M+K)^+$ in positive mode confirms the monoisotopic mass of para Impurity as 277 corresponding to molecular formula of $C_{14}H_{15}NO_5$ and the values were tabulated (Table 13). The mass spectrum of para Impurity was presented in Figure 23.

The adduct ions at m/z 300 (M+Na) and 316 (M+K) in positive mode confirms the monoisotopic mass of AZL-I as 277 corresponding to molecular formula of $C_{14}H_{15}NO_5$ and the same values as in Table 13 were obtained. The mass spectrum of AZL-I was presented in Figure 24.

The protonated molecular ion at m/z 583 (M+1) and deprotonated molecular ion at m/z 581 (M-1) confirms the monoisotopic mass of 4-Nitro Azelnidipine as 582 corresponding to molecular formula of $C_{33}H_{34}N_4O_6$ and the values were tabulated (Table 14). The mass spectra of positive and negative modes of 4-Nitro Azelnidipine were presented in Figures 25 and 26.

The protonated molecular ion at m/z 583 (M+1) and deprotonated molecular ion at m/z 581 (M-1) confirms the monoisotopic mass of 2-Nitro Azelnidipine as 582 corresponding to molecular formula of $C_{33}H_{34}N_4O_6$ and the same values as in Table 14 were obtained. The mass spectra of positive and negative modes of 2-Nitro Azelnidipine were presented in Figures 27 and 28.

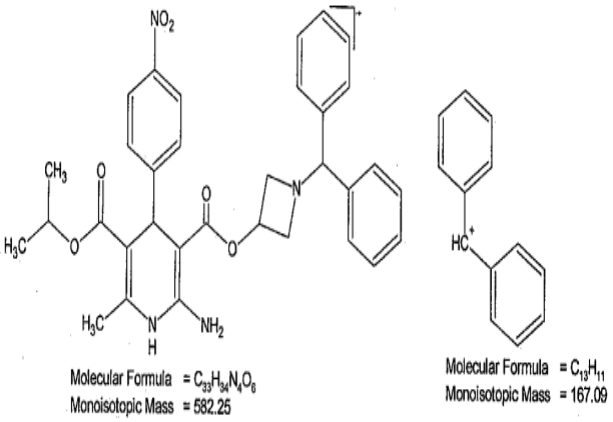
Table 13: m/z values of para Impurity & AZL – I

m/z	IONS
300	$(M+Na)^+$
316	$(M+K)^+$

Table 14: m/z values of 4 – Nitro and 2 – Nitro Azelnidipine

m/z	IONS
583	$(M+H)^+$
581	$(M-H)^-$
617	$(M-H+HCl)^-$
695	$(M-H+CF_3COOH)^-$

Major fragments are as shown below:



Applications of Impurity Profiling

Numerous applications have been sought in the areas of AZL drug designing and in monitoring quality, stability, and safety of pharmaceutical compounds, whether produced synthetically, extracted from natural products or produced by recombinant methods. The applications include alkanoids, amines, amino acids, analgesics, antibacterials, anticonvulsants, antidepressants, tranquilizers, antineoplastic agents, local anaesthetics, macro molecules, steroids etc.

Table 15: Batch numbers and IUPAC names of Impurities of Azelnidipine

Name of the Impurity	Batch No	Molecular formula	Molecular Weight	IUPAC name
Azelnidipine Stage-I (para Impurity)	AZL-SUN (A-766) 27	$C_{14}H_{15}NO_5$	277.2	Propan-2-yl 2-(4-Nitro benzylidene)- 3-oxo butanoate
Azelnidipine Intermediate (AZL-I)	AZL-SUN (A-766) 01 R/C	$C_{14}H_{15}NO_5$	277.27	Propan-2-yl 2-(3-Nitro benzylidene)-3-oxo butanoate
4-Nitro Azelnidipine (Azelnidipine Impurity)	AZL-CSK (A-819) 13	$C_{33}H_{34}N_4O_6$	582.65	3-[1-(diphenyl methyl) azetidin-3-yl] propan-2-yl 2-amino-6-methyl-4-(4- Nitro phenyl)-1,4-dihydro pyridine-3,5-di carboxylate
2-Nitro Azelnidipine (Azelnidipine Impurity)	AZL-CSK (A-819) 23	$C_{33}H_{34}N_4O_6$	582.65	3-[1-(diphenyl methyl) azetidin-3-yl] 5- propan-2-yl 2-amino-6-methyl-4-(2-Nitro phenyl)-1,4-dihydro pyridine-3,5-di carboxylate

Table 16: Data showing the instruments used for various spectra of AZL drug

Instrument used for IR Spectrum	Perkin- Elmer Spectrum One FT-IR Spectrometer using 1 % KBr pellets for all the Impurities of AZL
Instrument used for UV Spectrum	Perkin – Elmer Lambda 35 UV-Visible Spectrophotometer scanned from 200-400 nm with a conc. of 0.01 mg/mL solution in methanol for all the Impurities of AZL
Instrument used for NMR Spectrum	Bruker 300 MHz Avance NMR Spectrometer, ^1H and ^{13}C spectra were recorded in DMSO – d_6 at 300 MHz and 75 MHz for all the Impurities of AZL
Instrument used for Mass Spectrum	Agilent 1100 series LC-MSD-TRAP-SL system

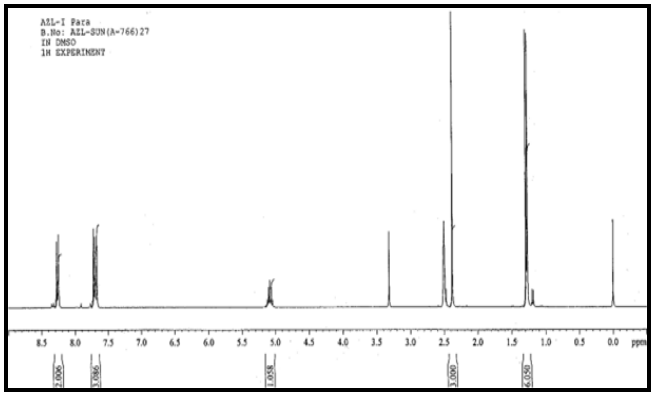


Figure 9: ^1H NMR Spectrum of Para Impurity AZL

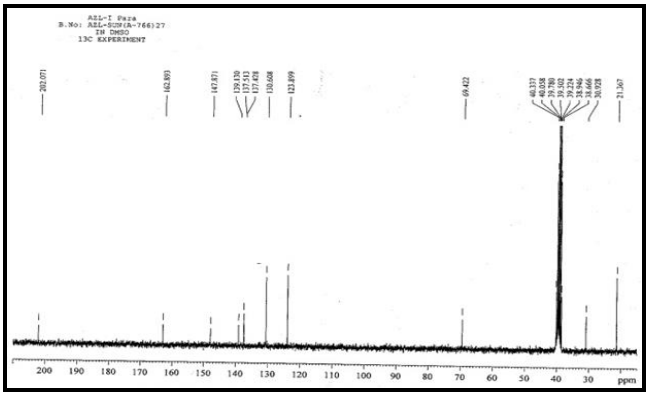


Figure 10: ^{13}C NMR Spectrum of Para Impurity AZL

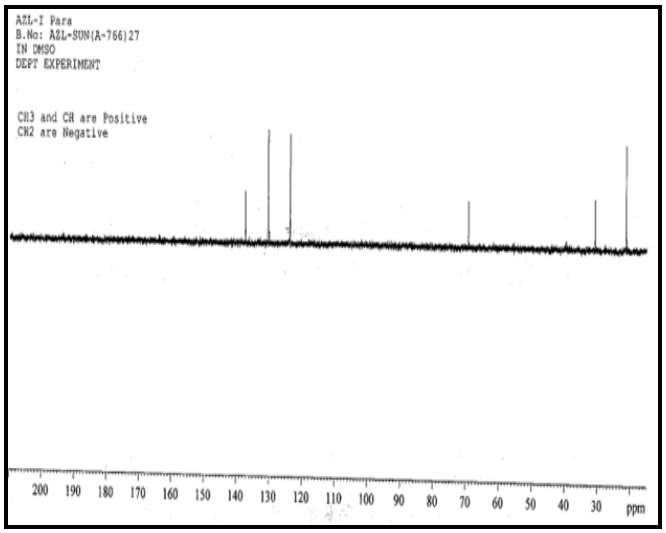


Figure 11: DEPT NMR Spectrum of Para Impurity of AZL

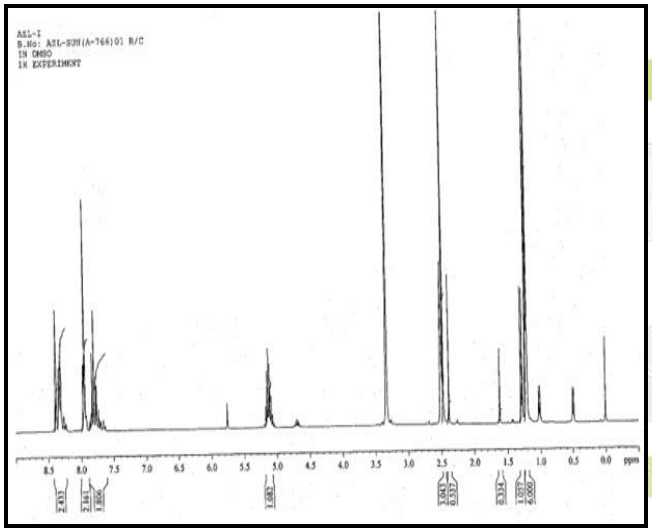


Figure 12: ^{1}H NMR Spectrum of AZL-1

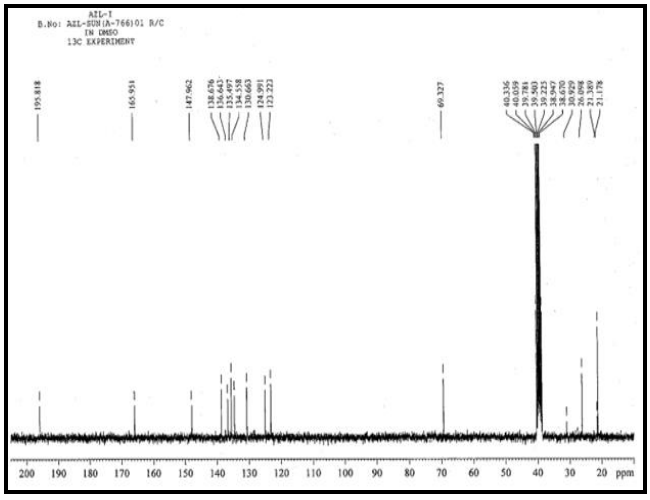


Figure 13: ^{13}C NMR Spectrum of AZL-1

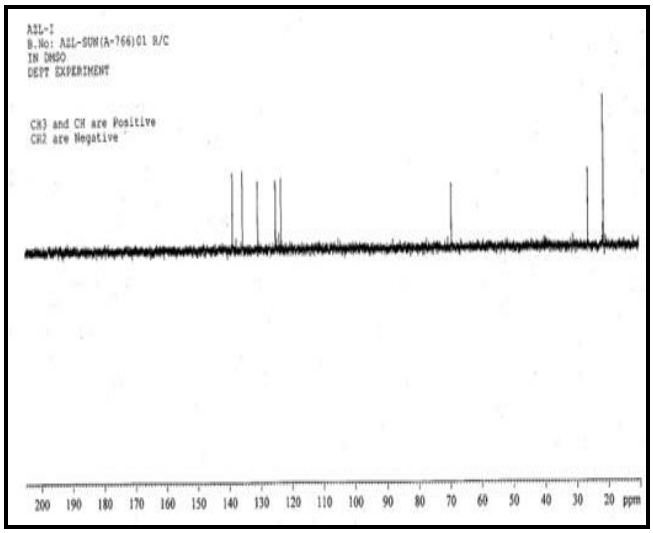


Figure 14: DEPT NMR Spectrum of AZL-1

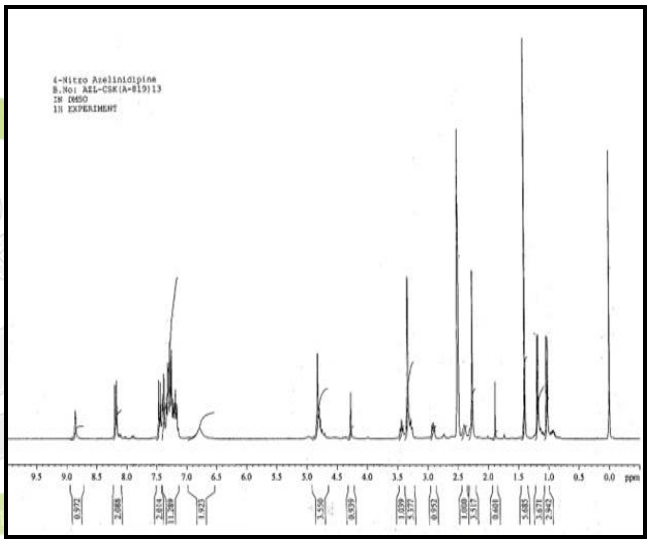


Figure 15: ^{1}H NMR Spectrum of 4- Nitro Azelnidipine

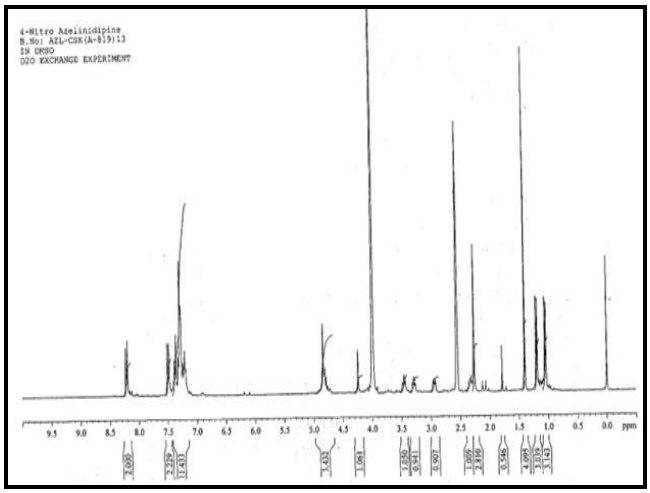


Figure 16: D_2O Exchange NMR Spectrum of 4-Nitro Azelnidipine

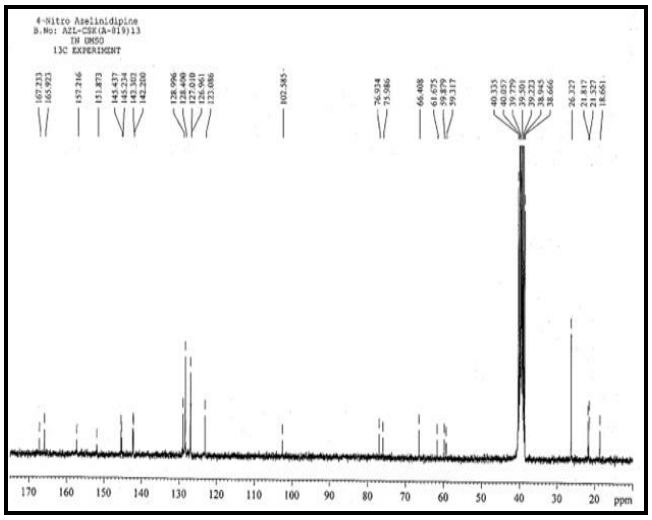


Figure 17: ^{13}C NMR Spectrum of 4- Nitro Azelnidipine

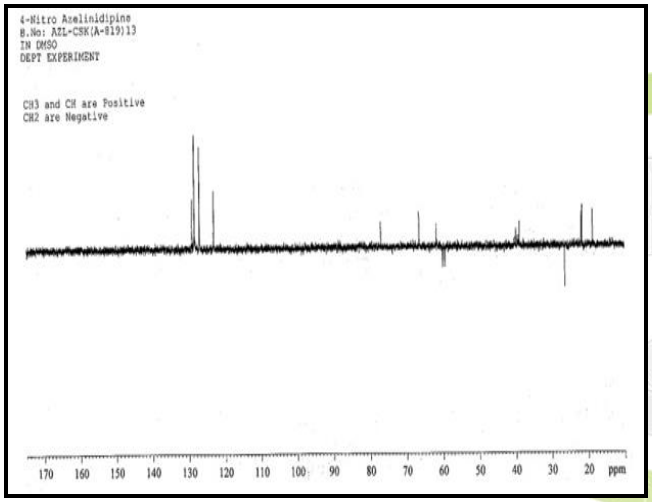


Figure 18: DEPT NMR Spectrum of 4- Nitro Azelnidipine

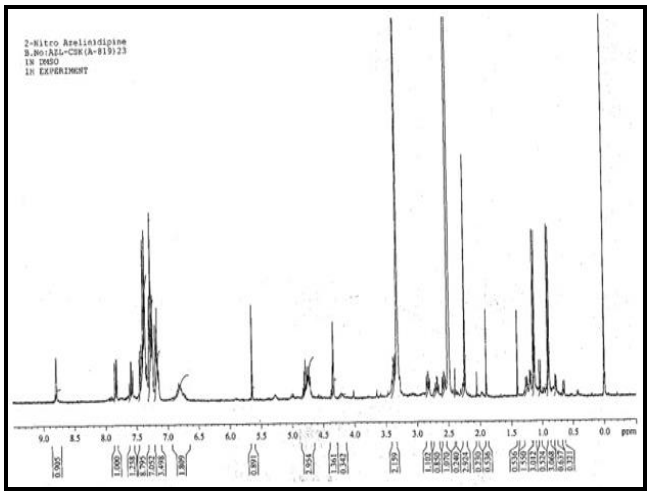


Figure 19: ^{13}C NMR Spectrum of 2- Nitro Azelnidipine

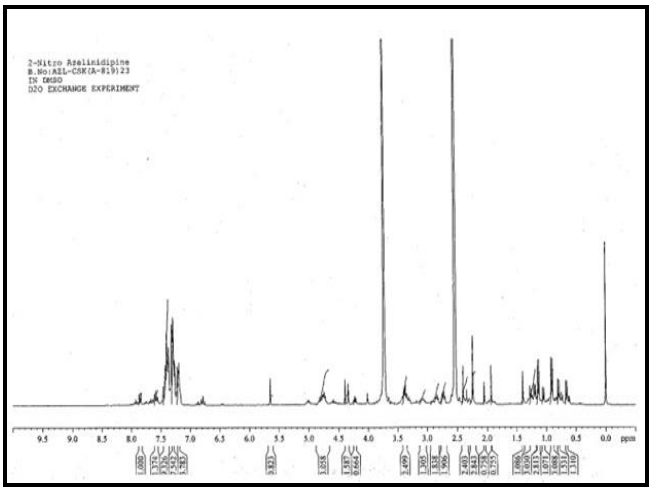


Figure 20: D_2O Exchange NMR Spectrum of 2-Nitro Azelnidipine

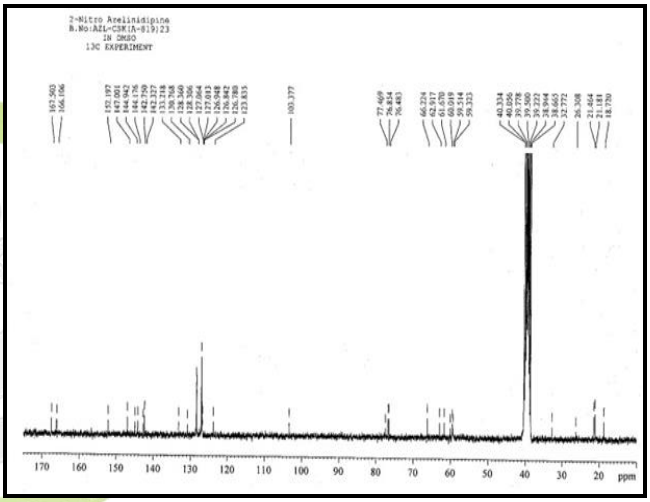


Figure 21: ^{13}C NMR Spectrum of 2- Nitro Azelnidipine

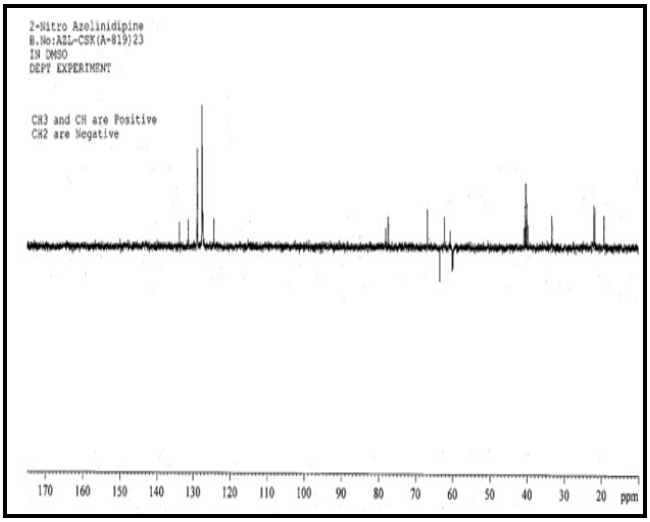


Figure 22: DEPT NMR Spectrum of 2- Nitro Azelnidipine

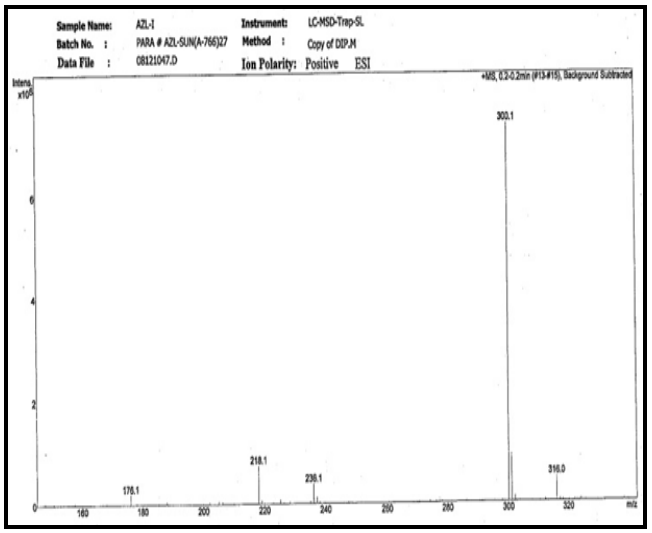


Figure 23: MS of Para Impurity

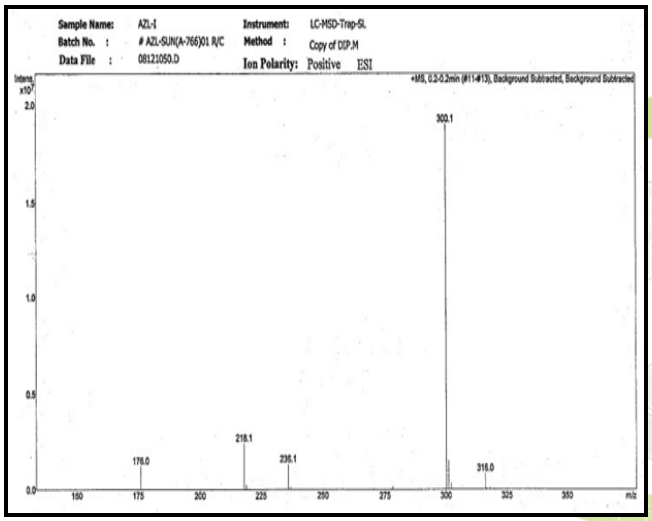


Figure 24: MS of AZL-1

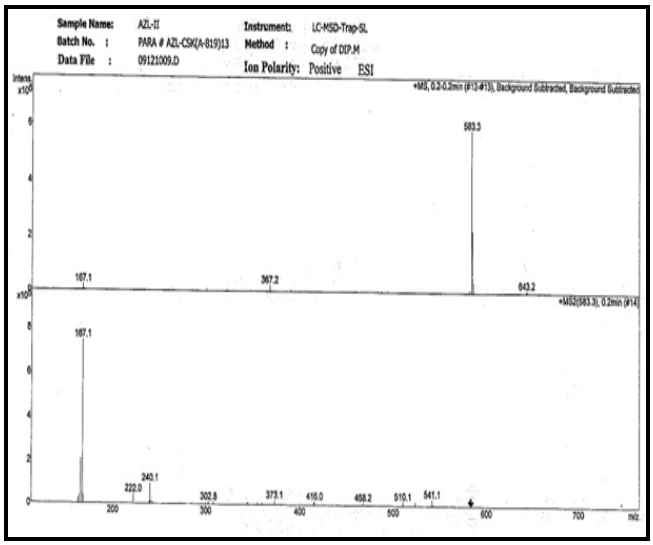


Figure 25: MS of Positive mode of 4-Nitro Azelnidipine

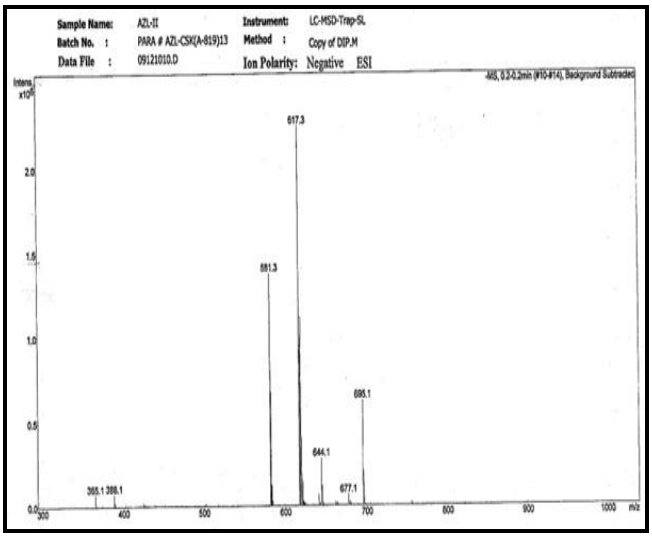


Figure 26: MS of Negative mode of 4-Nitro Azelnidipine

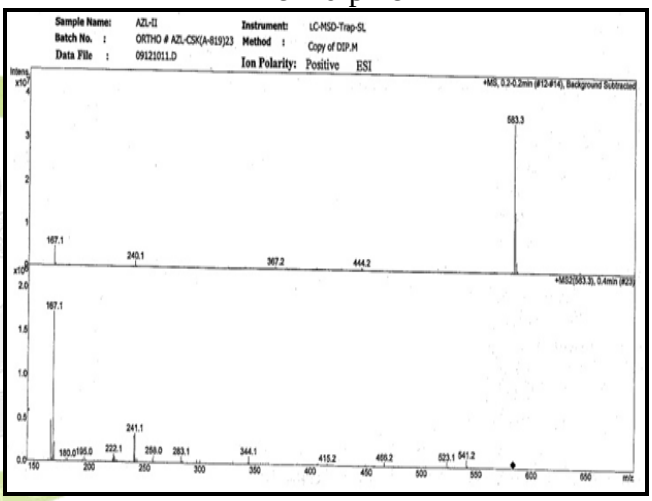


Figure 27: MS of Positive mode of 2-Nitro Azelnidipine

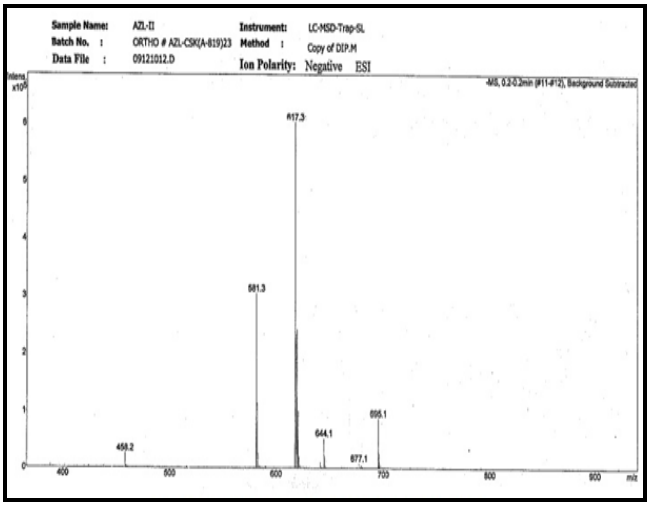


Figure 28: MS of Negative mode of 2-Nitro Azelnidipine

RESULTS AND DISCUSSION

Impurities 3 & 4 are the potential impurities present in bulk samples of AZL. The main target of chromatographic method is to get the separation of critical closely eluting impurities namely, Impurity 1, Impurity 2, Impurity 3 and Impurity 4. These impurities were eluted very closely to each other by using different stationery phases like CN, C_{18}, C_8 and phenyl and different mobile phases containing buffers like phosphate, sulfate and acetate with different p^H values (2.5-5) using organic modifiers like acetonitrile and methanol in the mobile phase. pH of the buffer has played a significant role in achieving the separation between Impurity 1, Impurity 2, Impurity 3 and Impurity 4. In the optimized conditions of AZL, these four impurities were well separated with resolution of greater than 3.

A typical analytical HPLC chromatogram of laboratory batch of AZL bulk drug recorded using the LC method was described in these studies. The target impurities under study were marked as impurity 1 (molecular weight 277.27), impurity 2 (molecular weight 277.27), impurity 3 (molecular weight 582.65) and impurity 4 (molecular weight 582.65). The LC-MS compatible method which is used to detect the impurities was described which helps to detect all the impurities of AZL (Figure a – d). Batch numbers, molecular formulae, and IUPAC names of these impurities of AZL were shown in Table 15. The instruments used for IR, UV, NMR and Mass spectra of AZL drug were shown in Table 16.

Impurities Identification by LC-MS

For identification of the four impurities, the ESI mass spectrum of impurities in positive ion mode showed a molecular ion peaks at m/Z 300, 300, 583 and 583 [$(mH)^+$], indicating the molecular weights of these compounds are 277.27, 277.27, 582.65 and 582.65 respectively. From this data we conclude that these four are intermediates, which were used in the manufacturing process of AZL and these four were also confirmed with photo diode array detector by comparing the spectra's with known standards. These four impurities further were characterized by UV, FT-IR, Mass and NMR (^{13}C, ^1H, DEPT).

ESI mass spectrum of major impurity formed during oxidative stress condition in positive ion mode showed a molecular ion peak at m/Z 583.3 [$(mH)^+$] indicating the molecular weight of the Impurity 3 & 4 as 582.65. This molecular ion mass was higher than that of AZL and this indicates the probability of the formation of Nitro compound. The same impurities were also formed in base, thermal and UV degradation studies but in smaller quantities. Further these impurities were synthesized and characterized by UV, FT-IR, Mass and NMR (^{13}C, ^1H, DEPT) spectroscopy studies.

CONCLUSION

Impurity profiling of a substance under investigation gives maximum possible account of impurities present in it. The establishment of guidelines for impurity levels in drug substances and products provides the quality criteria for manufacturers. Beginning with limit tests for impurities, this field of impurity identification and quantitation has progressed. Nowadays, it is mandatory requirements in various pharmacopoeias to know the impurities present in API's. Isolation and characterization of impurities is required for acquiring and evaluating data that establishes biological safety which reveals the need and scope of impurity profiling of drugs in pharmaceutical research. Keeping in view this regulatory requirement of AZL impurities, the process related impurities and metabolites in AZL bulk drug were identified, synthesized and characterized using IR, UV, NMR and Mass spectral data.

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