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Stability indicating method for estimation of Amlodipine besylate by RP-HPLC in tablet dosage form and characterization of the oxidation impurity of amlodipine besylate

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ABSTRACT

A simple, specific, accurate and precise stability indicating Reverse Phase High Performance Liquid Chromatographic method was developed for estimation of Amlodipine besylate in tablet dosage form on RP C_{18} BDS column (250mm×4.6 mm, 5µm) with a mobile phase consisting of A: Triethylamine (pH 3.0) adjusted with ortho phosphoric acid, B: ACN, with a timed gradient program of T/%B: 0/30, 7/70, 8/30, 10/30 with a flow rate of 1.4 ml/min, UV detection at 236 nm was used. The retention time for Amlodipine besylate was found to be 4.5 min. Proposed method was validated for precision, accuracy, linearity, range, robustness, ruggedness and force degradation study. The calibration curve of Amlodipine besylate was linear over the range of 12.5-37.5 µg/ml. The method was found to be sensitive with limit of detection was determined 0.16 and limit of quantitation was determined 0.49 µg/ml. The oxidative degraded product of Amlodipine besylate formed was investigated by electrospray ionization (ESI) time-of-flight mass spectrometry, NMR and IR spectroscopy. The found unknown impurity related to oxidative degradation was 2, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine.

INTRODUCTION

Amlodipine Besylate, chemically, (RS)-3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chloro-phenyl)-1, 4-dihydro-6-methyl- 3, 5-pyridinedicarboxylate benzenesulfonate (Figure 1),[1] is a long acting calcium channel blocker which is used as an antihypertensive agent. This medication block the transport of calcium into the smooth muscle cells lining the coronary arteries and other arteries of the body. Since calcium is important in muscle contraction, blocking calcium transport relaxes artery muscles and dilates coronary arteries and other arteries of the body. By relaxing coronary arteries, Amlodipine is useful in preventing chest pain (angina) resulting from coronary artery spasm.[2-4]

Literature survey revealed HPLC,[5-7] RP-HPLC,[8,9] HPTLC,[10-11] LC-MS/MS,[12] LC-MS,[13] and simultaneous UV-spectrophotometric methods,[14-16] are reported for the estimation of Amlodipine besylate alone or in combination with other antihypertensive agents. As no method is stability indicating method reported for Amlodipine besylate, the aim of the present study was to develop accurate, precise and selective reverse phase HPLC assay procedure for the analysis of Amlodipine besylate and characterization of oxidative degradation product by spectroscopic techniques.

EXPERIMENTAL

Material and reagents

Amlodipine besylate drug substance, drug product

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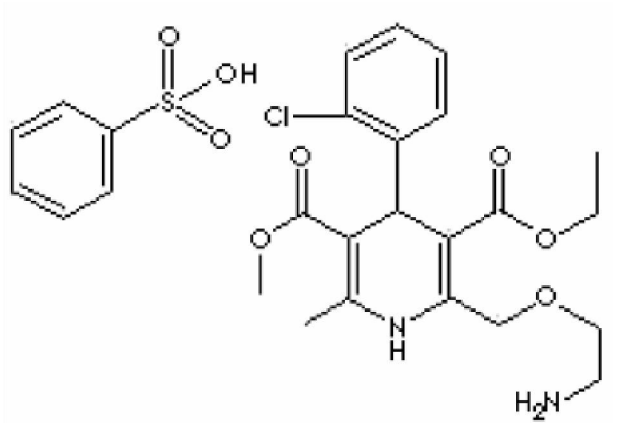


Figure 1 : Chemical structure of an Amlodipine besylate

(prepared from Amlodipine besylate) and samples enriched with this impurity was obtained from our laboratory. HPLC grade acetonitrile, AR grade triethylamine and AR grade orthophosphoric acids (85% v/v) were obtained from Merck India. Double distilled water obtained from water distillation assembly, was used.

High performance liquid chromatography (analytical)

Agilent HPLC system equipped with low pressure quaternary gradient pump along with photo diode array detector and manual rheodyne sample injector has been used for the analysis of samples. The data was collected and processed using Ezichrom Elite software. A LCGC RP-18, 5µ (250 x 4.5 mm) BDS column was employed for the separation of impurity from Amlodipine. The column eluent was monitored at 236 nm. The sample diluent was a mixture of 7 ml Triethylamine in 1000 ml water of pH 3.0 adjusted with orthophosphoric acid and acetonitrile in the ratio of 6:4 (v/v), filter through 0.45µ or finer porosity membrane filter.

High performance liquid chromatography (preparative)

An Agilent preparative HPLC system equipped with liquid controller pump, photo diode array detector, and manual sample injector fitted with 20 µL loop was used. The data was collected and processed using Ezichrom Elite software. An LCGC BDS C18 column (250 × 4.5 mm, 5-Micron) was employed for loading the sample. An analytical method was developed in gradient mode separately to resolve this impurity, followed by scaling up the same method for prep-HPLC to collect the required impurity fractions. The mobile phase A and B are 7 ml Triethylamine in 1000 ml water (pH adjusted to 3.0 with ortho phosphoric acid)/acetonitrile, respectively. The solvent composition was held at 70 % mobile phase A for 7 min. then increased mobile phase B from 30 to 70% for 8 min. and then held at 70% A for 10 min. The chromatographic run time was 10 min. The flow rate was set at 1.4 mL/min. Detection was carried out at 236 nm. Approximately 100 mg/mL of sample was prepared using a sample diluent. The sample diluent was a mixture of 7 ml Triethylamine in 1000 ml water of pH 3.0 adjusted with orthophosphoric acid and acetonitrile in the ratio of 6:4 (v/v)

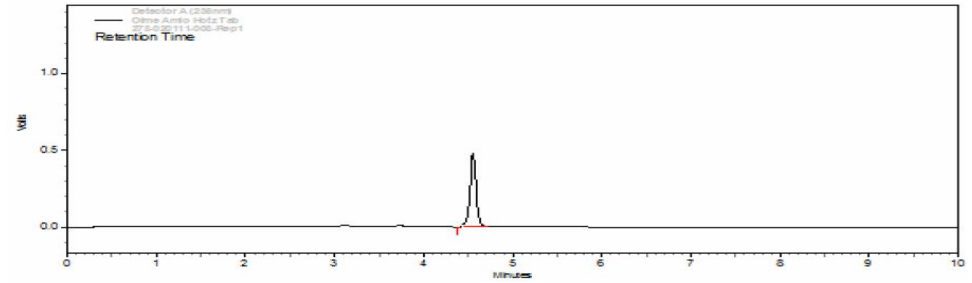
Mass spectrometry (LC/MS)

Initial LC/MS analysis has been performed on Varian Inc (USA) 410 Prostar Binary LC with 500 MS IT PDA Detectors. The analysis was performed in positive ionization mode with turbo ion spray interface. The parameters for ion source voltage IS = 5500 V, declustering potential, DP = 70 V, focusing potential, FP = 400 V, entrance potential, EP = 10 V were set with nebulizer gas as air at a pressure of 40 psi and curtain gas as nitrogen at a pressure of 25 psi in mass spectrometer. Further to get accurate mass, analysis was performed on high resolution mass spectrometer using electro spray ionization. The accurate mass obtained from the instrument, theoretical mass and mass error was mentioned in the TABLE no.1. An Inertsil ODS (50 x 4.6 mm, 5-Micron) column was used for the separation. The mobile phase A and B are 7 ml Triethylamine in 1000 ml water (pH adjusted to 3.0 with ortho phosphoric acid)/acetonitrile, respectively. The solvent composition was held at 70 % mobile phase A for 7 min.

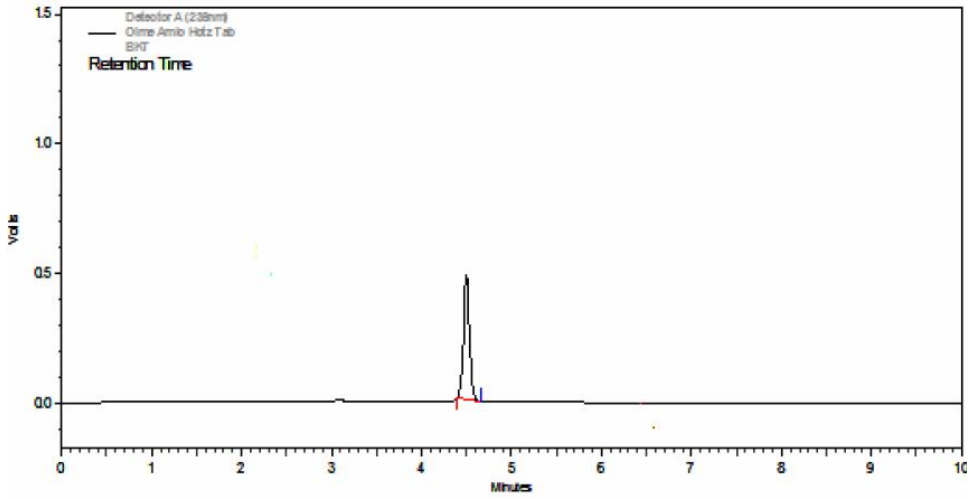
TABLE 1 : Results of system suitability parameters of the developed method for determination of Amlodipine besylate

Compound	Retention time	% RSD of retention time	% RSD of area	Asymmetry	Theoretical Plates
AMLO	4.28	0.20	0.90	1.23	5973

*** Average of five determinations**



(a)



(b)

Figure 2 : HPLC chromatogram of Amlodipine of standard (a) and tablet (b) graphs

then increased mobile phase B from 30 to 70% for 8 min. and then held at 70% A for 10 min. The analysis was performed at a flow rate of 1.4 mL/min with splitting.

NMR spectroscopy

The ^{1}H and ^{13}C experiment was carried out for unknown impurity at processional frequencies 400.1328 MHz at $25^{0}C$ on a Bruker Avance-300 FT NMR spectrometer. The ^{13}C experiment for Amlodipine maleate was performed in mixture of $CDCl_3$ and DMSO-d_6. The ^{1}H chemical shift values were reported on the δ scale in ppm, relative to TMS (δ = 0.00 ppm) and the chemical shift values were reported relative to $CDCl_3$ (δ = 77.00 ppm) and DMSO-d_6 (δ = 39.50 ppm) as internal standards, respectively.

FT-IR spectroscopy

The IR spectra were recorded in the liquid state using Shimadzu 1600 series FT-IR spectrophotometer.

Detection of impurities by HPLC

Typical HPLC chromatogram of Amlodipine besylate and its impurity observed in drug substance obtained by using the HPLC method (Figure 2). The observed impurity under study was 2, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine, eluted at retention time of about 3.90 min, while Amlodipine besylate eluted at about 4.55 min in drug substance HPLC method.

Chromatographic condition

The mobile phase- A containing of 7ml Triethylamine in 1000 ml buffer pH 3.0 and mobile phase-B consisting of Acetonitrile, with timed gradient program of T/%B: 0/30, 7/70, 8/30, 10/30, where a column BDS C_{18} (250mm×4.6 mm, 5µm) was found to resolve Amlodipine besylate. The mobile phase was filtered through 0.45-µ-membrane filter and the sonicated for 10 min. the flow rate was set at 1.4 ml/min. Amlodipine besylate showed good absorbance at 236 nm, which was selected as wavelength for further

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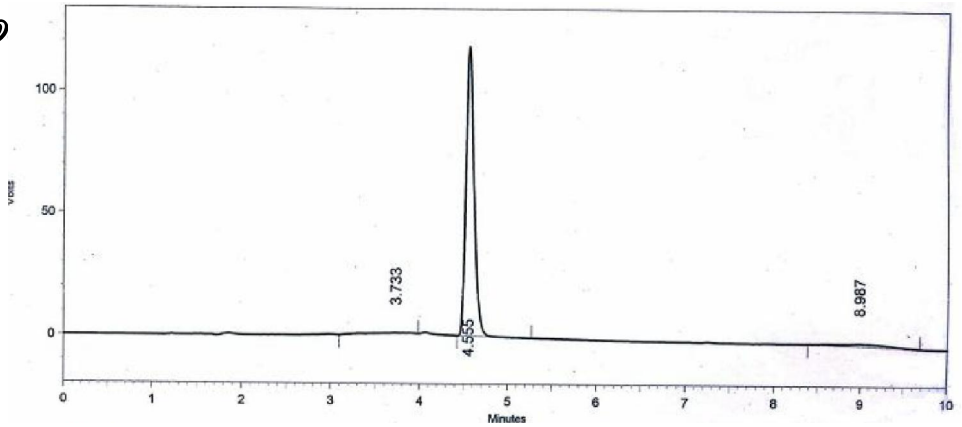

Figure 3 : HPLC Chromatograms of Acid degradation of Amlodipine besylate

TABLE 2 : Precision Studies

Sample number		
Assay of Amlodipine besylate as % of labeled amount	**Analyst 1(intra-day precision)**	**Analyst 2(inter-day precision)**
1	99.97	99.75
2	99.87	99.40
3	99.98	99.23
4	99.56	99.31
5	99.45	99.43
6	99.68	99.13
Mean	99.75	99.45
RSD	0.12	0.101

*** Average of six determinations**

analysis all determinations were performed at ambient column temperature. Diluent was used mobile phase-A and mobile phase-B in the ratio of 60:40 v/v.

Preparation of stock solution and standard solution

Accurately weighed 25mg of Amlodipine dissolved in 100 ml of volumetric flask with diluent (Stock solution), respectively. The stock solution was further diluted by using mobile phase to get the concentration of 25 µg/ml, of Amlodipine. (Figure 2a)

VALIDATION OF METHOD

The developed method was validated in terms of linearity, specificity, precision, accuracy, robustness and ruggedness[17, 18].

Preparation of the degradation products

The different stress conditions were used for the forced degradation studies of bulk drug and drug formulations (TABLE-2). In this procedure make one sample without drug i.e. placebo sample and sample with drugs were compared with force degradation sample. The stress sample was detected at 236 nm wavelength and run time was taken as same as assay sample.

Acidic condition

For Acid hydrolysis, 1N of hydrochloric acid was used for preparation of 25 µg/ml, solution. Amlodipine API taken 25 mg was dissolved in 100 of volumetric flask with 10 ml mobile phase, respectively and make sample preparation for tablet equivalent to 10 mg of Amlodipine besylate in 100 ml volumetric flask. Then add 5 ml of 1N Hydrochloric acid in each flask and exposed to 30 min at 105°C under water bath. After it add 5 ml of 1N sodium hydroxide in each flask for neutralization of reaction. Then make up with mobile phase. For further dilution take 5 ml of each sample in 50 ml of volumetric flask individually and for tablet degradation, 5 ml taken in 20 ml of flask and make up with mobile phase. (Figure 3)

Alkaline condition

For Base degradation, 2N sodium hydroxide was used for preparation of 25 µg/ml solution. Amlodipine API taken 25 mg was dissolved in 100 ml of volumetric flask with 10 ml mobile phase, respectively and makes sample preparation for tablet equivalent to 10 mg of in 100 ml volumetric flask. Then add 10 ml of 2N sodium hydroxide in each flask and exposed to 90 min at 105°C under water bath. After it add 10 ml of 2N hydrochloric acid in each flask for neutralization of reaction. Then make

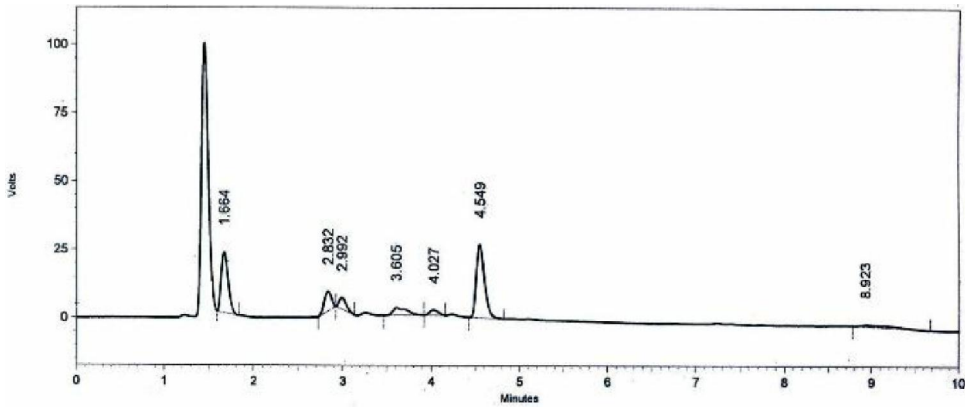

Figure 4 : HPLC chromatograms of Base degradation of Amlodipine besylate

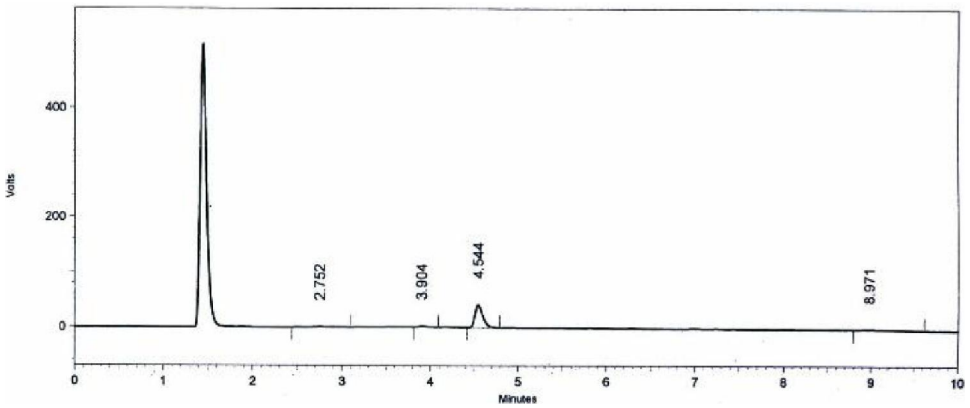

Figure 5 : HPLC chromatograms of oxidation degradation of Amlodipine besylat

up with mobile phase. For further dilution take 5 ml of each sample in 50 ml of volumetric flask individually and for tablet degradation, 5 ml taken in 20 ml of flask and make up with mobile phase. (Figure 4)

Oxidation condition

For Peroxide degradation, 10% Hydrogen Peroxide was used for preparation of 25 µg/ml solution. Amlodipine API taken 25 mg was dissolved in 100 ml of volumetric flask with 10 ml mobile phase and make sample preparation for tablet equivalent to 10 mg of Amlodipine besylate in 100 ml volumetric flask. Then add 5 ml of 10% Hydrogen peroxide in each flask and exposed to 45 min at 105°C under water bath. Then make up with mobile phase. For further dilution take 5 ml of each sample in 50 ml of volumetric flask individually and for tablet degradation, 5 ml taken in 20 ml of flask and make up with mobile phase. (Figure 5)

Assay preparation of amlodipne in tablet dosage forms

For assay preparation used strength of 10 mg of Amlodipine. Total of 20 tablets were taken and accurately weighed and finely powdered. All crushed powder was taken into 200 ml volumetric flask and dissolved in 150 ml of mobile phase, ultrasonicated for 25 min and filtered through whatman filter. Final volume was made up to 200 ml. From this solution 5 ml was taken and diluted to 200 ml in a volumetric flask. Final concentration was 25 µg/ml made. The diluted solution was analyzed under optimized chromatographic conditions. (Figure 2 b)

Isolation of 2, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine impurity by Prep HPLC

A simple reverse phase chromatographic system was used for isolating the unknown impurity 2, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine. In this chromatographic system, the 2, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine impurity eluted at about 3.90 min. So fractions eluting between 3.8 and 4.1 min. were collected, pooled and concentrated by evaporating ac-

TABLE 3: Recovery studies

Level of addition / (%)	Amount added / µg	Recovery /(%)	Average recovery / (%)
80	16	98.90	
100	20	99.81	99.6
120	24	100.1	

*** Average of three determinations**

etonitrile at room temperature under high vacuum on a Rotavapour. The aqueous layer was evaporated at room temperature. After drying oily material form, then add HPLC grade methanol to form a liquid sample. Purity was checked by HPLC, which was found to be 95%, and was characterized by NMR, Mass experiments.

RESULTS AND DISCUSSION

To develop a simple, specific, accurate and precise Reverse Phase High Performance Liquid Chromatographic method for simultaneous estimation of Amlodipine besylate, different mobile phases were tried and the proposed chromatographic conditions were found to be appropriate for the quantitative determination. System suitability tests were carried as per ICH guidelines and parameters are summarized in TABLE 1.

METHOD VALIDATION

Linearity

Linearity was studied by preparing standard solutions at different concentration levels. The linearity range was found to be as 12.5-37.5 µg/ml. The regression equation was found to be as y = 93447x - 15218 with correlation coefficient (R^2) 0.999.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Specificity of an analytical method is its ability to measure accurately and specifically the analyte of interest without interference from blank and placebo. Specificity the peak purity was assessed by comparing the retention time of standard and sample, good correlation was obtained between the retention time of standard and sample. Placebo and blank were injected and there were no peaks. There were no interferences hence method is specific.

TABLE 4 : Results of robustness study

Parameter		Retention time	Asymmetry	Theoretical Plates
pH of	2.8	4.55	1.21	5917
mobile phase	3.2	4.43	1.27	5841
Flow rate	1.3	4.58	1.26	5846
(ml/min)	1.5	4.47	1.24	5138

*** Average of six determinations**

Precision

Precision was evaluated by carrying out six independent sample preparations of a single lot of formulation. The sample preparation was carried out in same manner as described in sample preparation. Percentage relative standard deviation (%RSD) was found to be less than 2% that proves method is precise shown in TABLE-2.

Accuracy (Recovery studies)

To check the degree of accuracy of the method, recovery studies were performed in triplet by standard addition method at 80%, 100% and 120% concentration levels. Known amounts of standard Amlodipine besylate was added to the pre-analyzed samples and were subjected to the proposed HPLC method. The % recovery was found to be within the limits of the acceptance criteria with average recovery of 98.9-100.1%. Results of recovery studies are shown in TABLE-3.

Limit of quantitation and limit of detection

LOQ and LOD can be determined based on visual evaluation, signal-to-noise approach, standard deviation of the response and slope. Limit of detection was determined 0.16 and Limit of quantitation was determined 0.49.

TABLE 5 : Force degradation study results

Stress conditions	time / h	Degradation/(%)	Peak purity[a]
Acid hydrolysis	8	7.54	999
Base hydrolysis	8	29.73	999
Oxidation	8	74.40	999

[a]Peak purity values in the range of 990-1000 indicate the homogenous peak

TABLE 6 : Mass fragmentation of impurity mass m/z 239.27

Name of the compound	Mass observed in LCMS/Q-TOF system	Theoretical Mass	Molecular formula
2, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine impurity	239.2	239.27	$C_{12}H_{17}N_1O_4$
FRAGMENT 1	407.1	407.5	$C_{20}H_{24}N_2O_5Cl$
FRAGMENT 2	328.2	327.78	$C_{14}H_{19}N_1O_4Cl$
FRAGMENT 3	297.0	298.0	$C_{14}H_{21}N_2O_5$
FRAGMENT 4	348.5	347.79	$C_{18}H_{19}N_1O_4Cl$
FRAGMENT 5	165.1	169.6	$C_8H_{12}N_1OCl$
FRAGMENT 6	503.4	505.97	$C_{24}H_{23}N_1O_7SCl$
FRAGMENT 7	458.1	454.49	$C_{20}H_{26}N_2O_8S$

Robustness of the method

To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in optimized method parameters were done. The effect of change in flow rate and change in pH retention time, tailing factor and theoretical plates were studied. The method was found to be unaffected by small changes like +/- 10% in flow rate, +/- 0.2 change in pH, shown in TABLE-4.

Intermediate precision (ruggedness)

Different analyst carried out precision studies in a similar manner carried out by first analyst. The % Assay was found to be 97.4-99.1 %. Percentage relative standard deviation (%RSD) was found to be less than 2% that proves method is rugged, shown in TABLE-3.

Forced degradation study

Degradation was not observed for Amlodipine besylate drug sample during stress condition like acid, base, and oxidation. Amlodipine was degraded into acid, base and oxidation and forms polar impurities. In the acidic condition 7.54%, in the basic condition 29.73% after 8 h and in the oxidative condition 74.40% after 8 h, degradation was observed for Amlodipine. Peak purity results greater than 990 indicate that the Amlodipine peak is homogeneous in all stress conditions tested (TABLE- 5).

Characterization of oxidative degradant

Amlodipine besylate drug substance and Amlodipine besylate tablets (prepared from Amlodipine besylate, sustained release and orally

TABLE 7 : ^{1}H NMR assignments for Amlodipine besylate and Impurity

Position	Amlodipine besylate	2, 6-dimethyl-3-ethoxycarbonyl-5-methoxycarbonyl 1, 4-dihydropyridine impurity
	^{1}H ppm / ^{1}H /multiplicity	^{1}H ppm / ^{1}H / multiplicity
1	–	--
2	–	--
3	7.34/1H/dd	--
4	7.23/1H/dt	--
5	7.13/1H/dt	--
6	7.28/1H/dd	--
7	6.03/1H/s	6.10/2H/s
8	–	--
9	–	--
10	4.79/NH/s	4.70/ NH/ s
11	–	--
12	–	--
13	–	--
14	3.51/3H/s	3.50/3H/ s
15	–	--
16	3.98/2H/m	3.96/2H/m
17	1.12/3H/t	1.11/3H/t
18	2.31/3H/s	2.30/3H/s
19	4.64/2H/q	2.13/3H/s
20	3.66/2H/t	--
21	3.09/2H/t	--
22	7.81/NH_3/s	--
23	–	--
24	6.03/1H/s	--
25	6.03/1H/s	--
26	–	--
27	--	--

s-singlet, d-doublet,t-triplet, m-multiplet, dd-double of doublet, dt-double of triplet

TABLE 8 : ^{13}C NMR assignments for Amlodipine besylate and impurity

Position	Amlodipine besylate	2, 6-dimethyl-3-ethoxycarbonyl-5-methoxycarbonyl 1, 4-dihydropyridine impurity
C1	131	-
C2	144	-
C3	130	--
C4	126	-
C5	126	-
C6	128	-
C7	36	45
C8	101	101
C9	143	165
C10	-	-
C11	144	144
C12	102	102
C13	166	166
C14	49	49
C15	165	165
C16	58	58
C17	13	13
C18	17	17
C19	66	15
C20	65	-
C21	38	-
C22	-	-
C23	168	-
C24	135	-
C25	135	-
C26	168	-

disintegrating tablets) were subjected to stability as per ICH guidelines. The LC-MS analysis showed the m/z value for this unknown impurity as 239.2 $[M+H]^+$ in HPLC method. The melting point of the impurity was found to be 155^{0}C. To further investigate the chemical structure of the unknown impurity, Amlodipine besylate drug substance sample was kept at 105 °C for 3 h wherein impurity with m/z 239.2 got enriched to 0.45%. This sample was subjected to LCMS / ESI Q-TOF. The high resolution mass analysis using Mass Lynx fragmentation tool, proposed the following two probable elemental compositions / molecular formula: $C_{12}H_{17}N_1O_4$. This is due to the fact that this impurity is being derived from Amlodipine besylate molecule, which contains one nitrogen atom. Based on the high resolution mass fragmentation study in comparison to the reported fragmentation pattern of Amlodipine besylate, the chemical structure of the unknown impurity of m/z 239.2, assigned as 2, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine impurity. The observed LCMS Q-TOF fragments of Amlodipine besylate oxidation impurity m/z 239.2 is shown in TABLE 6. Subsequently, ^1H NMR spectra of the isolated compound of unknown impurity 2, 6-dimethyl-3-ethoxycarbonyl-5-methoxycarbonyl 1, 4 - dihydropyridine compared with that of Amlodipine besylate was described in TABLE 7. The ^{1}HNMR data indicate the absence of aromatic protons of chlorophenyl ring and the signals because of 2-amino ethoxy moiety. Based on the above high resolution mass spectral data and NMR data, it is proposed that the unknown impurity is 2, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine. We believe that more precisely the

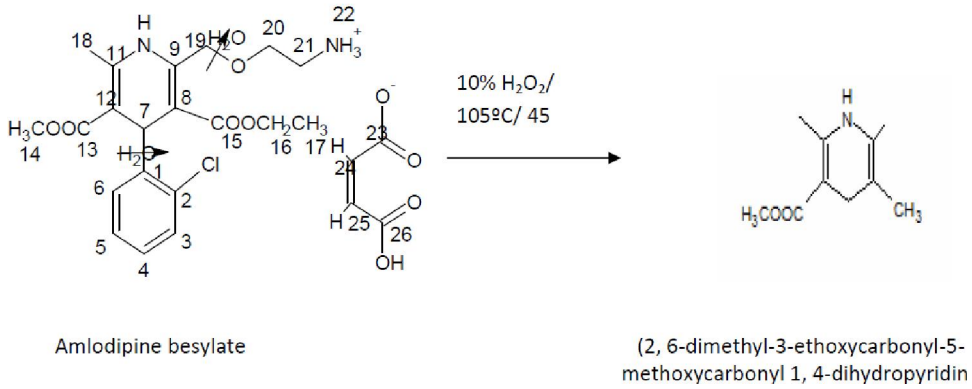

Figure 6 : Oxidative pathway of Amlodipine besylate

chloro phenyl ring of Amlodipine besylate was very much prone to degradation in the presence of oxygen. The IR spectrum of Amlodipine was characterized by the absorption frequency of N-H stretching and bending of amide band at 3254 and 1554 cm^{-1} also sharp peak of chlorine group at 818cm^{-1}. On the other hand, the IR spectrum of impurity lacked the characteristic bands. The ^{13}CNMR spectra showed twelve carbon atoms for the impurity where as there are twenty six carbon atoms were present in Amlodipine besylate (TABLE 8) confirmed the impurity were formed. The possible pathway for the formation of this impurity is shown in Figure 6.

CONCLUSIONS

The present study was conducted to develop and validate a simple, sensitive and reproducible stability-indicating RP-HPLC method for quantitative determination of Amlodipine besylate. The identification of degraded product of Amlodipine was a challenging task. Amlodipine was found to be unstable to alkaline and peroxide degradation, and were less stable to acidic condition.. The developed chromatographic assay fulfilled all the requirements to be identified as simple, specific, selective and reliable method, including accuracy, linearity, recovery and precision data.

The data generated from the performed forced degradation studies enabled the evaluation of Amlodipine stability under a variety of ICH recommended conditions. Such data is valuable for the safety and potency assessment of a drug product. Furthermore, this simple and rapid RP-HPLC method can also be used successfully for the determination of Amlodipine in pharmaceutical formulations without any interference from the excipients. So, the proposed chromatographic procedure confirmed its applicability as a stability indicating method. We have identified one impurity in aged and stressed samples of Amlodipine drug substance. This is characterized as, 6-dimethyl-3-Ethoxycarbonyl-5-Methoxycarbonyl 1, 4-Dihydropyridine by analytical data.

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