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SYNTHESIS, CHARACTERIZATION AND DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF PROCESS-RELATED IMPURITY DIETHYL 4-(4-CHLOROPHENYL)-2, 6-DIMETHYL-1, 4-DIHYDROPYRIDINE-3, 5-DICARBOXYLATE FROM AMLODIPINE BULK AND FORMULATION

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ABSTRACT

The research subscribed in this article is directed towards the identification of process related impurities from Amlodipine drugs and formulation. The identified impurities are then synthesized and the structure of the same was established using FTIR, ^{1}H-NMR, ^{13}C-NMR, GCMS and elemental analysis. After structural establishment of the synthesized impurity a RP-HPLC method was developed for the identification and quantitation of process related impurity diethyl 4-(4-chlorophenyl)-2, 6-dimethyl-1, 4-dihydropyridine-3, 5-dicarboxylate from Amlodipine bulk and formulations. The impurity was synthesized using Hantzsch pyridine synthesis method.

KEY WORDS: Impurity, Hantzsch synthesis, validation, ICH guidelines.

1. INTRODUCTION

ICH defines impurities profile of a drug materials is, "A description of the identified and unidentified impurities present in a new drug

substance." For Pharmaceutical products, impurities are defined as, "substance in the product that are not the API itself or the excipient used to manufacture it " i.e. impurities are unwanted chemical that remains within the formulation or API in small amounts which can influence Quality, Safety and Efficacy, thereby causing serious health hazards.[1] Qualification of the impurities is the process of acquiring and evaluating data that establishes biological safety of an individual impurity; thus, revealing the need and scope of impurity profiling of drugs in pharmaceutical research.[2] Identification of impurities is done by a variety of Chromatographic and Spectroscopic techniques, either alone or in combination with other techniques. There are different methods for detecting and characterizing impurities with TLC, HPTLC, and HPLC etc. Conventional Liquid Chromatography, particularly, HPLC has been exploited widely in field of impurity profiling; the wide range of detectors, and stationary phases along with its sensitivity and cost effective separation have attributed to its varied applications. Various regulatory authorities like ICH, USFDA, Canadian Drug and Health Agency are emphasizing on the purity requirements and the identification of impurities in Active Pharmaceutical Ingredient's (API's). According to ICH guidelines on impurities in new drug products, identification of impurities below the 0.1% level is not considered to be necessary, unless potential impurities are expected to be unusually potent or toxic. According to ICH, the maximum daily dose qualification threshold is considered as follows; ≤ 2g/day 0.1% or 1 mg per day intake (whichever is lower) ≥ 2g/day 0.05%.[3]

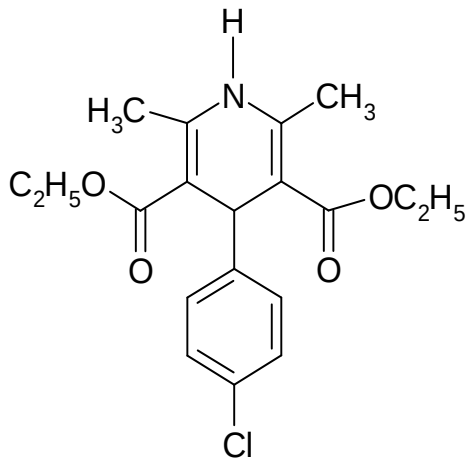

Fig 1: STRUCTURE OF IMPURITY AMLODIPINE

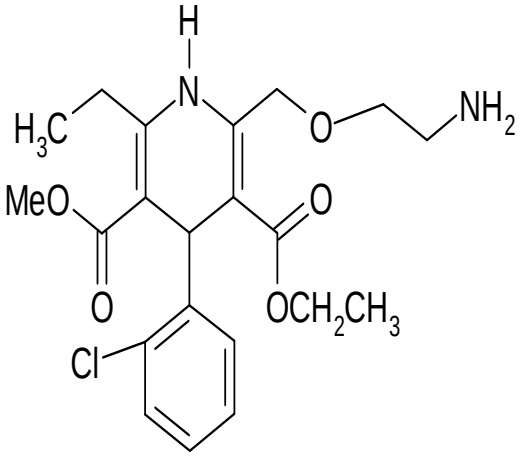

Fig 2: STRUCTURE OF

2. MATERIALS AND METHODS

2.1. Chemicals: p-chlorobenzaldehyde (AR), Ethylacetoacetate (AR), Ammonia (AR), Methanol(AR), Acetonitrile (HPLC grade), Methanol (HPLC grade), Water (HPLC grade) were purchased from Merck chemicals, India.

2.2. Methods and insruments

2.2.1. UV- Visible Spectrophotometer

The UV detection at wavelength 240 nm was selected by using UV- Vis Spectrophotometer (UV- 1650 PC) SHIMADZU INC.

2.2.2. FT-IR

The IR spectra were recorded by using Fourier Transform Infrared spectrophotometer by KBr press pellet technique.

2.2.3. NMR

Characterization of impurities was achieved by NMR using $CDCL_3$ as a solvent. The ^{1}H and ^{13}C NMR chemical shift values were reported on the delta scale in ppm.

2.2.4. GC-MS

The Q- TOF Micro mass (YA-105) spectrometer capable of recording High Resolution Mass Spectrum (HRMS) both in atomic pressure chemical ionization (APCI) and Electron spray Ionization (ESR) were used for characterization of Amlodipine impurity.

2.2.5. RP-HPLC

The HPLC method was developed by using LC20AD Prominence Liquid Chromatography SPD 20-A Shimadzu, Japan. The UV- Vis detector and C18 column with dimension on 250x 4.6 mm was used for the HPLC method Development having flow rate of 1 ml/min at wavelength 240nm. The water: methanol: acetonitrile in proportion of (50v:10v:40v) as a mobile phase was selected for development of validated method of amlodipine impurity and various parameters according to ICH guidelines (Q2B) were studied.

3. EXPERIMENTAL

The quantization of diethyl 4-(4-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate from bulk and formulation was carried out by HPLC method. The LC20AD Prominence Liquid Chromatograph SPD20-A Shimadzu, Japan with UV-Vis detector and C18 column with dimension on 25 x 0.6 cm was used for the method development with flow

rate 1.0 ml/min at wavelength 240 nm. The water: methanol: acetonitrile in proportion of (50v:10v:40v) as a mobile phase, for development of chromatogram. The method was validation for synthesized compound and various parameters according to ICH guidelines (Q2B) were studied.

3.1Synthesis of Impurity for Amlodipine

0.01mole of p-chloro benzaldehyde, 0.02 mole of ethylacetoacetate were added in round bottom flask. To it add 5ml of ammonia and 10ml of methanol stir vigoursly. Refluxed for 4 hours and pour the solution in cold water and the solution was kept overnight in freezer. Filter at the vacuum and recrystallized from methanol. Purity was checked by TLC used cyclohexane: ethyl acetate: formic acid in the ratio of 6:3:1 was selected as mobile phase for quantification of impurity.[4-6]

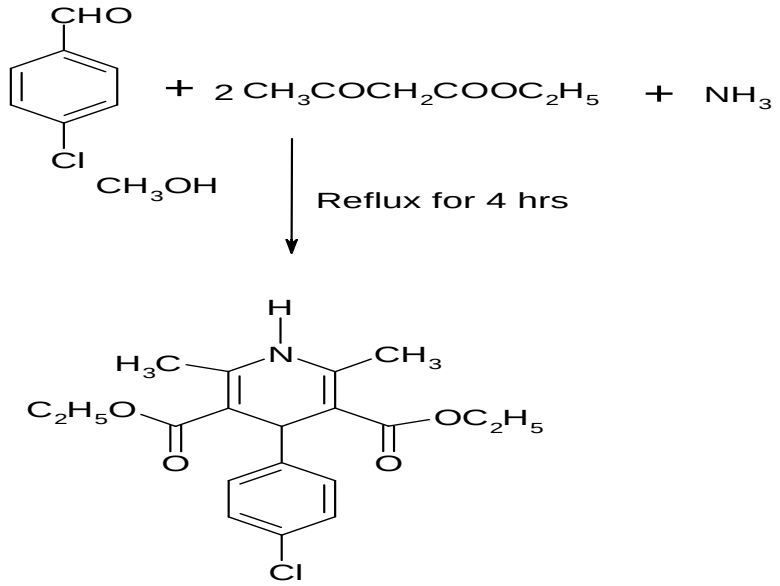

Fig 3: Synthesis scheme of 4-(4-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate

3.2 Preparation of Mobile phase

The selection of mobile phase was according to polarity and non-polarity of solvents. The water: methanol: acetonitrile in proportion of (50v:10v:40v) was selected as mobile phase. It was filtered on membrane filter (0.45 µ) to remove degassing and were stirred for 10-15 min.

3.3 Preparation of Stock solution standard

The stock solution was prepared according to the standard procedure viz., 10 mg of synthesized compound was accurately weighed on analytical balance and using mobile phase

it was dissolved to make volume up to 100 ml stock solution. The sample was prepared in the ppm in the range of 1-20 ppm in concentrations respectively for the method validation by HPLC.

3.4 Preparation of sample solution (formulation)

Stock solution of bulk Amlodipine, Two different batches of Amlodipine marketed formulation of 100 ppm in 100 ml volumetric flask were prepared. Dissolve equivalent weight of tablet formulations required for 100 ppm and dissolve that quantity in 100 ml diluents. 1ml of this stock was diluted to 10 ml to prepare 10 ppm stock solution. For the tablet formulation 20 tablets from each tablet batch were crushed respectively. Further dilute to 1 ppm, 2 ppm, and so on, were prepared by taking 0.1 ml, 0.2 ml and so on of standard test solution and diluting it to 10 ml. Validation experiment was performed to demonstrate system suitability, linearity, precision, accuracy study, ruggedness and robustness as per ICH guidelines.[7]

3.5 System Suitability Parameters

The area of respective concentrations, theoretical plates, number of theoretical plates per height and the peak symmetry was recorded.

3.6 Linearity

Dilution of standard impurity in the range of 1-6 µg/ml were prepared by taking suitable aliquots of working standard solution in different 10 ml volumetric flasks and diluting upto the mark with mobile phase. 20 µl was injected from it each time into the column at flow rate of 1 ml/min. The standard from elute was monitored at 240 nm and corresponding chromatogram were obtained from these chromatograms peak area were calculated. A plot of peak area over concentration was constructed. Regression of the plot was computed by least square regression method.

3.7 Precision

Precision of analytical method was studied by multiple injections of homogenous samples. 6 replicate of 4 ppm solution were prepared and injected for precision at the same flow rate of 1ml/min. The intra-day and inter-day precision was used to study the variability of the method. SD and RSD were calculated for both.

3.8 Accuracy

Accuracy of the method was studied using the method of standard addition. Standard impurity solutions were added to the unknown bulk and tablet formulation of Amlodipine. The percent recovery was determined at three different levels (50%, 75% and 100%). Impurity content was determined and the percent recovery was calculated.

3.9 Robustness

Robustness was studied by changing parameters like change in flow rate. The SD and RSD between the change parameter were calculated.

3.10 Ruggedness

Ruggedness was studied was carried out by using different analysts. The SD and RSD were calculated.

3.11 LOD and LOQ

Limit of detection and limit of quantitation of the method was calculated by formula given below

LOD= 3.3xSD/Slope

LOQ= 10xSD/Slope

3.12 Quantitation of Impurity

The total amount of impurity present in Amlodipine bulk and formulations was calculated for the synthesized compound and the result was compared to ICH limit for impurities in new drug substance is 0.1%.

3.13 Statistical Calculations

The standard curve, slope and intercept were determined by statistical software version 0.5. Regression curve analysis was carried out by using of Microsoft Excel 2007 software, with intersecting through zero. Means, standard deviations, one way ANOVA test carried out by using Graph pad PRISM software version 6.0.

4. RESULTS AND DISCUSSION

4.1. Physicochemical properties

Table 1: Physicochemical Properties of Amlodipine Impurity

Molecular Formula	Molecular Weight	M.P.^{o}C	Rf Value
$C_{19}H_{22}ClNO_4$	363.84	160-162 ^{o}C	0.74

4.2UV Data

The λmax for Amlodipine impurity was found to be 240nm.

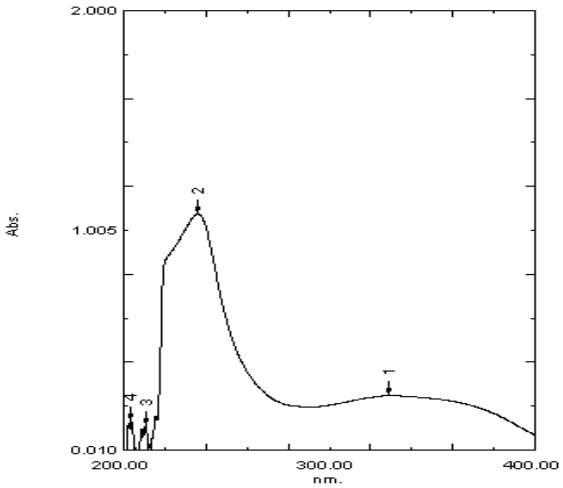

Fig 4: UV spectra of impurity

4.3. IR Data

The major functional groups are primary amine, nitro and carbonyl groups. Obtained peaks in IR spectrum are as follows.

IR (KBr) cm^{-1}: 3327(NH-Stretch), 2937, 2978, 3078(C-H Stretch for aromatic), 2802, 2874(C-H Stretch for aliphatic), 1683(C=O Stretch), 1610(C=C Stretch), 1487, 1049(C-O-C stretch), 1452(CH_3 Bend), 831.35(Substitution of chlorine at para position of benzene ring).

4.4. NMR Data

4.4.1. ^{1}HNMR (CDCL3)

δ = 5.830(1H,NH of 1,4-dihydropyridine), 1.159(6H,CH_3 of 1,4-dihydropyridine), 4.066 (4H,CH_2 proton of ester), 2.303(6H,CH_3 proton of ester), 6.498(1H, CH of 1,4-dihydropyridine ring)

4.4.2. ^{13}CNMR (CDCL3)

δ= 13.99(2C, CH_3 Carbon attached to CH_2), 50.86(2C, CH_2 Carbon attached to CH_3), 167.34 (2C, Carbonyl carbon attached to 1,4- dihydropyridine ring), 19.15(2C, CH_3 Carbon attached to 1,4- dihydropyridine ring), 131.15(2C, C=C of 1,4- dihydropyridine ring), 132.75(2C, C=C of 1,4- dihydropyridine ring), 34.41(1C, Carbon of 1,4- dihydropyridine), 147.49(6C, Carbon of phenyl ring).

HPLC method validation

Optimized chromatographic conditions

Optimized chromatographic conditions for RP-HPLC

I. Range: The range of the impurity was found to be 1-20µg/ml.

II. Linearity

The linearity of the proposed method was estimated by regression analysis at six concentration levels in the range of 1-6 µg/ml for intermediate. The correlation coefficient (R2) was found to be 0.993 and intercept Y=35.92x + 92.57 was linear.

Table 02: Results of linearity by HPLC

Sr. No.	Concentration (ppm)	Area at 240nm
1		
2	2	202.76
3	3	260.43
4	4	328.67
5	5	420.54
6	6	497.56

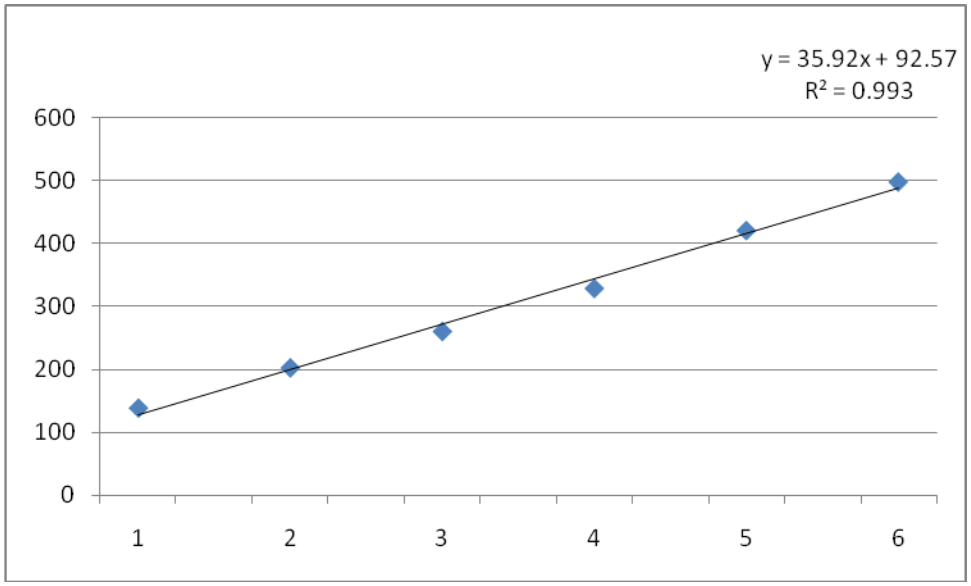

Figure 05: Calibration curve for impurity by HPLC:

III. Precision: The precision of the intermediate was quantified for repeated concentration of 4 µg/ml in range and was reliable with their area of chromatogram as shown in above table. The Standard deviation (SD) and Relative standard deviation (RSD) was found to be 0.8093 and 0.65507 respectively. The intra and interday precision was carrying out and difference in % RSD was found not much varies and remains less than 2% indicate preciseness of method.

Table 03: Precision study

Sr.No	Concentration (ppm)	Peak Area (mV) at 240 nm	Mean	Standard Deviation	RSD
1	4	328.99	328.595	0.8093	0.65507
2	4	328.67			
3	4	328.56			
4	4	327.34			
5	4	329.78			
6	4	328.23			

Table 04: Interday Precision: Intraday readings were taken after 4 hours.

Sr.no	Concentration (ppm)	0 hrs study at 240nm	after 5 hrs study at 240 nm
1	4	328.76	326.54
2	4	329.45	330.34
3	4	327.43	328.67
4	4	328.13	328.45
5	4	328. 90	329.80
6	4	326.54	328.35
7	4	328.42	329.89
mean		328.232	328.86
SD		0.9798	1.2898
RSD		0.96006	1.6635
Mean SD	1.6288		
Mean RSD	1.6049		

Table 05: b)Interday readings were taken after 24 hours.

Sr.no	Concentration (ppm)	0 hrs study at 240nm peak area	after 24 hrs study at 240 nm(peak area)
1	4	328.76	330.65
2	4	329.45	330.54
3	4	327.43	331.65
4	4	328.13	329.78
5	4	328. 90	331.89
6	4	326.54	332.23
7	4	328.42	329.56
mean		328.232	330.90
SD		0.9798	1.045
RSD		0.96006	1.0922
Mean SD	1.5023		
Mean RSD	1.5061		

IV) Robustness: The robustness of the Intermediate was performed for change in flow rate from 0.5ml/min to 0.7 ml/min and method was robust with standard deviation 1.1406 and relative standard deviation 1.5153.

Table 06: Robustness study

Sr. No	Conc. (ppm)	Peak Area (mV) 0.5ml/s	Peak Area (mV) 0.7ml/s	Mean	S.D	% R.S.D
1	1	138.78	137.67	138.225	0.784	0.6160
2	2	203.67	202.76	203.215	0.6434	0.4140
3	3	260.45	258.43	259.44	1.4283	2.040
4	4	328.89	327.12	328.00	1.2515	1.5664
5	5	420.76	418.45	419.83	1.3152	1.7298
6	6	497.71	495.70	496.70	1.4212	2.020
Average				307.566	1.1406	1.5153

V) Ruggedness: The ruggedness of the Intermediate was carried out for change in Analyst and method was found to be rugged.

Table 07: Ruggedness study

Analyst	SD	%RSD
Analyst-I	0.8956	0.8783
Analyst-II	0.7845	0.7987

VI) Accuracy: Accuracy study was performed by the recovery method. The results demonstrate that the percentage recovery in tablet was more than bulk due to the presence of impurity in the tablet. Percentage recovery was found to be more at higher concentration level a compare to lower concentration level.

Table 08: Accuracy study

Sr no.	Sample	% amount recovered			mean	SD	RSD
		50%	100%	150%			
1	Bulk	90.56	91.78	89.56	90.633	1.111	1.236
2	Tablet I	93.89	96.67	95.07	95.213	1.395	1.946
3	Tablet II	97.45	95.78	95.67	96.333	0.8356	0.6982

VII) Limit of detection

The LOD by HPLC was 74.34 ng and that of LOQ 225 ng the method is more sensitive and selective. To verify that analytical system is working properly and can give accurate and precise results the system suitability parameters are to be set and it was found to be in stated range.

Table 9: LOD and LOQ study

Sr.No	Parameter	Observation
1	LOD	74.34ng/ml
2	LOQ	225 ng/ml

VIII) Assay or Quantitation of Synthesized Compound

Quantization of process related impurity of amlodipine in bulk and tablets was carried out. Impurity was found in bulk and in tablet I & II it was found to be 0.223%, 0.236%.and 0.312% respectively. As per the ICH limit the amount of impurity is more than 0.1% indicates that the impurity found in bulk and tablet formulations is potential impurity.

Table 10: Assay study

Bulk/Formulation	% Quantity Found
Bulk	0.223%
Tablet I	0.236%
Tablet II	0.312%

IX) System Suitability Parameters

Table 11: System Suitability Parameters

Property	Values	Official limits
Retention time (t_R)	7.860	-
Theoretical plates (N)	5813	N≥ 2000
Resolution (R)	3.320	R≥ 2
Tailing factor (T)	0.18	T≤ 2

Table 12: ANOVA study

% RECOVERY		
Observation	**Tablet I**	**Tablet II**
1	92.50	93.23
2	90.89	90.96
3	90.56	91.67
Mean	91.296	91.953
SD	1.0602	1.1612
%RSD	1.161	1.262
Variance	1.124	1.348

ANOVA					
Observation	SS	DF	MS	F Value	P Value
Between the groups	0.647	1	0.647	0.524	0.509
Within the groups	4.945	4	1.236		
Total	5.592	5			

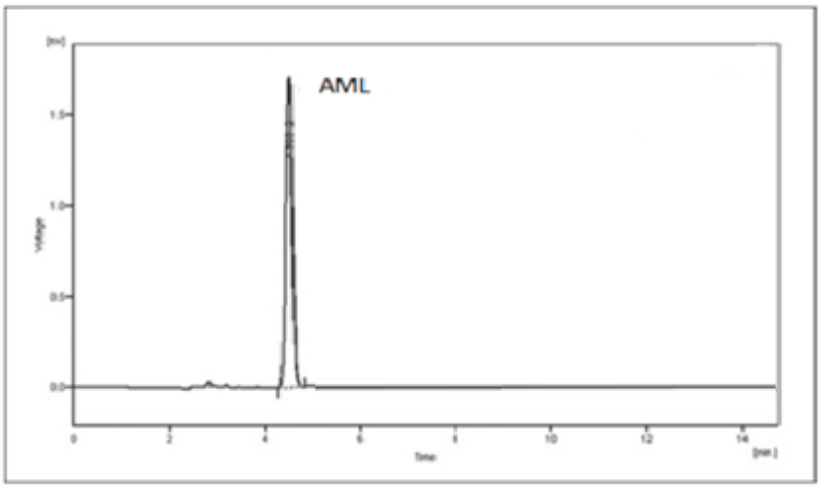

Figure 06: Chromatogram of Amlodipine

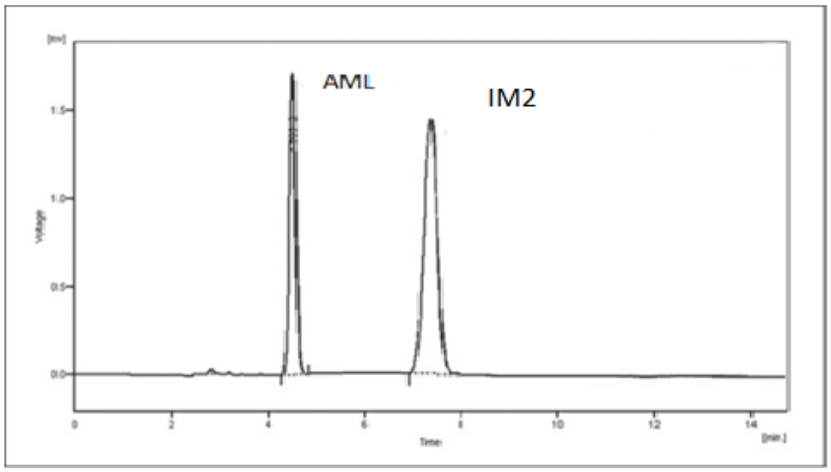

Figure 07: Chromatogram of Amlodipine and Impurity

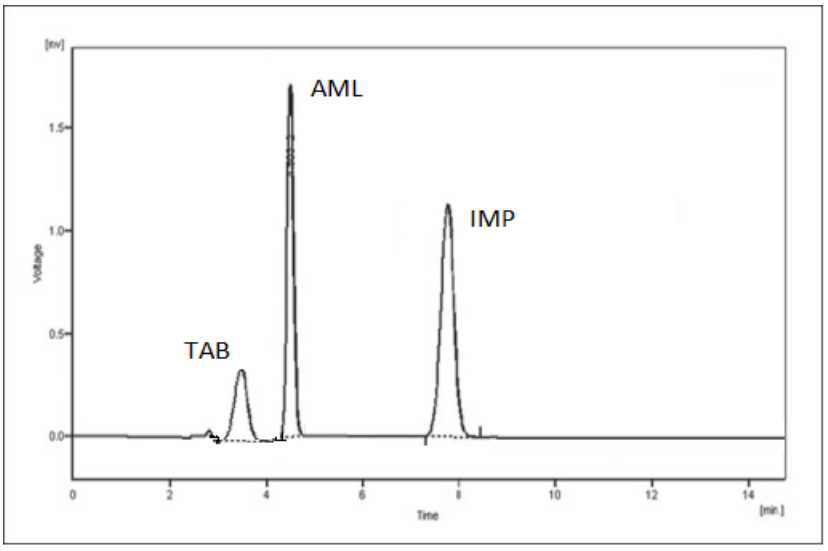

Figure 08: Chromatogram of Amlodipine formulation, Amlodipine and impurity

5.0 CONCLUSION

A successful hantzsch pyridine synthesis method was developed for the synthesis of diethyl 4-(4-chlorophenyl)-2, 6-dimethyl-1, 4-dihydropyridine-3, 5-dicarboxylate impurity. The impurity was recrystallized and preliminary evaluation was done on lab scale viz. Melting point, TLC and elemental analysis. The melting point of impurity was found to be 160-162^{0}C. The TLC of impurity was carried out by using cyclohexane: ethyl acetate: formic acid in the ratio of 6:3:1was selected as mobile phase for quantification of impurity. The Rf value of the impurity was found to be 0.74. The developed RP-HPLC method was found to be linear, precise, accurate, robust and rugged. Quantization of process related impurity of Amlodipine in bulk and tablets was carried out. Impurity was found in bulk and in tablet I & II it was found to be 0.223%, 0.236%.and 0.312% respectively. From the ANOVA study the p value is not less than 0.05 therefore the null hypothesis is accepted for the study. As per the ICH limit the amount of impurity is more than 0.1% indicates that the impurity found in bulk and tablet formulations is potential impurity.

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