IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF PROCESS RELATED IMPURITIES FROM LACIDIPINE BULK AND FORMULATION

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ABSTRACT

The process related impurity of Lacidipine 3-{2-[3, 5-bis(ethoxycarbonyl)-1,4-dihydropyridin-4-yl]phenyl}prop-2-enoic acid in bulk and formulations was synthesized, characterized and the RP-HPLC method was developed according to ICH Q2B guidelines for quantitation of impurity in bulk and formulations. The synthesis of intermediate was carried out by Hantzsch pyridine process using substituted aldehyde, EAA and Urea and methanol as catalyst. The percentage yield was found to be 69.89 %. The impurity was recrystallized and purified. The preliminary evaluation was done on lab scale viz. melting point, TLC and elemental analysis. The melting point of impurity was found to be 156^0C. The TLC of impurity was carried by using Benzene and Methanol (6:1) and the R_f was found to be 0.80.

Key-words: Impurity, Lacidipine, Process, HPLC, Validation.

INTRODUCTION

There has been ever increasing interest in impurities present in Active Pharmaceutical Ingredient's (API's). Nowadays, not only purity profile but also impurity profile has become mandatory according to the various regulatory authorities.[1-3] In the pharmaceutical world, an impurity is considered as an inorganic or organic material, or residual solvents other than the drug substances, or ingredients, arising out of synthesis or unwanted chemicals that remain with APIs. Impurity profiling includes identification, structure elucidation and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulation. The

control of impurities in Formulated products and API's were regulated by various regulatory authorities like ICH, USFDA, Canadian Drug, and Health Agency. Impurity profiling is very important in the modern pharmaceutical analysis due to the fact that unidentified, potentially toxic impurities are hazardous to health and in order to increase the safety of drug therapy, impurities should be identified and determined by the selective method. Nowadays, [4-6]it is a mandatory requirement in various pharmacopeias to know the impurities present in APIs and finished drug products. Thus, impurity profiling can act as a Quality Control tool. It can provide crucial data regarding the toxicity, safety, various limits of detection and limits of quantitation of several organic and inorganic impurities, usually accompany with APIs and finished products. There is a strong requirement to have unique specifications/standards with regard to impurities. The major aim of bulk drug industries and pharmaceutical industries is to produce the best quality product. [7,8] As drugs are meant for saving lives and even minute quantities of impurities are unacceptable. Hence, impurity profiling has become very important. Impurity can be defined as any substance that exists with the original drug; it can be starting material or intermediates that are formed due to any side reactions. Our aim is to provide details regarding impurity and its profiling, which is very important in the Pharmaceutical sector. As per the, International Conference on Harmonization (ICH), "any component of the drug product that is not the chemical entity defined as the drug substance or an excipient in the drug product is considered an impurity.[9-10]

Materials and Methods

Material

3-(2-formylphenyl)prop-2-enoic acid, Ethyl acetoacetate, Urea, Acetone, silica gel all the chemical were purchased from Merck Chemicals Pvt. Ltd. Nasik, MS, India are of AR grade.Methanol, benzene, pyridine, ammonia, ethylacetoacetate of AR grade and the acetonitrile, methanol and water of HPLC grade were purchased from Merck Chemicals Pvt. Ltd. Nasik, MS, India.

Methodology

Synthesis of 3-{2-[3, 5-bis(ethoxycarbonyl)-1,4-dihydropyridin-4-yl]phenyl}prop-2-enoic acid [11]

0.01 mole of 3-(2-formylphenyl)prop-2-enoic acid, 0.02 mole of ethyl acetoacetate and 0.01 mole of urea in presence of 10 ml of ethanol was subjected for MW irradiation at 250W for 2 minutes in an interval of 30 seconds. The resultant mixture is the transferred to ice cold water to offer yellow crystalline product. Filtered recrystallized from alcohol to offer title compound.

HPLC Method Development and Validation [12-14]

The quantitation of 1, 4-DHP 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 236 nm. The methanol: water in proportion of (60:40) 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.

1. Analytical Method Validation

A suitable analytical method was developed and validated for identification. New drug development requires meaningful and reliable analytical data to be produced at various stages of development.

2. Preparation of Mobile phase

The selection of mobile phase was according to polarity and non-polarity of solvents. The methanol: water was selected as mobile phase in ratio of 60:40 and was filtered on membrane filter (0.45 µ) to remove degassing and were stirred for 10-15 min.

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 1-6 ppm in concentrations respectively for the method validation by HPLC.

4. Preparation of sample solution (formulation)

Stock solution of bulk Lacidpine, 2 different batches of Lacidpine marketed formulation of 100 ppm in 100 ml volumetric flask were prepared. Dissolve 10 mg of test sample 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 2 tablet batch were crushed respectively. The powder of this formulation equivalent to 10 mg of the drug was used to prepare the stock solution. 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.

1. System Suitability Parameters:

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

2. 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 up to 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 264 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. 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.

4. 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 Lacidpine. The percent recovery was determined at three different levels (50%, 75% and 100%). Impurity content was determined and the percent recovery was calculated.

5. Robustness

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

6. Ruggedness

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

7. 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

5.5 Quantitation of Impurity

The total amount of impurity present in Lacidipine 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%.

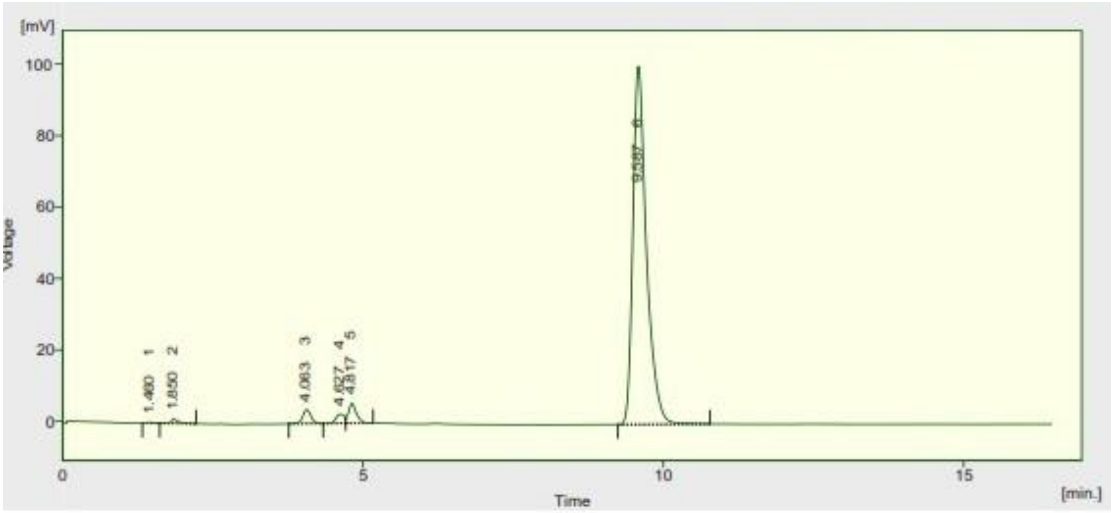
RESULT AND DISCUSSION

HPLC Method Validation

The ICH Q2B guidelines discuss the analytical method validation on HPLC. Currently the vast majority of process-related impurity determinations are performed by HPLC. It offered the desired sensitivity for trace level determinations with a high degree of automation. A wide variety of stationary phases and operation modes make HPLC applicable to all drug classes. The typical detection limits for process-related impurities by HPLC are 0.1% or lower and this can be routinely met in the majority of circumstances using conventional UV detectors. These methods involved the prediction of likely impurities within the synthetic process, their isolation and identification by suitable analytical techniques.

The last step of the present study was to develop, validated HPLC method for detection and quantification of synthesized impurity in bulk and tablet formulations.

HPLC Chromatograph of Lacidipine



Result Table

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.460	2.424	0.289	0.1	0.3	0.13
2	1.850	11.705	1.278	0.7	1.1	0.11
3	4.063	37.267	3.919	2.2	3.4	0.15
4	4.627	25.732	2.608	1.5	2.3	0.17
5	4.817	52.839	5.630	3.1	4.9	0.14
6	9.587	1568.718	100.231	92.3	88.0	0.23
	Total	1698.684	113.955	100.0	100.0	

Figure No.1:HPLC Chromatogram of Lacidipine

The Retention time of Lacidipine was 9.5 min.

HPLC Chromatogram of synthesized compound

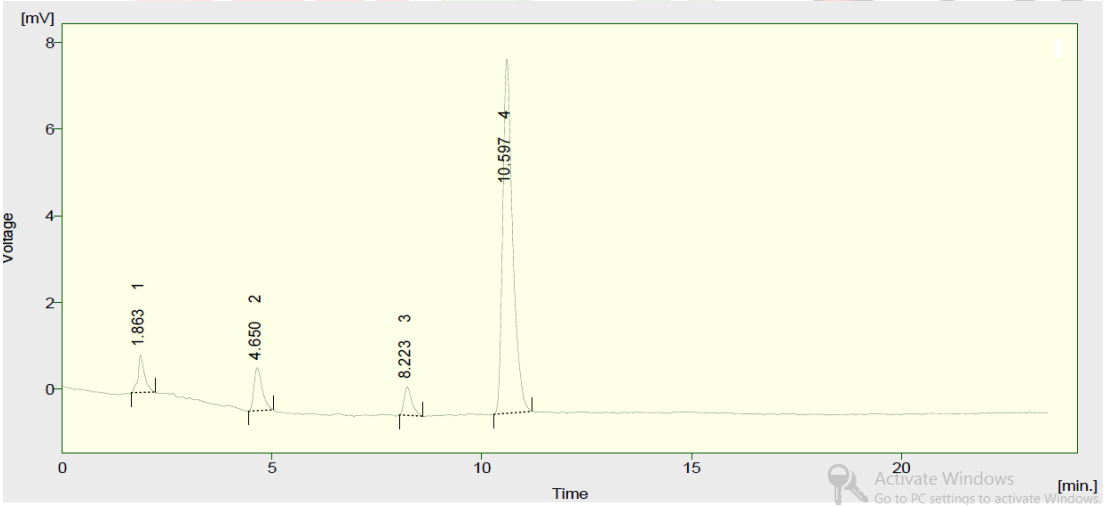


Figure No.2 HPLC chromatogram of synthesized compound

The retention time of impurity was 10.5 min and it shows a single peak which indicates purity of compound.

HPLC chromatogram of Lacidipine and synthesized compound combination

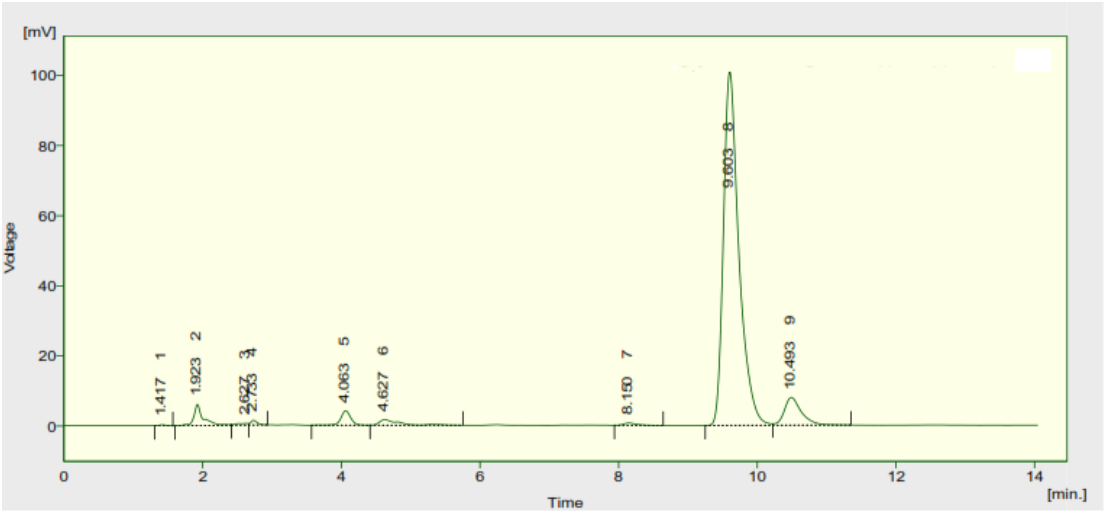


Figure No. 3 HPLC Chromatogram of Lacidipine and synthesized compound mixture.

The retention time of Lacidipine and synthesized compound in lab mixture was found at 9.60 and 10.49 min respectively.

HPLC Chromatogram of Tablet

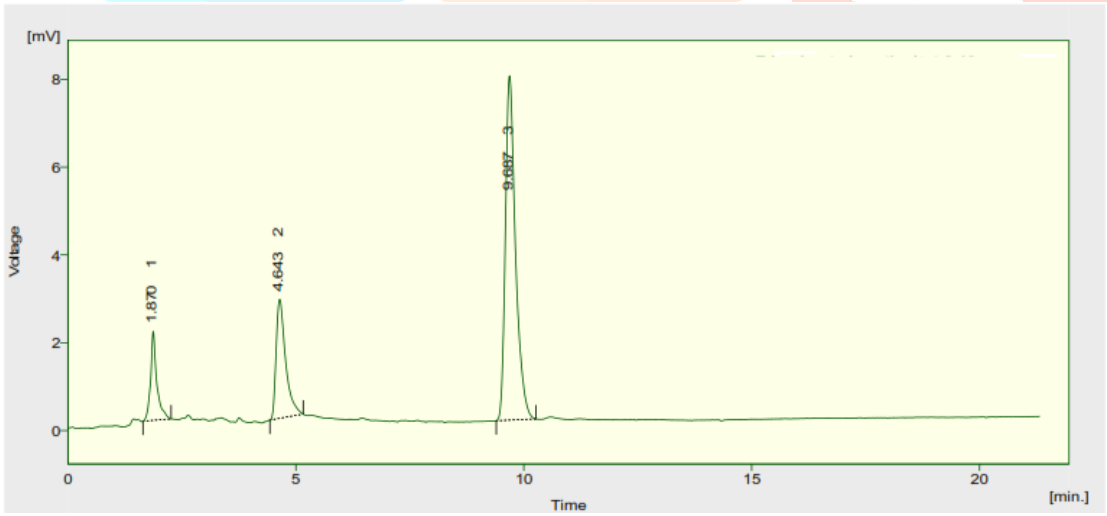


Figure No.4 HPLC Chromatogram of Lacidipine Tablet

The retention time of Lacidipine tablet was found at 9.6 min.

HPLC Chromatogram of Tablet and synthesized compound mixture

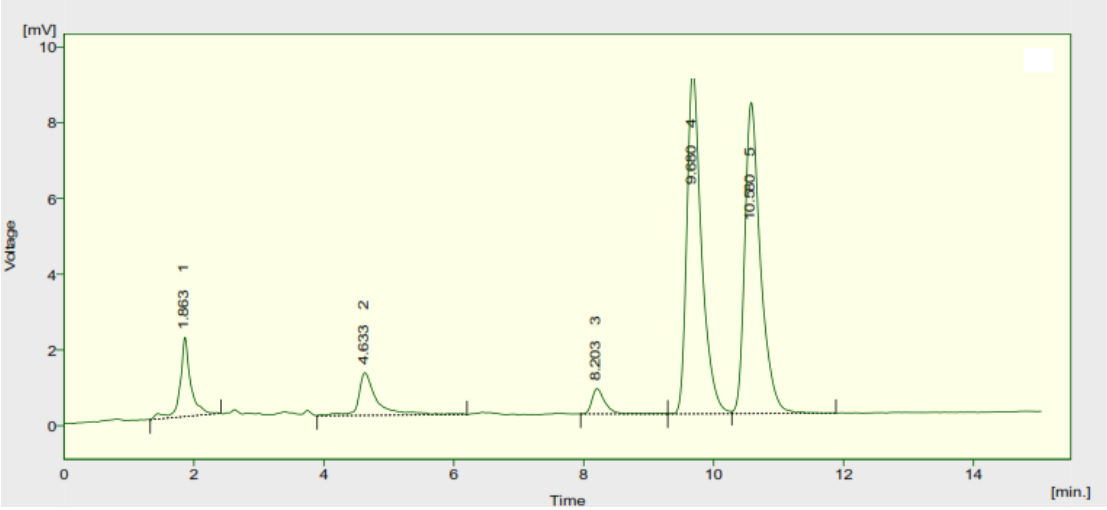


Figure No.5 HPLC Chromatogram of Tablet and synthesized compound mixture.

The retention time of Lacidipine Tablet and synthesized compound in lab mixture was found at 9.60 and 10.58 min respectively.

Optimized chromatographic condition

Table No.1 Optimized chromatographic condition for RP-HPLC

Chromatographic Conditions	**SHIMADZU HPLC System**
Mobile phase	Methanol: Water (60:40)
Column	ARP-C18 (250 mm X 4.6 mm), 5µ column
Flow rate	1 ml/min
Wavelength detection	264 nm
Injection volume	20µl
Temperature	Ambient
Retention time	10.5 min
Run time	15 Min

1. Linearity

Table No. 2 Result of Linearity by HPLC (Peak area vs. Conc.)

Sr. No	**Concentration (ppm)**	**Area (mill volts) at 245 nm**
1.	1	123.17
2.	2	215.68
3.	3	312.27
4.	4	419.19
5.	5	513.62
6.	6	618.54

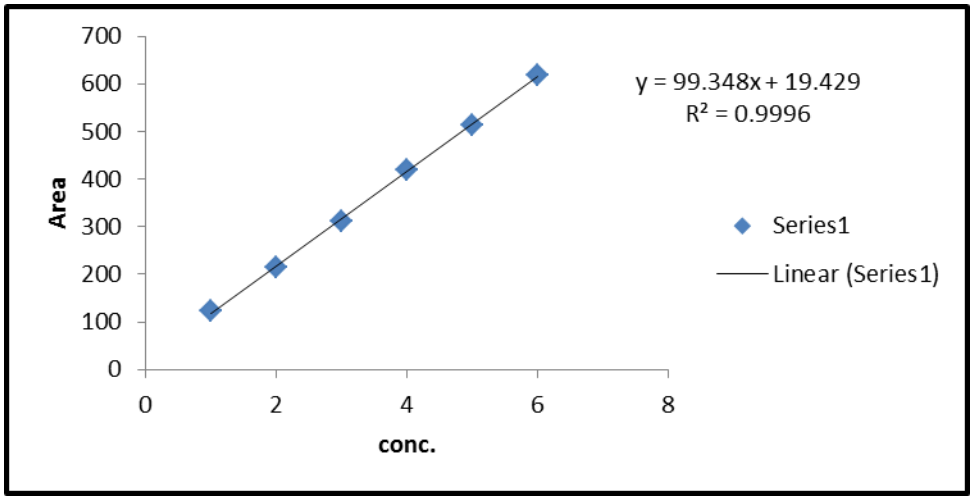


Figure No.6 Graph of linearity of synthesized compound by HPLC

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 (R^2) was found to be 0.999 and intercept Y= 99.34x + 19.42 was linear.

2. Precision

Table No. 3 Precision by HPLC

Sr.No	Concentration (ppm)	Peak area (mV)at 264 nm	Mean	SD	% RSD
1.	4	419.19	419.89	1.331	0.317
2.	4	418.98			
3.	4	421.90			
4.	4	419.90			
5.	4	418.39			
6.	4	421.01			

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 1.331 and 0.317 respectively.

a. Intraday precision after 4 hours

Table No.4 Result of Intraday precision after 4 hours

Sr. No	Conc. (ppm)	Peak area after 4 hour at 264 nm	Mean	S.D	%RSD
1.	4	418.83	417.0	2.250	0.539
2.	4	413.15			
3	4	418.39			
4.	4	417.12			
5.	4	415.86			
6.	4	418.99			

b. Interday precision after 24 hours

Table No.5 Intraday precision after 24 hours

Sr. No	Conc. (ppm)	Peak area after 24 hour at 264 nm	Mean	S.D	%RSD
1.	4	423.12	424.42	1.760	0.414
2.	4	422.60			
3.	4	426.66			
4.	4	424.24			
5.	4	423.42			
6.	4	426.53			

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.

3. Robustness

Table No.6 Results of Robustness study by change in flow rate.

At flow rate of 0.8 ml/min

Sr. No	Conc. (ppm)	Peak area (mV) 0.8 ml/min	Mean	S.D	%RSD
1.	4	787.12	789.07	3.122	0.395
2.	4	784.96			
3.	4	793.21			
4.	4	786.68			
5.	4	791.48			
6.	4	789.07			

The robustness of the Intermediate was performed for change in flow rate upto 0.8 ml/min and method was robust with standard deviation 3.122 and relative standard deviation 0.395.

5. Ruggedness

Table No.7 Results of Ruggedness study by change in analyst.

Sr. No	Conc. (ppm)	Peak Area in mV		Mean		S.D		%RSD	
		Analyst I	Analyst II	I	II	I	II	I	II
1.	4	419.19	418.80	419.94	420.66	1.392	1.481	0.3314	0.3520
2.	4	418.98	420.18						
3.	4	421.90	420.36						
4.	4	419.86	421.79						
5.	4	418.39	419.93						
6.	4	421.34	422.98						

The ruggedness of the Intermediate was carried out for change in analyst and method was found to be robust.

Table No.8 Summary of Precision

Sr .No	Parameter	SD	%RSD
1.	Precision	1.331	0.317
2.	Intraday precision	2.250	0.539
3.	Interday precision	1.760	0.414
4.	Robustness	3.122	0.395
5.	Ruggedness	1.436	0.341

The summary of the precision is given in the above table and % RSD was found to be ≤ 2.

5. Accuracy

Table No.9 Result of recovery study by HPLC

Sr.No.	Drug / Formulation	Percentage recovery			Mean	S.D.	%RSD
		50%	75%	100%			
1.	Bulk	98.18	99.07	99.89	99.04	0.852	0.863
2.	Tablet I	99.22	101.30	103.79	101.43	2.288	2.255
3.	Tablet II	99.25	101.68	103.13	101.30	1.969	1.934

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.

6. Limit of detection

$$LOD = \frac{3.3 \times \text{Standard deviation}}{\text{Slope}}$$

$$LOD = \frac{3.3 \times 1.3313}{98.44}$$

LOD = 0.4462

7. Limit of quantitation

$$LOQ = \frac{10 \times \text{Standard deviation}}{\text{Slope}}$$

$$LOQ = \frac{10 \times 1.3313}{98.44}$$

LOQ = 0.1352

The LOD by HPLC was 446.2 ng and that of LOQ 135.2 ng the method is more sensitive and selective.

System Suitability Parameters

Table No.10 System Suitability Parameters of synthesized compound

Property	**Values**	**Required limits**
Retention time (t_R)	10.59	RSD ≤ 1%
Theoretical plates (N)	10224	N ≥ 2000
Resolution (R)	6.36	R ≥ 2

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.

6. Quantitation of Synthesized Compound

Quantitation of process related impurity of Lacidipine in bulk and tablets was carried out.

Table No.10 Quantitation of process related impurity of Lacidipine in bulk and tablets.

Sr.No.	**Bulk/ Formulation**	**Quantisation of Impurity**
1.	Bulk Lacidipine	Absent
2.	Lacidipine tablet I	0.28%
3.	Lacidipine tablet II	0.33%

Impurity was not found in bulk and in tablet I & II it was found to be 0.28%.and 0.33% respectively. As per the ICH limit the amount of impurity is more than 0.1% indicates that the impurity found in tablet formulations is potential impurity.

CONCLUSION

This project study was focussed toward synthesis, characterization and quantification of 3-{2-[3, 5-bis(ethoxycarbonyl)-1,4-dihydropyridin-4-yl]phenyl}prop-2-enoic acid impurity in Lacidipine and its marketed formulations by Reverse Phase High Performance Liquid Chromatography method. The synthesis of a process related impurity of Lacidipine was successfully carried out by Hantzsch pyridine procedure. The impurity was purified by column chromatography. Characterization was done by I.R, ^{1}H-NMR, ^{13}C-NMR and GC-MS. Based on the spectral data, the structure of impurity was characterized as 3-{2-[3, 5-bis(ethoxycarbonyl)-1,4-dihydropyridin-4-yl]phenyl}prop-2-enoic acid. An efficient isocratic RP-HPLC was developed and validated according to ICH guidelines with respect to specificity, accuracy, linearity and precision. The validated HPLC method was used for detection and quantitation of 3-{2-[3, 5-bis(ethoxycarbonyl)-1,4-dihydropyridin-4-yl]phenyl}prop-2-enoic acid, a process related impurity of Lacidipine, from Lacidipine bulk and tablet formulations. The above method was found to be specific, accurate, precise, rugged and robust and can be used for routine analysis.

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