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Development and Validation Method for Marketed Formulation of Amlodipine by Different Analytical Technique

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ABSTRACT

In the current study, the author presents HPLC, HPTLC, and UV methodologies for the quick, accurate, and precise determination of amlodipine in dose form. Using a mobile phase mixture of Phosphate Buffer: ACN in a ratio of 65: 35%v/v adjusted to pH 3 with 0.1% v/v potassium di hydrogen phosphate at a flow rate of 1.0 mL/min, the separation was carried out on a C-YMC pack pro C18 (1004.6mm) 5µm at 354 nm, the detection was made. Amlodipine's retention period was discovered to be 3.4 minutes. Over the Amlodipine concentration range of 1–5 µg/mL, the calibration curve was linear. Using a pre-coated silica gel G60 F254 plate and the mobile phase chloroform: methanol: toluene in a ratio of 1.5: 3: 3.5 v/v/v, the HPTLC method was carried out, followed by analysis at 254 nm and 366 nm. For the HPTLC approach, linearity was discovered over the concentration range of 1–5 g/spot. The UV method's chosen wavelength for methanol measurement was 354 nm. The concentration range of 1 to 5 µg/mL showed linearity for the detector response. The analysis's findings have undergone statistical validation.

INTRODUCTION:

Amlodipine is a long-acting, lipophilic, third-generation dihydropyridine (DHP) CCB that works by preventing calcium from entering cardiac and vascular smooth muscle cells. This lowers peripheral vascular resistance (PVR). Amlodipine is prescribed to treat angina and high blood pressure (BP)/HTN. Additionally, several randomized trials have confirmed its effectiveness in treating angina pectoris. [1, 2] Due to its lengthy half-life, amlodipine is often dosed once daily, which is advantageous for patient compliance. Typically, a starting dose of 5 mg and a daily dose cap of 10 mg are advised. A starting dose of 2.5 mg is advised for the elderly and people who have hepatic insufficiency. Since amlodipine takes time to take effect, there is minimal reflex neuroendocrine activation. Lipid and carbohydrate metabolism may suffer when reflex mechanisms, such as high heart rate and PVR, are activated. The first generation -blockers (BBs), such as atenolol and metoprolol, and early generations of DHPs are among the medications that frequently cause these significant side effects. Amlodipine undergoes hepatic metabolism and exhibits considerable decreased clearance in the presence of liver cirrhosis, but no buildup in the presence of renal failure. Its bioavailability is high, ranging from 60% to 80%. Additionally, the slow rate of removal of amlodipine takes 40 to 60 hours. If amlodipine is stopped, blood pressure typically returns to normal within a week without any risky rebound spikes (unlike clonidine). [3-5]

HPLC [6, 7, 9–11], HPTLC [8], Colorimetric [6] and UV techniques were found in bulk medication and pharmaceutical dosage forms, according to a literature review. Instead of using a complex, expensive, and time-consuming LC-MS/MS or GC-MS/MS approach, laboratories lacking specialized analytical equipment can use the straightforward HPLC UV method because of its simplicity. In the current study, the author presents HPLC, HPTLC, and UV methodologies for the quick, accurate, and precise determination of amlodipine in dose form.

MATERIALS AND METHODS

Chemicals and Reagents: Amlodipine working standard of the pharmaceutical grade was gifted from Nuray Chemicals Pvt. Ltd, Tamil Nadu and all chemicals and reagents such as methanol, acetonitrile, sodium hydroxide pellets of analytical grade were provided by the college.

Instrumentation and Analytical conditions: The HPLC method was performed by using Shimadzu Prominence binary gradient, high pressure liquid chromatographic instrument. The instrument was provided with a YMC pack pro c18 (100×4.6mm,5µ), an LC 20 AD pump and an SPD 20A UV-Visible detector was employed in the study. A 10 µL Hamilton injection syringe was used for sample injection. Data acquisition was done by using LC solutions software. HPLC grade ACN and Analytical grade Potassium dihydrogen phosphate were used in the study. A freshly prepared binary mixture of Phosphate buffer: ACN (65:35 %v/v) adjusted to pH 3 with 0.1 % v/v Potassium dihydrogen phosphate was used as the mobile phase. The mobile phase was filtered through a 0.45 µ membrane filter and degassed before use. The flow rate of mobile phase was maintained at 1.0 mL/min. The detection of the drug was carried out at 354 nm. The analysis was carried out at 22 °C.

Chromatography was performed on 10 × 10 cm Aluminum TLC plates 60 F254 precoated with 250 µm layers of silica gel. Samples were applied in the form of bands, under a continuous flow of nitrogen, by means of a CAMAG-LINOMAT-5 (Switzerland) sample applicator fitted with 100 µL applicator syringe. A constant application rate of 0.1 µL per second was used and the distance between the adjacent bands were also optimized. The plates were then conditioned for 10 minutes in a presaturated twin-trough glass chamber (10 x 10 cm2). The spotted plate was then dipped in mobile phase chloroform: methanol: toluene (1.5: 3: 3.5) and ascending development was performed to a distance of around 80 mm from the point of application at ambient temperature. Subsequently, plates were dried in a current of air with the help of an air dryer, and spots were visualized in Camag UV cabinet copper formed at 254 nm with Camag TLC scanner III operated in reflectance-absorbance mode and controlled by Win Cats software. The slit dimensions (4 × 0.2 mm) were also optimized and kept constant throughout the analysis

The UV method was performed on a double-beam Shimadzu UV-Visible Spectrophotometer 1800, with spectral bandwidth of 1.0 nm, wavelength accuracy ± 0.1 nm and a pair of 1-cm matched quartz cells were used to measure absorbance of the resulting solution. Methanol (Sd fine) and double distilled water were used as solvents in the UV method. Detection was done at 354 nm.

ASSAY BY HPLC [11, 12, 13]

Reagents	Grade
Potassium di hydrogen phosphate	AR
Formic acid	AR
Acetonitrile	HPLC
Water	HPLC/MILLI-Q

Chromatographic Conditions:

- Column: YMC pack pro c18(100×4.6mm,5µ)
- Column temperature: Ambient
- Flow rate: 1.0ml/min
- Wavelength: 242nm
- Injection volume: 10 µl
- Run time: 8min
- pH: 3
- Mobile phase: Phosphate Buffer: ACN (65: 35)

Solution Preparations:

i) Preparation of Buffer Solution:

- Dissolve about 6.80g of potassium di hydrogen phosphate in1000 ml of HPLC/ Milli – Q water.
- Adjusts the pH to 3.0 ± 0.05 with formic acid.

ii) Preparation of Mobile Phase: Prepare a mixture of buffer and acetonitrile in the ratio of 60:40 Filter through 0.45µ Membrane filter and degas.

iii) Preparation of Standard Solution: Weigh accurately about (5 + 50) mg of amlodipine and atenolol reference /working standard in to a 100 ml volumetric flask, add 30 ml of methanol sonicate to dissolve. Then make up to the volume with methanol. Pipette out 10ml

of this solution into a 100 ml volumetric flask and dilute up to the mark with mobile phase. Filter through 0.45 µ membrane filter.

Prepare the standard solution in duplicate calculate the similarity factor for standard-I and Standard -II solutions by using the following formula.

$$\frac{\text{Area of standard solution-1} \times \text{weight of STD (in mg) solution-2}}{\text{Area of standard solution-2 (1st injection)} \times \text{weight of STD (in mg) solution-1}}$$

Note: similarity factor for both the standard solutions should be between 0.98 to 1.02.

iv) Preparation of Sample Solutions:

For 5 mg tablets: Weigh and transfer 5 tablets into a100 ml volumetric flask. Add 50 ml of methanol and sonicate to dissolve then make up to the volume with methanol, pipette out 5ml of this solution into a 100 ml volumetric flask and dilute up to the mark with mobile phase. Filter through 0.45 µ membrane filter.

Procedure: Inject 10 µl portions of blank, standard solution and sample solutions into the chromatograph and record the chromatograms. Record the peak responses for the major peaks.

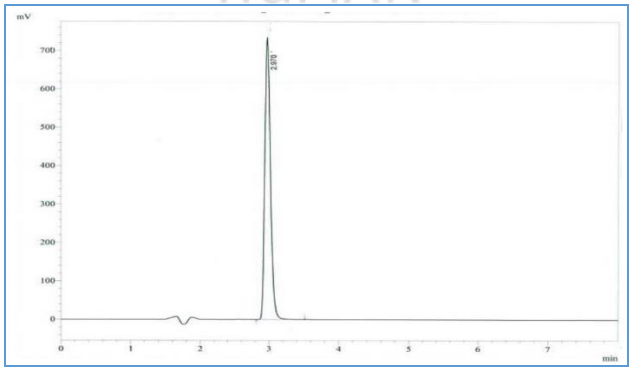

Figure 1: HPLC Chromatogram of Amlopress (Amlodipine)

Validation Program for Assay: [14]

System Suitability: Five replicate injections of the standard solutions were injected the percentage RSD for the peak area and tailing factor for Amlodipine were calculated.

Table 1: System Suitability

No. of injection	Area	Tailing Factor
1	4132232	1.543
2	4123452	1.532
3	4112965	1.523
4	4135421	1.526
5	4119764	1.521
Average	4124766.8	1.529
SD	92367.53	
% RSD	0.21	

Specificity: Blank, placebo, standard, sample solution injected into HPLC system. There was no interference from the blank and placebo at the retention time of Amlodipine peak. Peak purity reveals that Amlodipine peak was homogeneous and there were no co-eluting peaks at the retention time of Amlodipine peak.

Placebo Preparation: Weighed and transferred 1.812 gm of placebo into 100ml volumetric flask, 50 ml of methanol added and sonicated to dissolve. Then made up to the volume with methanol. Pipette out 5 ml of this solution into a 100 ml volumetric flask and diluted up to the mark with mobile phase. Filtered through 0.45 µ membrane filter and injected into the chromatogram.

Accuracy/Recovery: Known amount of Amlopress spiked with placebo at about 80%, 100%, and 120% of working concentration in triplicate and analyzed as per testing procedure. The percentage recovery was calculated from the amount found and actual amount added.

Table 2: Accuracy

Level	Amount found in µg	Actual amount added in µg	% Recovery	Mean	% RSD
Level-I 80%	19.87 19.88 19.92	19.12 19.12 19.12	100.53 100.57 100.61	100.57	0.07
Level-II 100%	25.65 25.67 25.71	25.45 25.45 25.45	100.62 100.63 100.65	100.23	0.07
Level-III	29.76 29.78 29.80	29.65 29.65 29.65	100.2 100.2 100.2	100.15	0.11

Linearity And Range: The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The range of the analytical procedure is the interval between the upper and lower concentration (amount) of analyte in the sample (including the concentrations). For which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

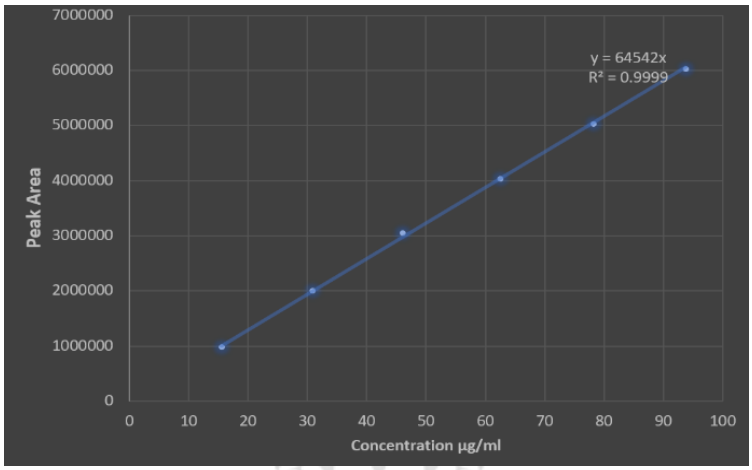

Figure 2: Calibration Curve for Amlodipine

Precision:

a) Repeatability: Prepare the standard and sample solutions. Inject standard and sample preparations and record the chromatograms. Calculate the % content of amlodipine.

Table 3: Repeatability

Sample	Weight (mg)	Area	% Assay
1	1287.76	4123875	97.54
2	1304.98	4117432	96.74
3	1309.65	4164332	98.53
4	1312.45	4159743	97.32
5	1305.76	4175421	97.53
6	1309.43	4195133	98.56
Average			97.23
SD			0.35
% RSD			0.34

Intermediate Precision (Ruggedness): Ruggedness of the method was verified by analysing the six samples of same batch which was used for method precision as per testing procedure. This study was performed by different analyst using different instrument and different column on different day. Calculated the percentage assay and percentage relative standard deviation for six assay results.

Table 4: Ruggedness

Sample No.	Percentage of Assay (w/w)	
	Analyst-I	Analyst-II
1	98.64	99.02
2	99.53	99.76
3	98.89	99.32
4	100.4	99.89
5	99.55	99.45
6	99.46	99.34
Average	99.67	99.64
% RSD	0.56	0.87

System Precision: Five replicate injections of standard solution were injected. The mean and percentage RSD for the peak areas of Amlodipine were calculated.

Table 5: Precision

S. No.	Peak areas
1	4230402
2	4176493
3	4199246
4	4267211
5	4198313
Mean	4189643
% RSD	0.19

Method Precision: Six samples of 5mg tablets were analyzed as per test method. The percentage of assay and percentage RSD of six results were calculated.

Table 6: Method Precision

S. No.	Percentage assay
1	99.3
2	97.4
3	97.6
4	98.6
5	99.1
6	98.9
Mean	98.3
% RSD	1.2

Robustness: Carry out the analysis, mentioned under deliberately modified conditions mentioned in the table.

Table 7: Conditions of Robustness

S. No.	Parameters	Normal Condition	Higher Side	Lower Side
1	pH	3.0	3.3	2.9
2	Flow rate	1.0 ml/min	1.2 ml/min	0.8 ml/min
3	Wavelength	354 nm	350 nm	360 nm

Procedure: Injected all the standard and sample preparations and recorded the chromatograms. Calculated the % content of Amlodipine.

Table 8: Robustness

S. No.	Sample Type	% Assay
1	Flow 0.8 ml (spl-1)	100.4
2	Flow 0.8 ml (spl-2)	100.3
3	Flow 1.2 ml (spl-1)	100.4
4	Flow 1.2 ml (spl-2)	100.2
5	pH 2.9 (spl-1)	100.5
6	pH 2.9 (spl-2)	100.6
7	pH 3.3 (spl-1)	100.3
8	pH 3.3 (spl-2)	100.4
9	260 nm (spl-1)	100.5
10	260 nm (spl-2)	100.7
11	272 nm (spl-1)	100.4
12	272 nm (spl-2)	100.6
	Average	100.4
	SD	0.23
	RSD	0.21

METHOD DEVELOPMENT AND VALIDATION FOR AMLODIPINE BY HPTLC

Chromatograms: [15, 16, 17]

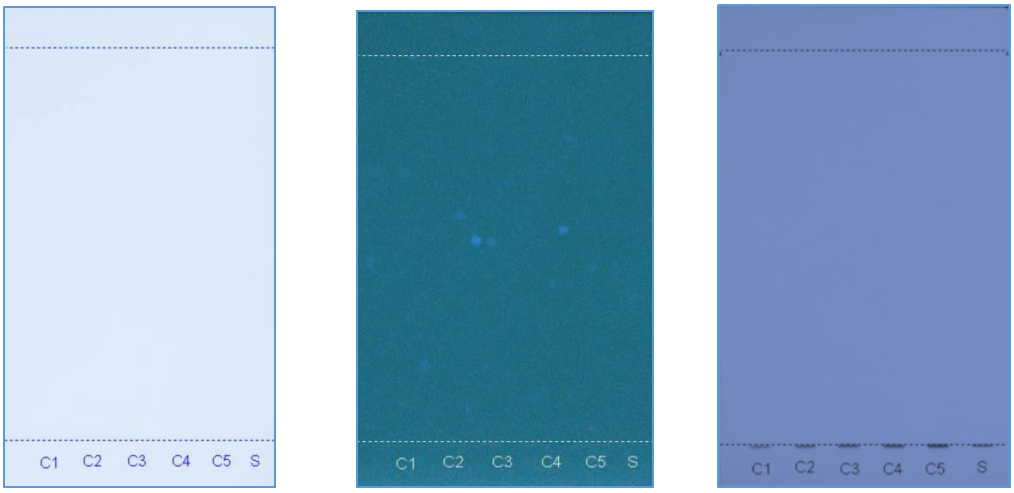

Figure 3. Daylight before mobile phase run **UV 366nm before mobile phase run** **UV 254 nm before mobile phase run**

3: Chromatogram of amlodipine

Analysis of Formulation:

Preparation of Sample Solution: Twenty tablets were powdered and weighed equivalent to 100 mg of Amlodipine which is transferred in to a100ml volumetric flask and extracted with methanol the extract was filtered through Whatman filter paper No.41 and residue washed with methanol and made up to 100ml with methanol. Aliquot of 0.3 µl solution of tablet formulation were applied and plate was developed with mobile phase.

Assay: The sample solutions were spotted along with the standard to check the specificity. Spotted 0.3 µl of sample solution allowed to develop in appropriate mobile phase and detect the spots as described earlier. From the peak area recorded the amount of the drug in the formulation was determined. [18]

Table 9: Analysis of Formulation

S. No.	Drug	Label Claim (mg)	Amount found (mg)	Assay % RSD
1	Amlodipine	5	4.763	98.45

Method Validation: [19]

1. Linearity: Aliquots of 0.1-0.5 µg/spot of standard solution of amlodipine is applied on the plate with the help of micro liter syringe using an automatic sample applicator. The plates were developed, dried and scanned densitometrically at 254 nm. The drug peak-area was calculated for each concentration level and a graph was plotted of drug concentration against the peak area.

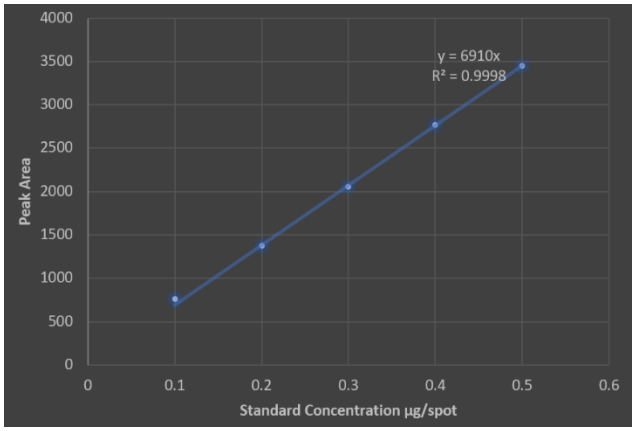

Figure 4: Calibration Curve of Amlodipine

2. Accuracy: Accuracy of the developed method was confirmed by doing a recovery study as per ICH guidelines at three different concentration levels (80%, 100% and 120%) by replicate analysis (n=3). Standard drug solutions were added to a preanalyzed sample solution, and then percentage of drug content was calculated. The results of the accuracy study are reported in Table 10. From the recovery study, it was clear that the method is very accurate for quantitative estimation of Amlodipine in tablet dosage form because all the statistical results were within the acceptance range (i.e., % RSD <2.0).

Table 10: Recovery studies for Amlodipine (n=3)

Label claim (mg/tablet)	Recovery level (%)	Amount Added (mg)	Amount recovered (mg) ± % RSD	% Recovery
5	80	4	3.56 ± 0.65	99.67
5	100	5	4.53 ± 0.65	98.32
5	120	6	6.07± 0.76	101.75

Precision: The precision of the method (system reproducibility) was assessed by spotting 0.3 µl of drug solution six times on a TLC plate, followed by development of plate and recording the peak area for 6 spots. The % RSD for peak area values of Amlodipine was found to be 0.58. The results were shown in Table 11. The method reproducibility (intra-day precision) was determined by analyzing standard solution in the concentration range of 0.1 µg/spot to 0.5 µg/spot of drug for 3 times on the same day and inter-day precision was determined by analysing corresponding standards daily for 3 day over a period of one week. The intra-day and inter-day coefficients of variation (%RSD) are in range of 0.13 to .36 and 0.30 to 0.56, respectively. [20]

Table 11: Precision of Amlodipine

S. No.	Concentration (µg/spot)	Peak Area
1	0.3	2045.87
2	0.3	2194.97
3	0.3	2078.98
4	0.3	2088.76
5	0.3	2198.86
6	0.3	2099.66
Mean		2088.8
% RSD		0.65

DEVELOPMENT OF UV SPECTROSCOPIC METHOD FOR THE AMLODIPINE

Selection of Solvents: The UV spectrum of Amlodipine was recorded in various solvents. The spectral pattern and absorbance maxima of amlodipine were thoroughly analyzed. It was found that significant spectra of amlodipine appeared in methanol and this solvent was selected for determining amlodipine content in formulation by UV spectroscopic method. Stock solution of amlodipine was prepared by dissolving 100mg of drug in 100ml of methanol to obtain the concentration of 1000 µg/ml. It was further diluted to obtain concentration ranging from 1-5 µg/ml. [21]

Selection of Wavelength: The stock solution was suitably diluted with methanol, so as to contain 10 µg/ml of amlodipine. This solution was scanned in the UV region and found that amlodipine exhibited maximum absorbance at about 354 nm. Hence 354 nm was selected for the proposed study.

Preparation of Standard Curve: Adequate dilutions were made from stock solution to get a concentration ranging from 1- 5 µg/ml for amlodipine using methanol. Absorbance of these solutions was measured at 354 nm. The measured absorbance was plotted against concentration. From the graph it was found that the Beer's law concentration for amlodipine lies between 1 -5 µg. The overlain spectra and the calibration graph are shown in fig (5).

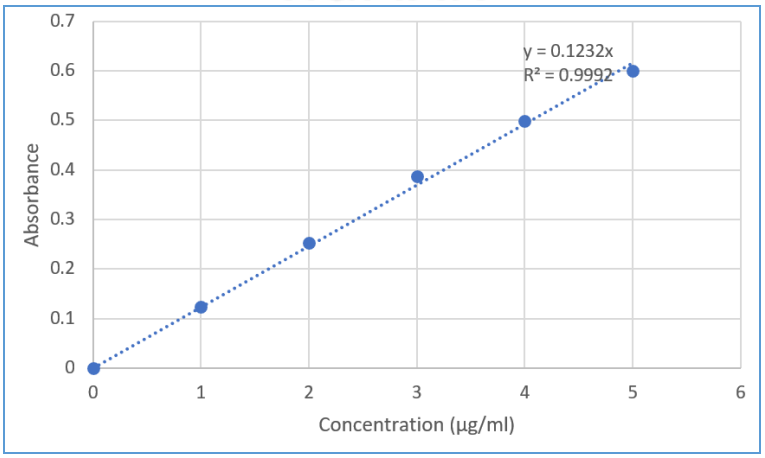

Figure 5: Absorbance of Amlodipine

Quantification Of Amlodipine in Formulation: The average weight of 20 tablets of amlodipine was weighed. An aliquot quantity equivalent to 100mg of amlodipine was weighed and transferred to 100 ml volumetric flask. The contents were shaken with methanol, so as to dissolve the active ingredients and filtered. Then it was made upto the

volume. It was further diluted to obtain the stock solution with a concentration 50 µg/ml. From this solution 3ml was taken and diluted to 50 ml to get a concentration of 3 µg/ml. The resultant solution was scanned in the wavelength range of 200-400 nm and the absorbance was measured. The concentration of drug was determined by single point standardization method and results are shown in table 12.

$$C_{test} = A_{test} \times C_{stnd} / A_{stnd}$$

Table 12 Analysis of Amlodipine formulation

Drug	Amount mg/tab Label	% Label Claim Found	% RSD
Amlodipine	5 mg	4.98 5.04 4.96 5.07 5.08 5.11	0.0498

Validation of the Method: The developed method was validated in terms of linearity, accuracy and stability studies.

1) Linearity: Amlodipine was found to be linear in a concentration range of 1-5 µg/ml. The absorbance of this solution was measured at 354 nm and a calibration graph was plotted using absorbances versus concentration. The correlation co-efficient value was found to be 0.9997.

2) Accuracy: The accuracy, specificity, suitability and validity of the present method were satisfied by conducting percentage recovery studies. A known quantity of the drug was added to the pre-analyzed sample formulation at 50% and 100% levels. The percentage recovery and standard deviation were calculated.

Table 13: Recovery Studies

Drug	Level	Amount found in µg	Actual amount added in µg	% Recovery	% RSD
Amlodipine	50%	2.54 2.98 2.68	2.45 2.53 2.48	100.5 100.3 100.6	0.14
	100%	4.65 4.61 4.73	4.55 4.65 4.49	100.4 100.2 100.5	0.12

3) Stability: The drug solution was found to be stable for about three hours at room temperature. Stability data reported in table 14.

Table 14: Stability Data

Concentration in µg/ml	Time (Min)	Absorbance
1	0	0.123
	30	0.126
	60	0.121
	90	0.119
	120	0.118
	150	0.117
	180	0.117

Table 15: Repeatability Studies

Concentration in µg/ml	Absorbance	% RSD
1	0.123 0.122 0.121 0.121 0.120 0.119	0.53
2	0.598 0.597 0.597 0.596 0.595 0.594	0.23

Table 16: Precision

Concentration in µg/ml	Absorbance	% RSD
1	0.123 0.122 0.121 0.121 0.120 0.119	0.53
2	0.598 0.597 0.597 0.596 0.595 0.594	0.23

RESULTS AND DISCUSSION:

Validated analytical methods are aimed for the estimation of Amlodipine in formulation. Simple, precise, rapid, accurate methods were developed for the estimation of Amlodipine in formulation by following methods.

- Estimation of Amlodipine by RP-HPLC
- Estimation of amlodipine by HPTLC
- Estimation of amlodipine by UV

In RP-HPLC method, a wavelength of 354 nm was selected and the mobile phase which consist potassium di hydrogen phosphate buffer: acetonitrile, in the ratio of (65:35). pH 3 adjusted with formic acid at a flow rate of 1ml/min were found to be optimum condition for analysis. The retention time was found to be 2.9 with optimized conditions.

Amlodipine showed the linearity in the range of 15.62 -93.75 µg/ml. Where the peak shape was symmetrical, and a good correlation coefficient value was obtained. The percentage label claim and recovery at three different levels, 80%, 100%, 120%, level was carried out. The suitability of the method was thus proved. Precision of the method was studied by making

repeated injection of the same sample and standard deviation was determined. Inter day and intraday precision was also carried out and % RSD was calculated.

In HPTLC during the stage of method development different mobile phase were tried and mobile phase comprising of ethyl chloroform: methanol: toluene in the proportion of 1.5: 3: 3.5 v/v/v for amlodipine, were found to be better and produced the Rf value of 0.72 for Amlodipine. The linearity of drug was determined by calibration curve and the linearity based on the area observed in the range of 0.1 – 0.5 µg/ ml. The regression coefficient value for Amlodipine is 0.999. Interday precision of the drugs was studied. No interference with the additives of the formulation was reported. Recovery studies were carried out for the accuracy parameter and were reported.

The proposed UV analytical method for the quantification of amlodipine in tablet formulation is simple, accurate, and rapid and can be employed for the routine analysis. Once the absorbance of the sample is determined, it requires only simple calculation. This method can be applied for the substances which obey Beer's law. The low standard deviation and good percentage recovery indicated the reproducibility and accuracy of the method.

The validated method was applied for the analysis of tablet containing 5 mg amlodipine drug as the label claim. The method developed was simple. It has showed a good peak and good Rf values.

These methods, RP-HPLC, HPTLC and UV Spectroscopy were found to be sensitive, precise, and accurate. However, these three methods can be used for the routine analysis of Amlodipine from formulation.

CONCLUSION:

In order to develop a RP-HPLC, HPTLC and UV effective most of the effect should be spent in method development and optimization as this will improve the final method performance. A well developed method should be easy to validate. A method should be developed with the goal to analyze rapidly, the preclinical samples, formulations and commercial samples.

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