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RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF AMLODIPINE AND LOSARTAN IN BINARY MIXTURE

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ABSTRACT

A Novel, selective and rapid Gradient Reversed Phase High Performance Liquid Chromatographic (RPHPLC) method for the analysis of Amlodipine and Losartan in binary mixture has been developed and validated. The chromatographic system consisted of a LC-20AT VP series model chromatograph equipped Spinchrome software. The separation was achieved from a Inertsil ODS 3V C18 (150 X 4.6 mm, 5µm) at ambient temperature with a mobile phase containing a mixture mobile Phase-A with 70% v/v of buffer pH-3.7 and 30%v/v of acetonitrile and mobile phase–B containing 70% v/v of acetonitrile and 30% v/v of buffer pH-3.7. The samples were monitored at 237 nm for detection at a flow rate of 1.0 mL/min and the retention time was about 5.13 and 11.11 mins for Amlodipine and Losartan respectively. The calibration curve was linear over the concentration range 1.25-7.5 µg/mL and 12.5-75 µg/mL for Amlodipine and Losartan respectively. The proposed method is accurate in the range of 99.95% - 100.133% recovery and precise (%RSD of intraday variation and %RSD of inter day variation were found to be within the acceptance criteria). Therefore, this method can be used as a more convenient and efficient option for the analysis of Amlodipine and Losartan in Quality control laboratory.

Keywords: RP-HPLC, Amlodipine, Losartan, Gradient.

1. INTRODUCTION:

A combination[1] of antihypertensive agents can better control blood pressure and reduce the number and severity of side effects than a monotherapy. To increase the compliance, combination therapy may have other advantages over monotherapy, such as synergistic mechanisms of action for controlling hypertension since both CCBs (calcium channel blockers) and ARBs (angiotensin II receptor type-1 blockers) are current and effective antihypertensive drugs, this study assessed the synergistic antihypertensive effects as well as the optimal combination ratio of these two drugs The present literature shows that there are only 2 RP-HPLC[2,3] are available in combination with Amlodipine and Losartan. Few analytical methods are in combination with other drugs including LC-MS/MS[4-6], HPLC[7-30], HPTLC[31-32] and spectroscopic[33] methods.

2. MATERIALS AND METHODS

Instrumentation and Chemicals

The liquid chromatographic system consisting of the following components was used for analysis. LC-20AT VP series model chromatograph equipped Spinchrome software. Detection of the drug was done by using a SPD-20A UV-Visible detector. The reference standard Amlodipine besilate and Losartan potassium were obtained from AIZANT Drug Research Solutions pvt. Ltd. Acetonitrile and orthophosphoric acid were of HPLC grade, while potassium dihydrogen phosphate is of GR grade (Merck Ltd. Mumbai, India) milli-Q water was used for the analysis.

Preparation of Buffer pH 3.70: About 3.4 gm of potassium dihydrogen phosphate was weighed accurately and transferred into 1000 mL of beaker containing milli-Q water, mixed well. The pH was adjusted to 3.70 ± 0.05 with

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dilute orthophosphoric acid and filtered through 0.45 µm membrane filter.

Preparation of Mobile phase:

Mobile Phase-A: pH 3.70 phosphate buffer was mixed with acetonitrile in the ratio 70:30 %v/v.

Mobile Phase-B: Acetonitrile was mixed with pH 3.70 phosphate buffer in the ratio 70:30 % v/v.

Diluent Preparation: Water and methanol was mixed in the ratio of 50:50%v/v was prepared and degassed for about 5 mins in a sonicator.

Preparation of Amlodipine besilate Standard Stock Solution: 10 mg of Amlodipine besilate working standard was weighed quantitatively and transferred into a 200 mL volumetric flask. 20 mL of methanol was added, sonicated to dissolve and volume was made up with diluent, mixed well.

Preparation of Losartan potassium Standard Stock Solution: 50 mg of Losartan potassium was weighed quantitatively and transferred into a 100 mL volumetric flask. 5 mL of methanol was added, sonicated to dissolve, volume was made up with diluent and mixed well.

Preparation of Mixed Standard: 5 mL of each Amlodipine besilate and Losartan potassium standard stock solutions was transferred into 50 mL volumetric flask and volume was diluted with diluent, mixed well.

Method Development: To develop a suitable (specific) and robust LC method for the determination of Amlodipine and Losartan, different mobile phases were employed to achieve the best separation and resolution. The method development was started with Inertsil ODS 3V C18 (150 X 4.6 mm, 5µm) with the Flow rate of 1.0 mL/min and the column temperature was monitored at 25ºC and the injection was 20 µL. UV detection was performed at 237 nm and the sample temperature was maintained at 25ºC. mobile phase containing a mixture mobile Phase-A with 70% v/v of buffer pH-3.7 and 30%v/v of acetonitrile and mobile phase–B containing 70% v/v of acetonitrile and 30% v/v of buffer pH-3.7 with following gradient programme.

Table: 1 Gradient Program for Amlodipine and Losartan

Time (mins)	Mobile Phase-A(%v/v)	Mobile Phase-B (%v/v)
0	90	10
5	85	15
6	80	20
10	50	50
13	50	50
14	0	100
18	0	100
19	90	10
25	90	10

The retention time of Amlodipine and Losartan is 5.13 and 11.11 mins and the peak shape was good. The chromatogram of Amlodipine and Losartan blank, placebo and standard were shown in Figure: 1.1, 1.2 and 1.3.

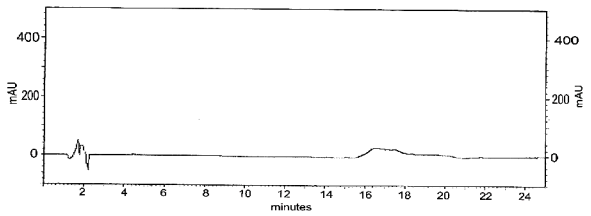


Figure:1.1 Atypical chromatogram of Amlodipine and Losartan Blank

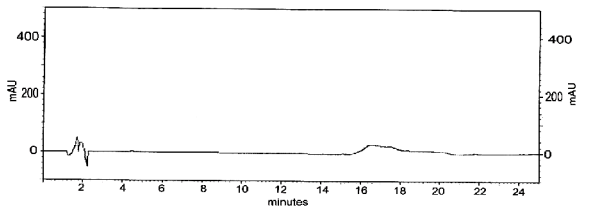


Figure:1.2 Atypical chromatogram of Amlodipine and Losartan Placebo

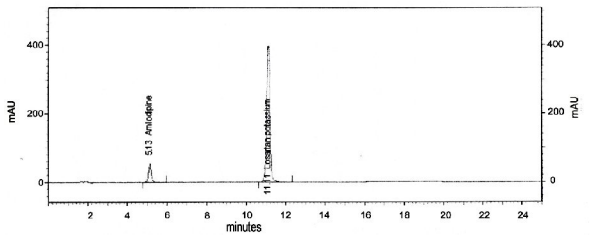


Figure:1.3 Atypical chromatogram of Amlodipine and Losartan Standard

3. METHOD VALIDATION

The developed LC method extensively validated for assay of Amlodipine and Losartan using the following parameters.

System Suitability: According to USP system suitability tests are an integral part of chromatographic method validation. The tests were used to verify that the reproducibility of the chromatographic system is adequate for analysis. To ascertain its effectiveness system suitability tests were carried out on freshly prepared standard stock solution and 6 replicates of working standard samples were injected into the optimized chromatographic system, parameters like retention time (RT), plate number (N), peak area and peak asymmetry of sample were calculated these results are presented in the Table: 2 and 3 for Amlodipine and Losartan respectively.

Table 2: System Suitability of Amlodipine

Injection	Retention time RT	Peak area	USP Plate Count	USP Tailing
1	5.13	513107	5912	1.5
2	5.14	513891	5918	1.5
3	5.13	518756	5906	1.4
4	5.13	516431	5911	1.4
5	5.12	514678	5830	1.5
6	5.13	517230	5804	1.4
MEAN	**5.13**	**515682**	**5880**	**1.5**
SD	**0.006**	**1968.633**	**-**	**-**
%RSD	**0.123**	**0.382**	**-**	**-**

Table 3: System Suitability of Losartan

Injection	Retention time RT	Peak area	USP Plate Count	USP Tailing
1	11.13	4850346	3826	1.1
2	11.11	4854539	3827	1.1
3	11.12	4880346	3825	1.3
4	11.11	4862346	3830	1.2
5	11.11	4954539	3820	1.3
6	11.14	4850158	3810	1.5
MEAN	**11.12**	**4875379**	**3823**	**1.3**
SD	**0.013**	**36873.413**	**-**	**-**
%RSD	**0.114**	**0.756**	**-**	**-**

Precision: The ICH documents recommended that repeatability should be assessed by using a minimum of nine determinations covering the specified range for the procedures (i.e., three concentrations and three replicates of each concentration).

Precision was studied to find out intra and inter day variations of the proposed method at three different levels (2.5, 5 and 7.5 µg/mL for Amlodipine and 25, 50 and 75 µg/mL for Losartan) on the same and on three different days. The results were interpreted by statistical analysis by calculating % RSD values and tabulated in the Table: 4.

Table 4: Summary of Intraday and Inter day precision

Conc. (µg/mL)	Intra-day			Inter-day		
	Mean Amount Found (µg/mL)	±SD	%RSD	Mean Amount Found (µg/mL)	±SD	%RSD
Amlodipine						
2.5	2.487	0.006	0.232	2.502	0.007	0.280
5	5.023	0.035	0.699	4.999	0.012	0.240
7.5	7.493	0.015	0.204	7.498	0.001	0.013
Losartan						
25	25.003	0.012	0.046	24.990	0.020	0.080
50	49.997	0.012	0.023	49.987	0.006	0.012
75	74.983	0.006	0.008	75.003	0.012	0.016

Accuracy : The accuracy of the HPLC method was confirmed by recovery studies by spiking 50, 100 & 150% of pure drugs to the pre analyzed samples and the samples after dilution injected into the system (n=3). The peak area of each drug was measured and the recovery data for Amlodipine and Losartan were given in the Table: 5 and 6.

Table 5: Accuracy of Amlodipine

Amount added (µg/mL)	Amount found (µg/mL)	% Recovery	Statistical Analysis of % recovery	
2.5	2.499	99.960	**MEAN**	99.987
2.5	2.498	99.920	**SD**	0.083
2.5	2.502	100.080	**%RSD**	0.083
5	5.020	100.400	**MEAN**	100.133
5	5.010	100.200	**SD**	0.306
5	4.990	99.800	**%RSD**	0.305
7.5	7.490	99.867	**MEAN**	99.956
7.5	7.510	100.133	**SD**	0.154
7.5	7.490	99.867	**%RSD**	0.154

Table 6: Accuracy of Losartan

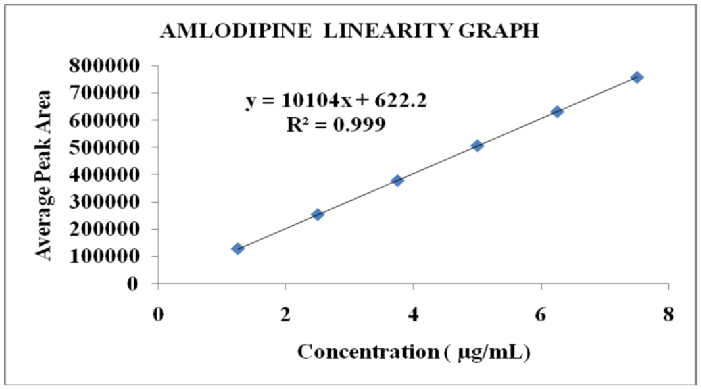
Amount added (µg/mL)	Amount found (µg/mL)	% Recovery	Statistical Analysis of % recovery	
25	24.99	99.960	**MEAN**	99.973
25	24.98	99.920	**SD**	0.061
25	25.01	100.040	**%RSD**	0.061
50	50.01	100.020	**MEAN**	99.987
50	49.99	99.980	**SD**	0.031
50	49.98	99.960	**%RSD**	0.031
75	74.98	99.973	**MEAN**	99.978
75	74.99	99.987	**SD**	0.008
75	74.98	99.973	**%RSD**	0.008

Linearity & Range: The linearity was established with a series of working standard solutions prepared by diluting the stock solution with mobile phase. A linear response of peak area was observed over the concentration range 1.25-7.5 µg/mL and 12.5-75 µg/mL for Amlodipine and Losartan respectively. 20 µL of each samples solution was injected (n=3) under above chromatographic conditions and average peak area for Amlodipine and Losartan was measured. Calibrated curves were constructed and presented in Figure: 4 and 5. The regression data is summarized in the Table: 7 and 8 for Amlodipine and Losartan.

Table 7: Linearity of Amlodipine

Linearity Conc. (µg/mL)	Average Area	SD	%RSD
1.25	127322	247.577	0.194
2.5	253311	1022.814	0.404
3.75	378298	1637.809	0.433
5	506622	2045.675	0.404
6.25	631944	1096.001	0.173
7.5	758599	908.614	0.120

Figure: 4 Linearity graph of Amlodipine



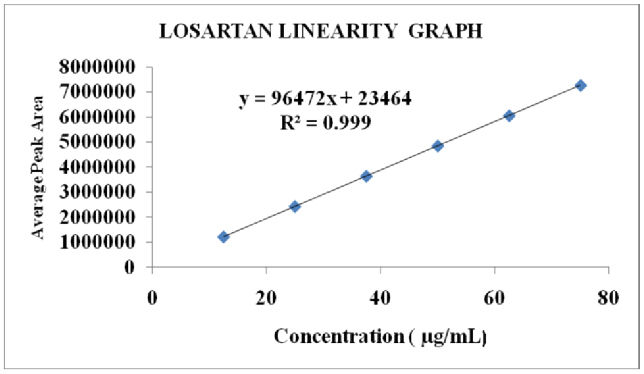


Figure: 5 Linearity graph of Losartan

Linearity Conc. (µg/mL)	Average Area	SD	%RSD
12.5	1226257	9714.509	0.792
25	2437649	22325.16	0.916
37.5	3642796	17922.52	0.492
50	4849014	16790.12	0.346
62.5	6050240	32206.68	0.532
75	7258772	57221.5	0.788

Table 8: Linearity of Losartan

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

A study to establish the limit of detection and limit of quantification for Amlodipine and Losartan. A series of solutions having Amlodipine and Losartan were injected were established by identifying the concentration which gives signal to noise ratio about 3. Limit of quantitation was established by identifying the concentration which gives signal to noise ratio about 10. The limit of detection was found to be 0.041 µg/mL for Amlodipine and 0.080 µg/mL for Losartan. The limit of quantitation was found to be 0.135 µg/mL for Amlodipine and 0.264 µg/mL for Losartan, data were summarized in Table: 9

Table 9: Limit of Detection and Limit of Quantitation

Parameter	LOD (µg/mL)	LOQ (µg/mL)
Amlodipine	0.041	0.135
Losartan	0.080	0.264

ROBUSTNESS

The robustness of the method was determined as per USP guidelines under a variety of conditions including changing the flow rate by ± 20% or ±0.2 mL, temperature by ± 5ºC and using different columns. No marked changes were observed in the system suitability parameters and peak area. The results obtained by deliberately variation in method parameters and data are summarized below as Table: 10.

Table 10: Robustness of Amlodipine and Losartan

Parameter		%RSD of Peak Area		Theoretical Plates		Asymmetry	
		AMLD	LOS	AMLD	LOS	AMLD	LOS
Flow rate ± 20% (0.2 mL/min)	0.8 mL/min	0.259	0.045	5893	3810	1.2	1.3
	1.2 mL/min	0.315	0.024	5835	3850	1.2	1.2
Temperature ± 5ºC	20ºC	0.148	0.548	5815	3825	1.2	1.3
	30ºC	0.179	0.249	5821	3826	1.2	1.2
Column variation (150X4.6mm, 5µm)	Xterra RP C18	0.028	0.019	5893	3842	1.2	1.3
	Symmetry C18	0.057	0.017	5886	3810	1.2	1.2

Estimation of Pharmaceutical Formulations: ACORD-L is a commercial formulation containing a Amlodipine and Losartan of 5 mg and 50 mg which has been taken up for checking the applicability of the proposed method to the formulation. 5 tablets were weighed and transferred into a 100 mL volumetric flask, 20 mL of diluent was added and sonicated with intermediate shaking for 15 mins. Made up to the volume with diluent and filtered through 0.45 µm nylon membrane filter. 2 mL of sample was pipette out and transferred into 10 mL volumetric flask and diluted up to the mark. 20 µL of sample was injected and chromatographed.

A typical chromatogram of ACORD-L tablet formulation is shown in Figure: 6

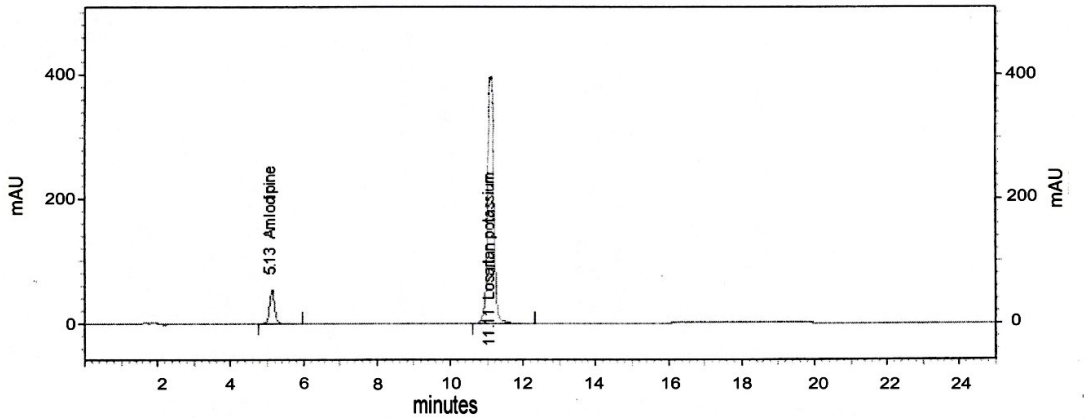


Figure 6: A Typical Chromatogram of ACORD-L tablet

Table 11: Recovery of ACRD-L Tablets

Drug	Label claim (mg/tablet) *N=3	Amount found (mg/tablet) ± SD	% Recovery	% RSD
Amlodipine	**5**	5.003 ± 0.012	100.067	0.231
Losartan	**50**	49.997 ± 0.012	99.993	0.042

RESULTS AND DISCUSSION

To optimize the mobile phase, various proportions of buffer with acetonitrile and were tested. Mobile phase containing a gradient mixture of mobile phase- A with buffer pH 3 and acetonitrile in the ratio of 70:30% v/v. Mobile phase-B containing a mixture of acetonitrile and buffer pH 3.7 in the ratio 70:30%v/v, resulted in peaks with good shape and resolution. A flow rate of 1.0 mL/min was found to be optimum in the 0.8-1.2 mL/min range resulting in the short retention time, baseline stability and minimum noise.

By applying the proposed method, the retention times of Amlodipine and Losartan were found to be 5.13 min and 11.11 min respectively. Quantitative linearity was obeyed in the concentration range of 1.25-7.5 and 12.5-75 µg/mL for Amlodipine and Losartan respectively. The regression equations of concentration Amlodipine and Losartan over their peak areas were found to be $y = 10104x + 622.2$ ($R^2 = 0.999$), and $y = 96472x + 23464$ ($R^2 = 0.999$) where y is the peak area and x is the concentrations of Amlodipine and Losartan (µg/mL). The numbers of theoretical plates obtained 5880 and 3828 for Amlodipine and Losartan respectively, which indicates the efficiency of the column. The limit of detection and limit of quantitation were found to be 0.041 and 0.080 µg/mL, 0.135 and 0.264 µg/mL for Amlodipine and Losartan respectively, which indicates the sensitivity of the method. The high percentage recovery indicates that the proposed method is highly accurate. No interfering peaks were found in the chromatogram indicating that excipients used in tablet formulations did not interfere with the estimation of the drug by the proposed HPLC method.

CONCLUSION

A simple, specific, accurate, precise, stability indicating reverse phase high performance liquid chromatography method has been developed which can be used for accurately quantitative estimation Amlodipine and Losartan for routine analysis of individual and combination of drugs. Method was validated as per ICH Q2 (R1) so it can be used by pharmaceutical industries.

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