WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 11, 310-327. Review Article ISSN 2277– 7105

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION ATENOLOL AND AMLODIPINE IN PHARMACEUTICAL DOSAGE FORMS

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Article Received on
09 April 2018,
Revised on 30 April 2018,
Accepted on 20 May 2018,
DOI: 10.20959/wjpr201811-12415

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ABSTRACT

A new, simple, precise, rapid and accurate RP-HPLC (Reverse Phase – High Performance Liquid Chromatography) method has been developed for the simultaneous estimation of Atenolol and Amlodipine in pharmaceutical dosage form. The chromatographic separation was achieved on Alliance Waters 2695 HPLC using HYPRSIL BDS, 150 x 4.6 mm, 5µ column maintained at ambient temperature with mobile phase, Buffer: water: Acetonitrile (50:50), flow rate 1.0 ml/min, load volume 10 µl and a run time of 08 min. Buffer was prepared with Triethylamine and adjusted pH to 3.1 with Ortho-Phosphoric Acid. The retention time and mean recoveries obtained for Atenolol was 1.8 min and 100.04%, for Amlodipine was 6.0 min and 99.73.% respectively. Linearity response was established over the concentration range of 50-150 µg/ml for Atenolol and 5-15 µg/ml for Amlodipine. The Assay for Atenolol and Amlodipine was found to be 99.46 and 99.45 respectively. The recovery studies ascertained the accuracy of proposed method and the results were validated as per ICH guidelines. Hence, the developed method can be successfully employed for routine quality control of Atenolol and Amlodipine in drug testing laboratories and pharmaceutical industries.

KEYWORDS: Atenolol, Amlodipine, RP-HPLC, Beta Blockers.

INTRODUCTION

Atenolol

Atenolol belongs to the class of drugs known as beta blockers.[1] It acts by blocking the action of CNS liberated chemicals such as epinephrine which acts on the heart and blood vessel and which in turns reduces the strain on the heart.[2] Atenolol mostly used with or without other medication. It is used in the treatment of hypertension and thereby prevents heart attack and kidney failure.[3] This drug is also used in the treatment of angina and improves the survival chances after the heart attack. It is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system.[4]

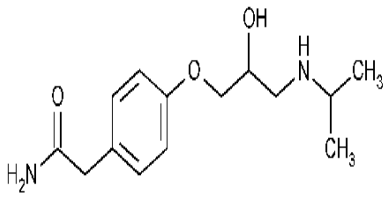

Fig 1: Chemical Structure of Atenolol.

Amlodipine

Amlodipine (AMD) is chemically a 2-[(2-Aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl- ,5- pyridine dicarboxylic acid- 3-ethyl 5-methyl ester and it belongs to the class of calcium channel blocker.[5] Several spectroscopic,[6] RP-HPLC,[7] HPTLC,[8] LC-MS/ MS[9] and LC-MS[10] have been reported for the estimation of amlodipine individually and in combination with other drugs. Amlodipine is an angioselective calcium channel blocker and inhibits the movement of the calcium ions into the vascular smooth muscles and cardiac muscles, this helps used in the treatment of high blood pressure and coronary artery diseases. Amlodipine was patented in 1986. It is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system. It is also used in the management of Vasospastic angina and without heart failure.[11] The common dose dependent side effects include Peripheral edema, dizziness, palpitations and flushing.[12]

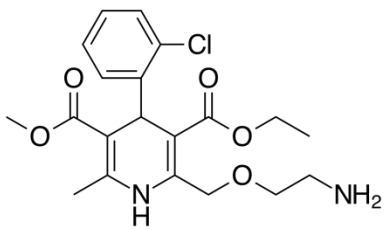

Fig 2: Chemical Structure of Amlodipine.

A combination product of the above two drugs is being marketed under the brand name of Adorant in India. Since there were no precise method available for the simultaneous estimation of the above two drugs in the combination product when we started our work.

AIM AND OBJECTIVE

Various analytical methods have been reported in literature to detect and quantify the individual drugs Atenolol and Amlodipine. But there is no proper official method reported for the simultaneous estimation of Atenolol and Amlodipine. Hence, a new analytical method development is developed which is simple, accurate and precise.

The main aim and objective of the present study is

- To develop a new Reverse Phase High Performance Liquid Chromatographic method for the simultaneous determination of Atenolol and Amlodipine in Pharmaceutical dosage form.
- To validate the developed method for the following parameters
  - System suitability
  - Specificity
  - Linearity
  - Accuracy
  - Precision
  - Limit of Detection
  - Limit of Quantification
  - Robustness
  - Solution stability
- To perform the assay of commercial product.

EXPERIMENTAL PROCEDURE

Instrumentation: Chromatography was performed with Alliance Waters 2695 HPLC provided with high speed auto sampler, column oven, degasser and & 2996 PDA detector to provide a compact and with class Empower-2 software.

Reagents and chemicals: The reference samples of Atenolol and Amlodipine were provided as gift samples from Spectrum Pharma Research Solutions, Hyderabad. HPLC grade acetonitrile, HPLC grade methanol and all other chemicals were obtained from Merck chemical division, Mumbai. HPLC grade water obtained from Milli-Q water purification

system was used throughout the study. Commercial formulations (Brand Name: Adorant Tablets; Label Claim: Amlodipine 5mg and Atenolol 50mg) were purchased from the local pharmacy.

Preparation of buffer Solution: Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1ml of Triethylamine then PH adjusted to 3.1 with dil. Orthophosphoric acid solution. Mobile phase**:** Buffer and Acetonitrile taken in the ratio 50:50.

Preparation of Standard Stock Solution: Accurately Weighed and transferred 50mg & 5mg of Atenolol and Amlodipine working Standards into a 10 ml clean dry volumetric flask, add 7ml of diluent, sonicated for 30 minutes and make up to the final volume with diluents (500µg/ml Atenolol & 50µg/ml Amlodipine). From the above stock solution, 1 ml was pipette out in to a 10ml Volumetric flask and then make up to the final volume with diluent.

Sample preparation: 5 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 5 tablets was transferred into a 100 ml volumetric flask, 70ml of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 2ml was pipette out into a 10 ml volumetric flask and made up to 10ml with diluent.

Chromatographic condition

Flow rate	**:**	1.0ml/min
Column	**:**	HYPRSIL BDS, 150 x 4.6 mm, 5µ.
Detector wave length	**:**	237nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	8 min
Diluent	**:**	Water: Acetonitrile (50:50)

Method Validation

System Suitability Tests: Data from six injections of 10 µl of the working standard solutions of Atenolol (500µg/ml) and Amlodipine (50µg/ml) were used for the evaluation of the system suitability parameters like tailing factor, the number of theoretical plates,

retention time and resolution factor.

Specificity: The specificity of the method was performed by injecting blank solution, placebo solution and standard solutions of Atenolol and Amlodipine separately.

Linearity: By taking appropriate aliquots of the standard Atenolol and Amlodipine solutions with the mobile phase, six working solutions ranging between 500 µg/ml Atenolol, 50 µg/ml Amlodipine were prepared. Each experiment linearity point was performed in triplicate according to optimized chromatographic conditions. The peak areas of the chromatograms were plotted against the concentration of Atenolol and Amlodipine to obtain the calibration curve.

Accuracy: Previously analyzed samples of Atenolol and Amlodipine to which known amounts of standard Atenolol and Amlodipine corresponding to 50%, 100% and 150% of target concentration were added. The accuracy was expressed as the percentage of analyte recovered by the proposed method.

Precision: The repeatability and intermediate precision were determined by analyzing the samples of Atenolol and Amlodipine.

Limit of detection and the limit of quantification: Limit of detection (LOD) and limit of quantification (LOQ) of Atenolol and Amlodipine were determined by calibration curve method. Solutions of Atenolol and Amlodipine were prepared in linearity range and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using following equations.

$$LOD = (3.3 \times Syx)/b,\ LOQ = (10.0 \times Syx)/b$$

Where Syx is residual variance due to regression; b is slope.

Robustness: The robustness of the method was performed by deliberately changing the chromatographic conditions. The parameters included slight variation in organic phase percentage in the mobile phase (45, 55%), flow rate (0.9, 1.1 ml/min) and column temperature (25, 35°C).

Stability: The sample solutions were injected at 0hr (comparison sample) and after 24hr (stability sample) by keeping at ambient room temperature. Stability was determined by determining %RSD for sample and standard solutions.

RESULTS AND DISCUSSION

Method development

Initially RP- HPLC method of separation was attempted by using various ratios of methanol and water, Acetonitrile and water as mobile phases, in which both the drugs did not responded properly and also the peak shapes and separations were not achieved to the best of requirement. Hence, the organic content of mobile phase was further investigated to optimize the separation of both drugs. To improve the tailing factor, the pH of mobile phase was adjusted. Thereafter, buffer: Water; Acetonitrile were taken in ratio of 50:50%v/v and with a flow rate of 1.0 ml/min was employed which is ideal for the successful elution of the analytes. Preliminary development trials were performed with different analytical columns of different types from different manufacturers with different configurations. Among the analytical columns tried, HYPRSIL BDS, 150 x 4.6 mm, 5µ was selected as the stationary phase to improve resolution and the tailing of both peaks were reduced considerably and brought close to 1. The chromatograms obtained for blank injection, placebo injection and optimized method were shown in the Fig.4, 5 and 6 respectively and optimized chromatographic conditions were shown in Table 1.

Table 1: Optimized chromatographic conditions.

S. No.	Parameter	Condition
1.	Mobile phase	water: Acetonitrile (50:50)
2.	Diluents	Water: Acetronitrile (50:50)
3.	Column, make	HYPRSIL BDS, 150 x 4.6 mm, 5µ.
4.	Column temperature	30^0C
5.	Wave length	225nm
6.	Injection volume	10µl
7.	Flow rate	1.0ml/min
8.	Run time	08 min
9.	Retention time (Atenolol)	1.9 min
10.	Retention time (Amlodipine)	6.0 min

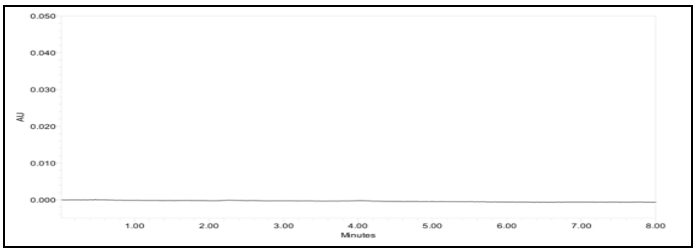

Fig. 4: Chromatogram of Blank.

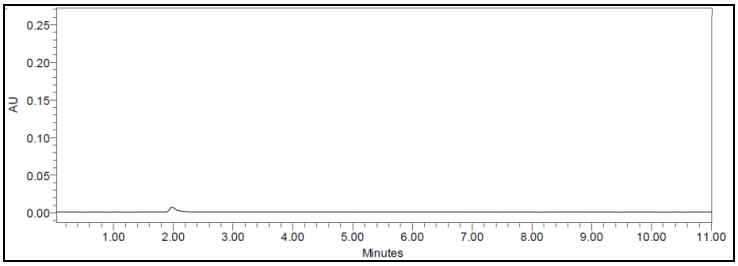

Fig. 5: Chromatogram of Placebo.

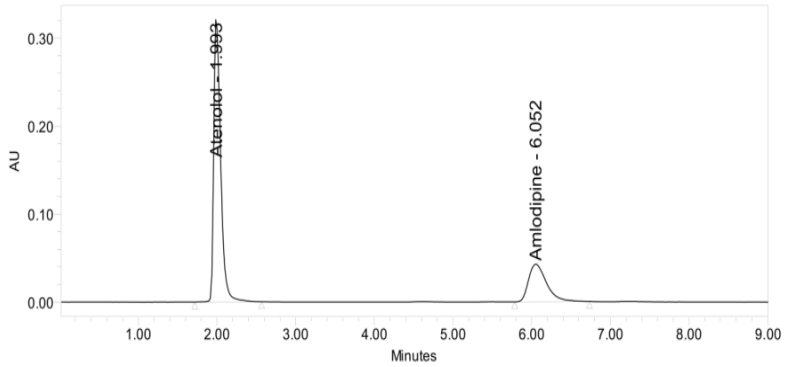

Fig. 6: Chromatogram of Atenolol and Amlodipine Standards.

Method Validation

System Suitability Test: Various system suitability parameters such as number of theoretical plates, peak tailing, and retention time and resolution factor were determined. The total run time required for the method is only 08 minutes for eluting Atenolol and Amlodipine. The results obtained were shown in Table No.2 and 3. The number of theoretical plates was found to be > 2000, USP tailing was < 2 and USP resolution is above 2. The % RSD of areas for Atenolol and Amlodipine were 0.5%, 0.3% and 1.1% respectively.

Table 2: System Suitability of Atenolol and Amlodipine.

S. No.	Area of Atenolol	Area of Amlodipine
1.	2029311	714700
2.	2019394	709008
3.	2016784	706927
4.	2001893	722684
5.	2036578	706397
6.	2049843	713203
Mean	2025634	712153
S.D	16734.80	6145.33
% RSD	0.80	0.86

Table 3: System Suitability parameters for Atenolol and Amlodipine.

Property	Atenolol	Amlodipine
Retention time (Rt)	2.2±0.3min	2.9± 0.3 min
Theoretical plates (N)	3360± 163.48	3690± 163.48
Tailing factor (T)	1.25± 0.117	1.20 ± 0.117

Specificity: The specificity of the method was performed by injecting blank solution, placebo solution and standard solutions separately. The chromatogram of the drug was compared with blank and placebo chromatogram to verify the interference. No interfering peak was observed at the retention time of Atenolol and Amlodipine. Hence, the method is specific for the determination of the above mentioned drugs.

Linearity: The linearity of Atenolol and Amlodipine were plotted and the curve obtained is found to be linear. The results were shown in the Table 4 and Fig 7-14.

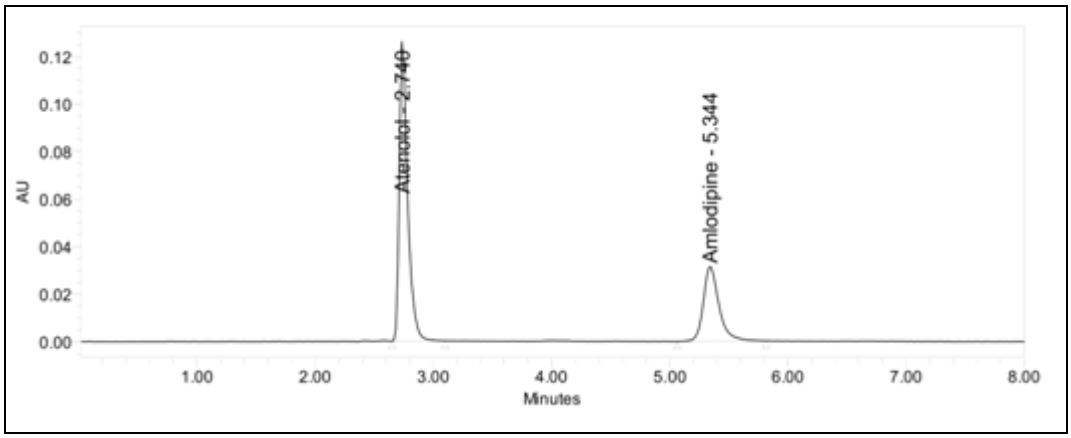

Fig. 7: Linearity 25% chromatogram of Atenolol and Amlodipine.

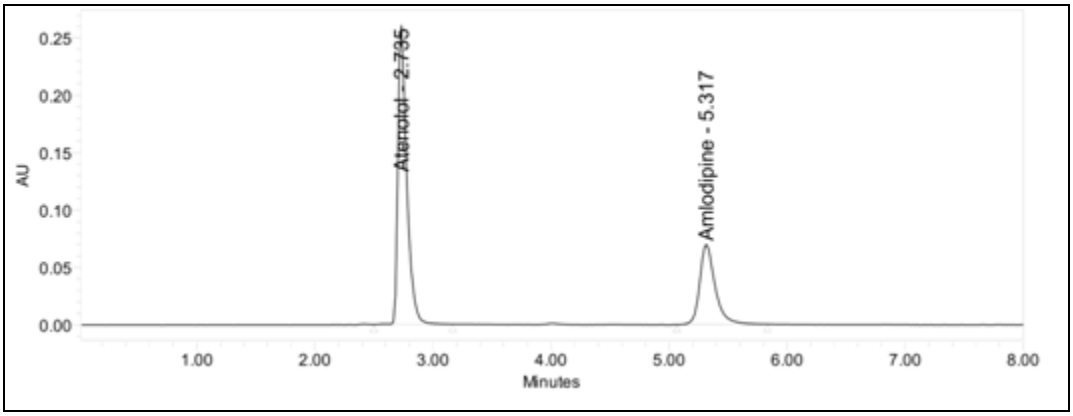

Fig. 8: Linearity 50% chromatogram of Atenolol and Amlodipine.

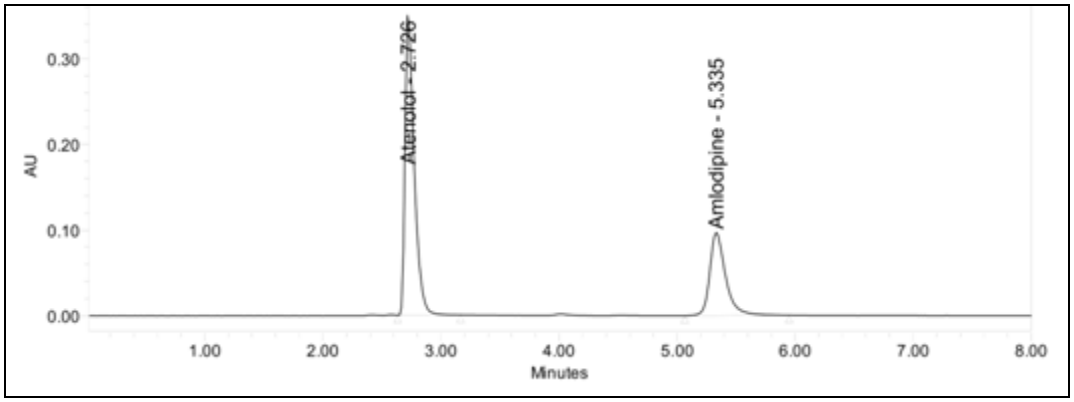

Fig. 9: Linearity 75% chromatogram of Atenolol and Amlodipine.

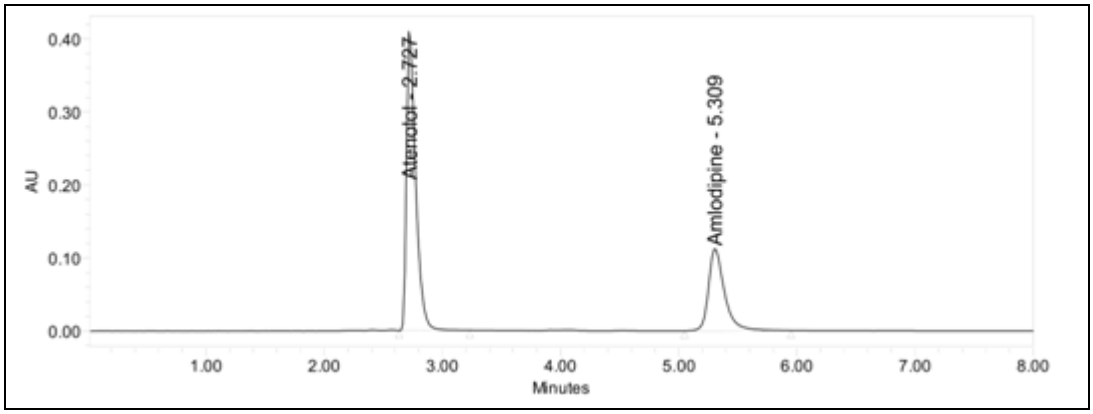

Fig. 10: Linearity 100% chromatogram of Atenolol and Amlodipine.

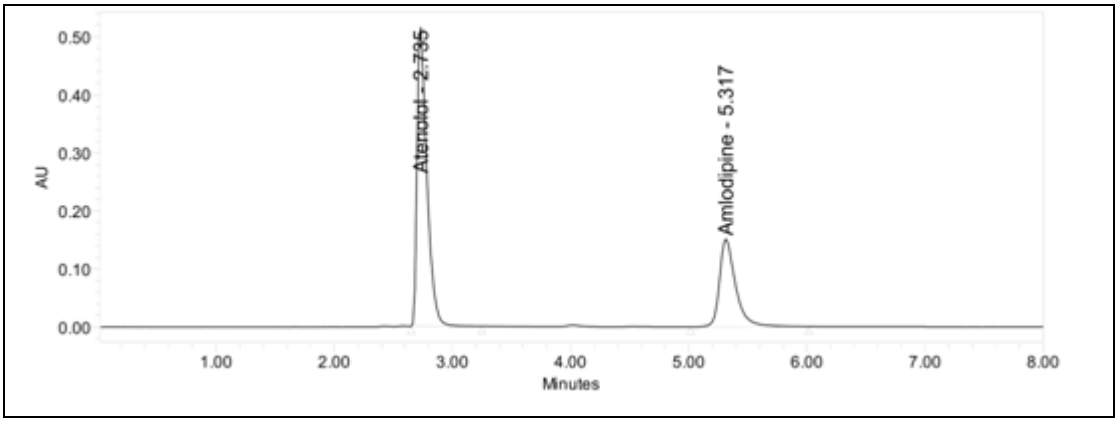

Fig. 11: Linearity 125% chromatogram of Atenolol and Amlodipine.

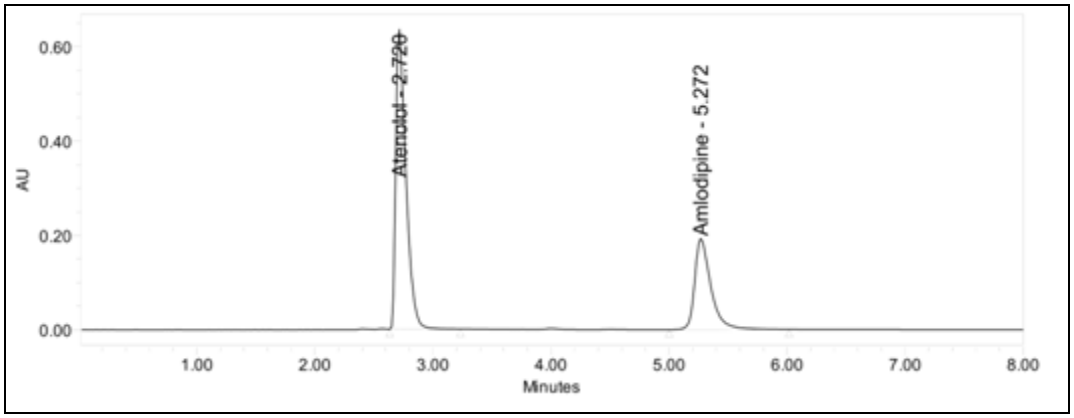

Fig. 12: Linearity 150% chromatogram of Atenolol and Amlodipine.

Table 4: Linearity data for Atenolol and Amlodipine.

S. No.	Pipette from stock (ml)	Volume of flask (ml)	Concentration in ppm(Atenolol)	Concentration in ppm(Amlodipine)	% Linearity Level
1	0.25	10	125	12.50	25
2	0.5	10	250	25.00	50
3	0.75	10	375	37.5	75
4	1	10	500	50	100
5	1.25	10	625	62.5	125
6	1.5	10	750	75	150

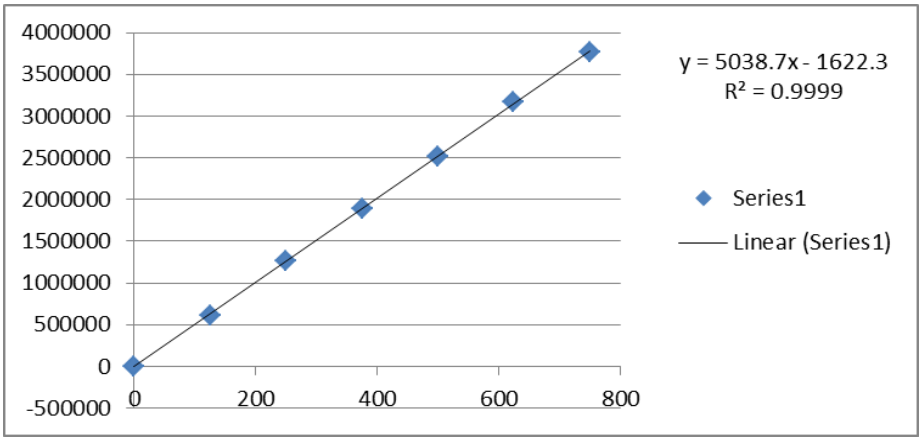

Fig. 13: Calibration curve of Atenolol.

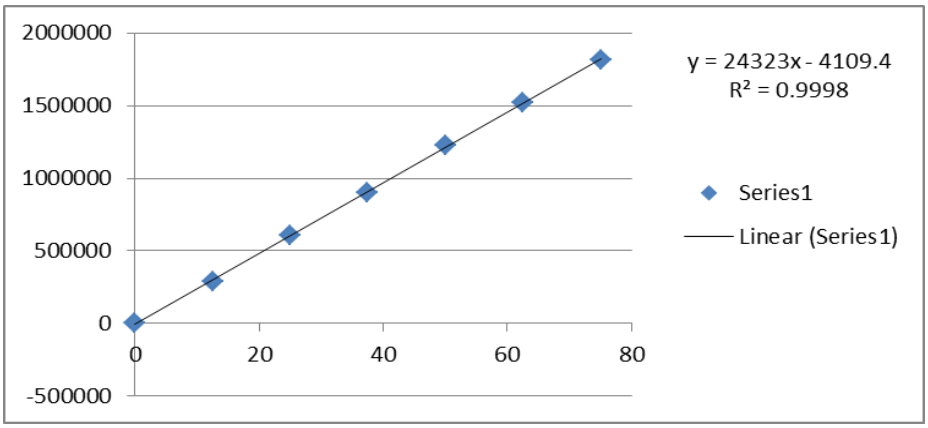

Fig 14: Calibration curve of Amlodipine.

Accuracy: To pre analyzed sample solution, a definite concentration of standard drug (50%, 100% & 150% level) was added and recovery was studied. The % Mean recovery for Atenolol and Amlodipine are 100.04% and 99.73% respectively and these results are within acceptable limit of 98-102. The % RSD for Atenolol and Amlodipine are 0.8 and 0.7 respectively and % RSD for Atenolol and Amlodipine are within limit of ≤2. Hence, the proposed method is accurate and the results are summarized in Table-5 and figure 16-18.

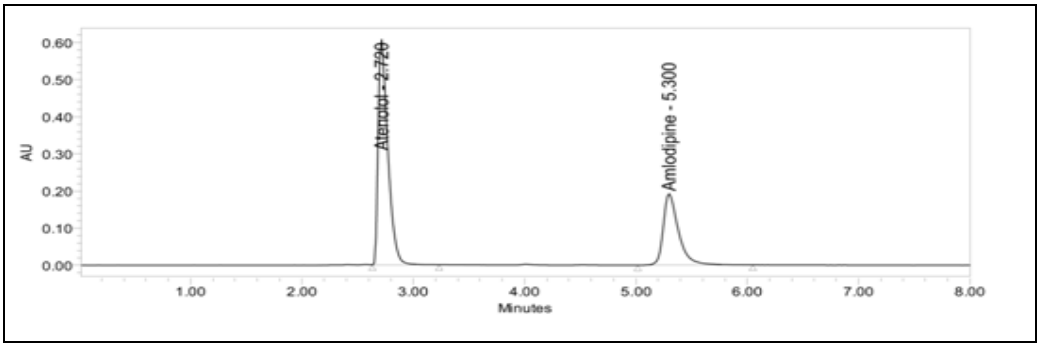

Fig. 16: Accuracy 50% chromatogram of Atenolol and Amlodipine.

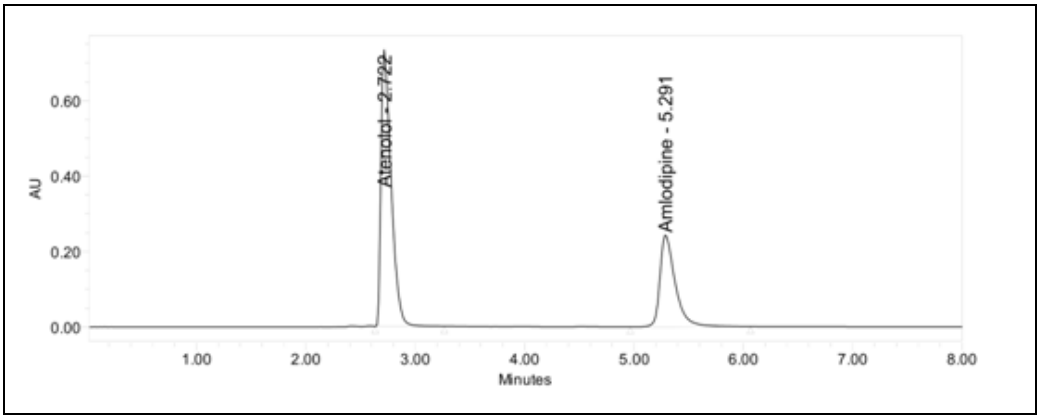

Fig. 17: Accuracy 100% chromatogram of Atenolol and Amlodipine.

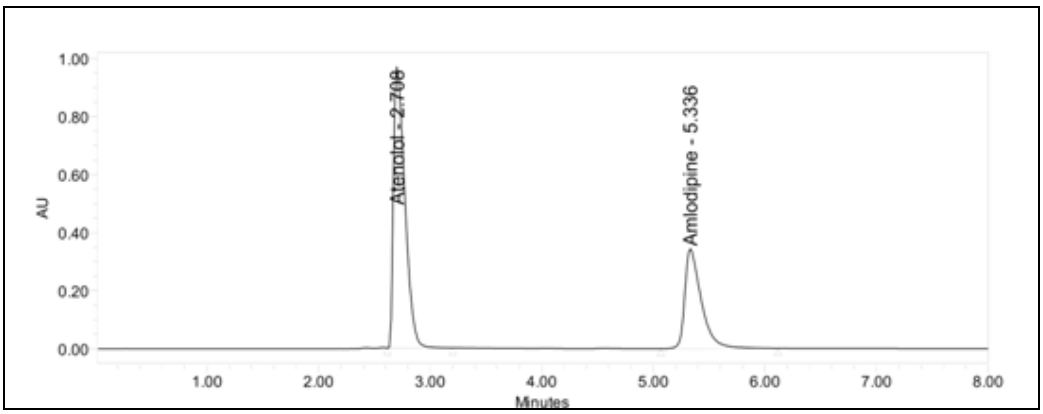

Fig. 18: Accuracy 150% chromatogram of of Atenolol and Amlodipine.

Table 5: Results of Recovery Experiments of Atenolol and Amlodipine.

Preanalysed amount (µg/ml)		Spiked Amount (µg/ml)		% Recovered	
Atenolol	**Amlodipine**	**Atenolol**	**Amlodipine**	**Atenolol**	**Amlodipine**
60	120	30	60	100.83	99.48
60	120	30	60	100.23	100.29
60	120	30	60	100.37	99.31
60	120	60	120	100.82	100.60
60	120	60	120	100.20	101.84
60	120	60	120	100.32	99.51
60	120	90	180	99.51	100.08
60	120	90	180	100.51	99.82
60	120	90	180	99.77	99.70
			MEAN	100.04	99.73
			SD	0.75	0.73
			%RSD	0.8	0.7

Precision: The repeatability and Intermediate precision data were summarized in Table 6 and 7, respectively and were assessed by the use of standard solutions of Atenolol and Amlodipine.

Repeatability: Six replicates injections in same concentration of Atenolol and Amlodipine were analyzed in the same day for repeatability and the % RSD for Atenolol and Amlodipine found to be 0.80 and 0.86 respectively and % RSD for Atenolol and Amlodipine found to be within acceptable limit of ≤2 and hence, method is reproducible. The results were shown in the Table 6.

Table 6: Results of Repeatability of Atenolol and Amlodipine.

S. No	Area of Atenolol	Area of Amlodipine
1.	2029311	714700
2.	2019394	709008
3.	2016784	706927
4.	2001893	722684
5.	2036578	706397
6.	2049843	713203
Mean	2025634	712153
S.D	16734.80	6145.33
%RSD	0.80	0.86

Intermediate precision (Day_ Day Precision): Six replicates injections in same concentration were analyzed on two different days with different analyst and column for verifying the variation in the precision and the % RSD for Atenolol and Amlodipine were found to be 1.0 and 1.2 respectively and it is within acceptable limit of ≤2. Hence, the method

is reproducible on different days with different analyst and column. This indicates that the method is precise. The results were shown in the Table7.

Table 7: Results of Intermediate precision of Atenolol and Amlodipine.

S. No	Area of Atenolol	Area of Amlodipine
1.	2295603	1105398
2.	2358474	1093129
3.	2337538	1115052
4.	2339458	1107504
5.	2319036	1086171
6.	2303808	1081391
Mean	2325653	1098107
S.D	23803.50	13230.90
%RSD	1.00	1.20

Robustness: Few chromatographic conditions were deliberately altered to evaluate the robustness of the developed HPLC method. The robustness was established by changing the flow rate, column temperature and composition of the mobile phase within allowable limits from actual chromatographic conditions. It was observed that there were no marked change in mean R_t and RSD is within limit of ≤2 .The tailing factor, resolution factor and number of theoretical plates were found to be acceptable limits for Atenolol and Amlodipine. Hence, the method is reliable with variations in the analytical conditions and the results were shown in the Table 8 and Fig 19-24.

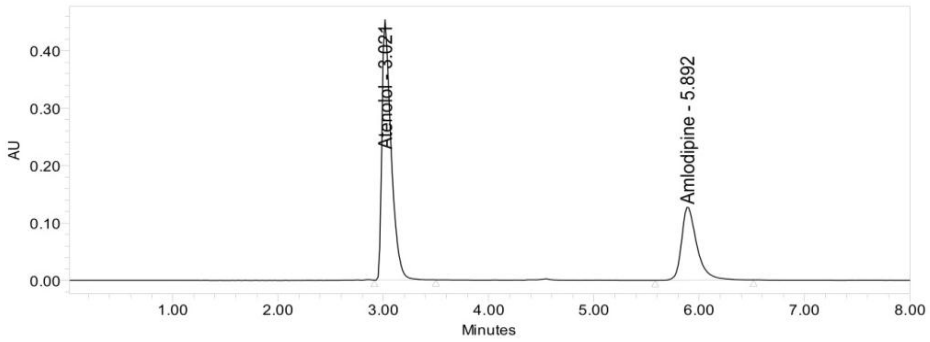

Fig. 19: Robustness (Flow Minus: 0.9ml/min) chromatogram of Atenolol and Amlodipine.

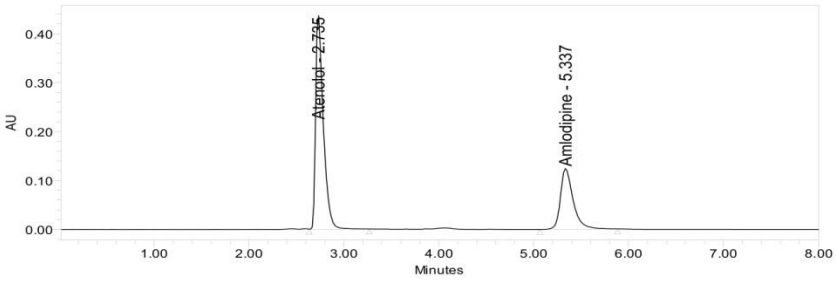

Fig. 20: Robustness (Flow Plus: 1.1ml/min) chromatogram of Atenolol and Amlodipine.

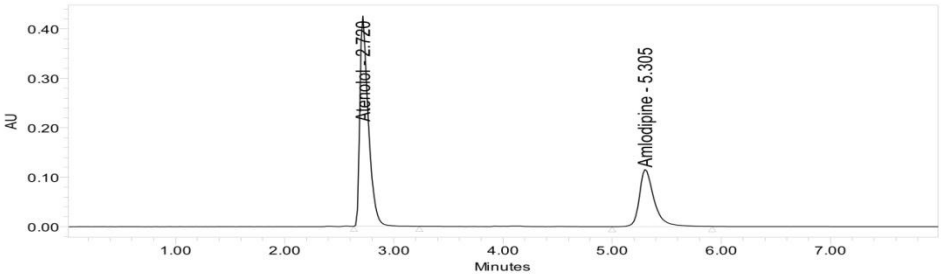

Fig. 21: Robustness (Mobile phase minus:45%) chromatogram of Atenolol and Amlodipine.

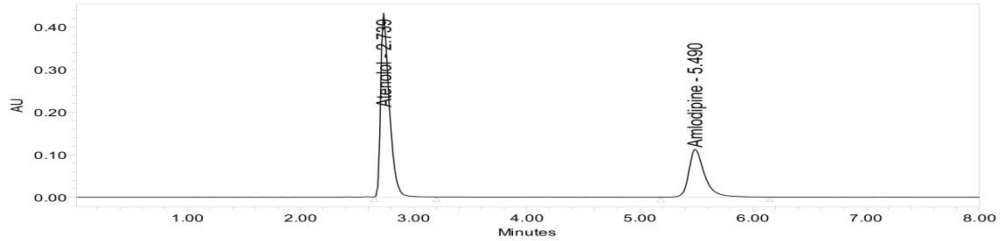

Fig. 22: Robustness (Mobile Phase Plus: 55%) chromatogram of Atenolol and Amlodipine.

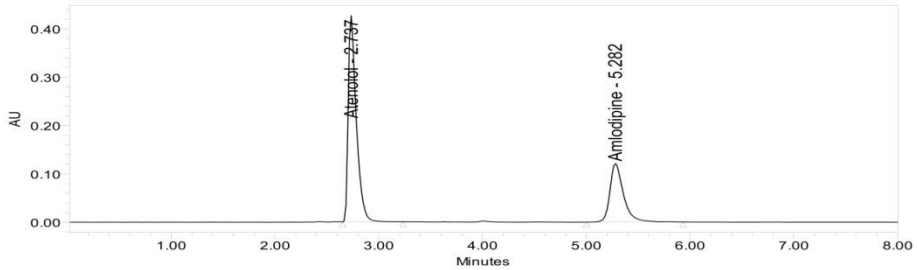

Fig 23: Robustness (Temperature Minus: 25 ^{o}C) chromatogram of Atenolol and Amlodipine.

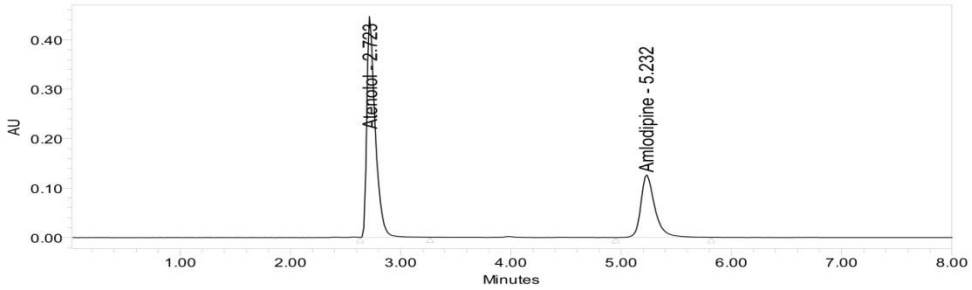

Fig. 24: Robustness (Temperature Plus: 35 ^{o}C) chromatogram of Atenolol and Amlodipine.

Table-8(a): Robustness – Flow Minus (n=6).

S. No.	Parameter	Atenolol	Amlodipine
1.	% RSD of area	0.8	0.8
2.	Tailing Factor	1.45	1.40
3.	Plate count	5526	8622

Table-8(b): Robustness- Flow Plus (n=6).

S. No.	Parameter	Atenolol	Amlodipine
1.	% RSD of area	0.5	0.9
2.	Tailing Factor	1.43	1.38
3.	Plate count	5344	8279

Table-8(c): Robustness - Mobile Phase Minus (n=6).

S. No.	Parameter	Atenolol	Amlodipine
1.	% RSD of area	1.4	1.5
2.	Tailing Factor	1.47	1.39
3.	Plate count	5623	8416

Table-8(d): Robustness – Mobile Phase Plus (n=6).

S. No.	Parameter	Atenolol	Amlodipine
1.	% RSD of area	0.6	0.9
2.	Tailing Factor	1.48	1.39
3.	Plate count	5882	8300

Table- 8(e): Robustness- Temperature Minus (n=6).

S. No.	Parameter	Atenolol	Amlodipine
1.	% RSD of area	0.8	0.9
2.	Tailing Factor	1.48	1.41
3.	Plate count	5576	8462

Table-8(f): Robustness – Temperature Plus (n=6)

S. No.	Parameter	Atenolol	Amlodipine
1.	% RSD of area	0.5	0.4
2.	Tailing Factor	1.45	1.35
3.	Plate count	5967	8808

Stability of sample solution: The sample solution injected after 24 hrs by keeping at ambient room temperature 30^{o}C did not show any appreciable change. The deviation in the assay is not more than 2 and the results are shown in Table 9.

Table 9: Stability data of Atenolol and Amlodipine.

Drug	%Assay at 0 hr*	%Assay at 24hr*	Deviation
Atenolol	99.46	99.15	0.52
Amlodipine	99.45	99.10	0.45

* n=6 for each parameter.

LOD and LOQ: LOD and LOQ for Atenolol is found to be 0.39 and 1.18 µg/ml; Amlodipine showed 0.28 and 0.87 µg/ml respectively. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive and the results were shown in Table-10.

Table 10: LOD and LOQ data of Atenolol and Amlodipine.

Atenolol			Amlodipine		
S. No	Slope	Y-Intercept	S. No	Slope	Y-Intercept
1	5011	2330	1	24323	4109
2	5049	2148	2	24534	6586
3	5043	3263	3	24602	8356
AVG	5034.333	2580.333	AVG	24486.33	6350.333
SD		598.1692	SD		2133.285
LOD		0.39	LOD		0.28
LOQ		1.18	LOQ		0.87

Assay: The percentage assay of labeled claim of Atenolol and amlopdipine present in the tablet Adorant Tablets was 99.46% and 99.45% respectively. % RSD values for Atenolol and Amlodipine were within limit of ≤2 and the results were shown in Figure No. Table 11 and Fig 25.

Table 11: Assay Data of Atenolol and Amlodipine.

S. No.	Drug Name	Amount injected (µg/mL)	Amount found (µg/mL)	% Assay ± SD*
1	Atenolol	50	49.73	99.46±0.57
2	Amlodipine	5	4.77	99.45±0.37

* n=6 for each parameter; Lable Claim: Adorant Tablets Atenolo 500mg and Amlodipine 5 mg

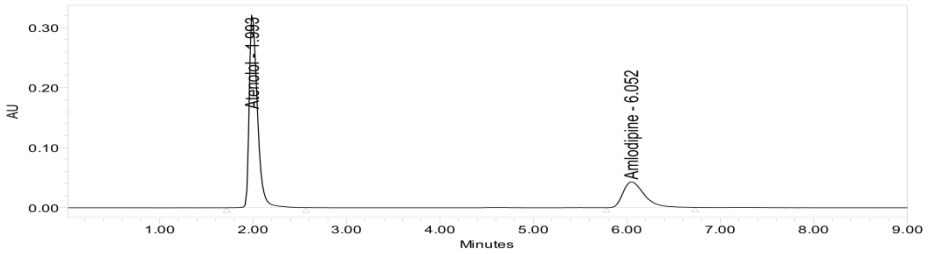

Fig. 9.25 Assay chromatogram of Atenolol and Amlodipine.

DISCUSSION

The present study involves HYPRSIL BDS, 150 x 4.6 mm, 5µ column as the stationary phase. dil. Orthophosphoric acid solution buffer (pH 3.1), Water: Acetonitrile were taken in the ratio 50:50%v/v and used as mobile phase at a flow rate of 1.0 ml/min. In this method, the numbers of theoretical plates were above 2000. The retention times of Atenolol and Amlodipine were found to be 2.2 min and 2.9 min respectively. Tailing factor is less than 2 and % RSD of peak area is less than 2, this indicates that the optimized method met the system suitability parameters. The regression coefficient r^2 value was 0.999 for Atenolol and Amlodipine and the response was linear. The percentage mean recovery of Atenolol and Amlodipine were found to be 100.04, 99.73 respectively and it showed that the proposed method is accurate. %RSD values of repeatability and intermediate precision were ≤2 and the method is precise. The lowest values of LOD and LOQ as obtained by the proposed HPLC method indicate that the method is sensitive. The solution stability studies of method indicate that the Atenolol and Amlodipine drugs were stable up to 24 hours. In robustness chromatographic conditions were changed as flow minus: 0.9 ml/min; flow plus: 1.1ml/min; temperature minus: 25ºC; temperature plus: 35ºC; mobile phase minus: organic phase 45%v/v; mobile phase plus: organic phase 55%v/v. These changes didn't show any variation in results and it showed the reliability of the method.

CONCLUSION

A new simple, precise and accurate HPLC method was developed and validated for the simultaneous estimation of Atenolol and Amlodipine in pharmaceutical dosage form.

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