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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF AMLODIPINE BESYLATE AND ATENOLOL IN TABLET DOSAGE FORM

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ABSTRACT

A simple, precise, accurate RP-HPLC method was developed and validated for the simultaneous determination of Amlodipine Besylate and Atenolol in pharmaceutical dosage form. A Thermohypersil-keystone C18, 150 x 4.6mm, 5µ, column was used and the mobile phase optimized was 0.05M of potassium di hydrogen ortho phosphate; pH adjusted to 7.0 ± 0.1 with 5N potassium hydroxide pumped at a flow rate of 1.5 ml/minute. Elutes were analyzed using a Photodiode array detectorat 230 nm. The retention time of Amlodipine and Atenolol were found to be 9.73 and 12.83 minutes respectively. The method was validated according to ICH guidelines. The developed method was found to be specific as there was no interference from the mobile phase. Results indicated that the response was linear over the range of 76.06 to 228.18 µg/ml for Amlodipine and 10.06 to 30.18 µg/ml for Atenolol with coefficient of regression, R^2, value 0.999 for Amlodipine and 0.998 for Atenolol peak responses respectively. The proposed method was found to be accurate as the individual recovery of Amlodipine ranged from 98.11 % to 101.16 % with mean recovery of 99.23 % and % RSD of 1.14. The individual recovery of Atenolol ranged from 98.9 % to 100.49 % with mean recovery of 99.42 % and % RSDof 0.5. Hence, it is concluded that the proposed method can be conveniently used for simultaneous determination of Amlodipine and Atenolol in a pharmaceutical dosage form.

Keywords: RP-HPLC, Simultaneous determination, ICH guidelines, Validation

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INTRODUCTION

Atenolol, having chemical structure $C_{14}H_{22}N_2O_3$, has molecularweight 266.34 g/mol. It is sparingly soluble in water, soluble in absolute Ethanol, slightly soluble in Dichloromethane and practically insoluble in Ether [1, 2]. AmlodipineBesylate, $C_{20}H_{25}ClN_2O_5 \cdot C_6H_6SO_3$, has molecular weight 567.1 g/mol. It is slightly soluble in water and in Isopropyl Alcohol, sparingly soluble in Ethanol and freely soluble in Methanol [3, 4]. Atenolol, an antihypertensive, anti-anginal and antiarrhythmic is widely used in combination with Amlodipine, a long-acting calcium channel blocker used for hypertension and angina pectoris. Use of this combination increases patient compliance as the dosage regimen is simplified and also gives synergistically improved control over hypertension [5].

Rolim et al reported a UV spectrophotometric and HPLC method for estimation of Atenolol in pharmaceutical preparations [6]. A UV spectrophotometric method for simultaneous estimation of AmlodipineBesylate and Atenolol as A.P.I. and in tablet dosage form by Vierodt's method is also reported [7]. A UV spectrophotometric method was also developed for simultaneous estimation of Ramipril and Amlodipine[8]. A stability indicating HPLC method was developed and validated for analysis of Atenolol and Hydrochlorthiazide in bulk drug and tablet formulation [9]. Literature survey also reports HPLC methods for simultaneous estimation of Amlodipine in combination with other drugs [10, 11]. There is a scientific research article also on RP-HPLC method for the simultaneous estimation of Atenolol and Amlodipine in tablet dosage form [12].

EXPERIMENTAL METHODS

Materials

The two active pharmaceutical substances were procured from Lupin Pvt. Ltd. Amdepin-AT, Amoldipine 5mg, Atenolol 50mg tablets were obtained from Cadila. The HPLC system consisted of Waters Series 600E pump Quaternary Gradient, Waters online degasser module and 996 Photodiode array (PDA) detector and a 515 autosampler. The data was acquired and processed by using EMPOWER software. Solvents used for HPLC method development were of HPLC grade and all other chemicals were of AR grade. HPLC grade water was obtained from Milli Q purification system.

Chromatographic Conditions

Thermohypersil-keystone C18, 150 x 4.6mm, 5µ (U.K.) column was used and the mobile phase comprised of 0.05M of Potassium di hydrogen ortho phosphate in water. The pH of the buffer solution was adjusted to 7.0 ± 0.1 with 5N potassium hydroxide. The buffer solution was filtered and then mixed with acetonitrile in the ratio of 70:30. Resultant solution was filtered and degassed prior to use. The mobile phase was pumped at a flow rate of 1.5 ml/minute. Detection was carried out using a PDA detector at 230 nm. The run time was 40 minutes and injection volume was 20 µL.

Preparation of standard stock solution of Atenolol

The standard stock solution of Atenolol was prepared by transferring about 50 mg of Atenolol working standard to a 250 ml volumetric flask. The contents of the flask were sonicated and then diluted up to the mark with methanol.

Preparation of standard stock solution of Amlodipine About 10 mg of Amlodipine working standard was accurately weighed and added to a 100 ml volumetric flask, sonicated to dissolve. Further, 1 ml of this solution was withdrawn and again diluted to 100 ml with methanol. A volume of 5 ml of Atenolol stock solution and 5 ml of Amlodipine stock solution were pipetted into a 25 ml volumetric flask and diluted up to the mark with mobile phase and mixed well.

Sample preparation

The average weight of twenty Amdepin-AT tablets (containing 5 mg Amlodipine and 50 mg Atenolol) was calculated. The tablets were then crushed and powdered. Quantity of powder equivalent to 50 mg of Atenolol (and 5 mg of Amlodipine) was transferred to a 100 ml volumetric flask containing about 60 ml of methanol. The flask was shaken for about 15 minutes and the volume was made up with methanol. The contents of the flask were sonicated and then centrifuged at 2500 rpm for 10 min. About 5 ml of the supernatant solution was then diluted to 25 ml with mobile phase.

Procedure

HPLC system was set as described under chromatographic conditions. Standard and sample solutions were prepared, injected and mean area counts for each sample was calculated.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

During method development following attempts was made with respect to columns, mobile phase and wavelength optimization.

A) Variation in column

Four different columns were tried, namely

1. Luna C8 (octylsilane) phenomenax, USA, 250 x 4.6mm, 5µ.
2. Luna C18 (octadecylsilane), phenomenax, USA, 250 x 4.6mm, 5µ.
3. Hypersil Phenyl, Thermoquest, USA, 250 x 4.6mm, 5µ.
4. Thermohypersil Keystone C18 (octadecylsilane), Thermoquest, USA, 150 x 4.6mm, 5µ.

The chromatographic conditions were as mentioned above. The chromatogram showing the separation on Thermohypersil keystone C18 (150 x 4.6 mm, 5µ) is shown in Figure 1 and the results obtained for the chromatographically important parameters are summarized as peak report in the Table 1. The retention time of Amlodipine and Atenolol were found to be 9.73 and 12.83 minutes respectively. The summary of parameters obtained on using different columns is tabulated in Table 2, 3 and 4 respectively.

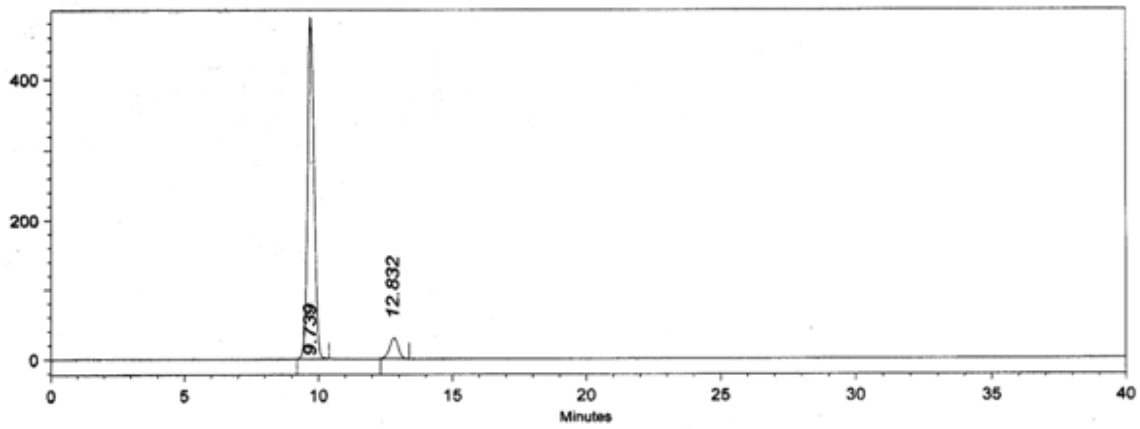

Figure1: Separationof Amlodipine and Atenolol onThermohypersil keystone C18 (150 x 4.6 mm, 5µ)

Table 1: Peak reports of Amlodipine and Atenololon Thermohypersil Keystone C18 (150 x 4.6 mm, 5µ) Thermoquest USA

Name	R.T. Min.	Area	% Area	Asym-metry	Theoretical Plates	Resolution	3 point peak purity
Amlodipine	9.73	8170349	92.40	0.98	7625.85	0.00	0.99837
Atenolol	12.83	671974	7.60	0.93	7228.27	5.89	0.99390

Table 2: Peak reports of Amlodipine and Atenololon Luna C8 (250 x 4.6mm, 5µ)phenomenax (USA)

Name	R.T. Min.	Area	% Area	Asym-metry	Theoretical Plates	Resolution	3 point peak purity
Amlodipine	12.363	8167608	92.24	1.08	13578.71	0.00	0.99715
Atenolol	15.733	687085	7.76	0.98	12763.43	6.87	0.91941

Table 3: Peak reports of Amlodipine and Atenololon Luna C_{18} (octadecylsilane) 250 x 4.6 mm, 5µ phenomenax (USA)

Name	R.T. Min.	Area	% Area	Asym-metry	Theoretical Plates	Resolution	3 point peak purity
Amlodipine	16.373	8332826	92.36	1.34	11397.50	0.00	0.99883
Atenolol	20.160	689604	7.64	1.11	11521.64	5.55	0.96725

Table 4: Peak reports of Amlodipine and Atenololon Hypersil Phenyl, 250 x 4.6 mm,5µ Thermoquest (USA)

Name	R.T. Min.	Area	% Area	Asym-metry	Theoretical Plates	Resolution	3 point peak purity
Amlodipine	7.563	715189	7.61	0.90	8678.14	0.00	0.93809
Atenolol	11.680	8673492	92.34	0.89	9671.33	14.95	0.87953

From the obtained chromatograms and relevant data it was observed that the Luna C_8 column gave the good resolution between Amlodipine and Atenolol but the peak purity of Atenolol was low. Luna C_{18} column had higher retention time for both Amlodipine and Atenolol and gave good resolution but the peak purity of Atenolol was on less. The order of elution was changed in Hypersil phenyl column with Atenolol eluting first followed by Amlodipine. Both the peaks had

good resolution between them but the peak purity was lower, indicating that both the peaks are impure. Thermohypersil keystone C_{18} column gave best resolution between Amlodipine and Atenolol and excellent peak purity with low retention time.

B) Variation in mobile phase

An additional mobile phase in the ratio of 0.05M NaH_2PO_4 (pH7 with dil. NaOH solution): Acetonitirile :: 68 : 32 was attempted to observe effect on resolution and peak purity for both drugs. The obtained chromatograms indicated good resolution between Amlodipine and Atenolol but peak purity of Atenolol was low. The results are shown in Table 5.

Table 5: Mobile phase in the ratio of 0.05M NaH_2PO_4 (pH 7 adjusted with dil.NaOH)Acetonitirile :: 68 : 32

Name	R.T. Min.	Area	% Area	Asym-metry	Theoretical Plates	Resolution	3 point peak purity
Amlodipine	12.128	6122627	92.34	1.23	9311.08	0.00	0.99855
Atenolol	15.147	507544	7.66	1.06	9093.86	5.30	0.97456

From experimental variations it was finally concluded that using Thermohypersil Keystone C18 (octadecylsilane) (150 x 4.6 mm, 5µ) Thermoquest USA along with A 0.05M of Potassium dihydrogen orthophosphate aqueous buffer solution adjusted to pH 7.0with 5N Potassium hydroxide and Acetonitrile in the ratio of 70:30 gave best results with retention times of 9.73 and 12.83 for Amlodipine and Atenolol respectively.

Validation parameters

Specificity

Specificity of the method was established by demonstrating that there was complete peak separation from any potential interference. This was demonstrated by injecting a mobile phase as blank and then injecting a standard preparation of Amlodipine followed by injecting standard preparation of Atenolol and chromatogram was obtained underpreviously described chromatographic conditions. No peak was observed at the retention time of Amlodipine or Atenolol thereby indicating that there is no interference from mobile phase.

System Precision (System Suitability)

System suitability parameters i.e. percent relative standard deviation of replicate injections, theoretical plates, resolution and tailing factors were determined before any analysis was carried out.Six replicate injections of the standard preparation were made into HPLC system and mean and percentage relative standard deviation (%RSD) of area counts was calculated. Table 6 below shows the value obtained and parameters for system precision for Amlodipine and Atenolol peak respectively with resolution, theoretical plates and tailing factor.

Table 6: System Precision of Amlodipine and Atenolol

Sr. No.	Parameters	Values	
		Amlodipine	Atenolol
1.	Mean	8495640.66	716572
2.	%RSD (n=6)	0.99	0.91
3.	Resolution	-	6.17
4.	Theoretical Plates	8306.05	7969.56
5.	Tailing Factor	1.04	0.97

Linearity and Range

The linearity of response was determined by preparing and injecting solutions with concentrations of about 76.06 µg/ml to 228.18 µg/ml for Amlodipine working standard and concentrations of 10.06 µg/ml to 30.18 µg/ml for Atenolol working standard respectively. Thelinearity and range graphs are shown in Figure 2 and 3. Table 7summarizes the data for linearity and range.

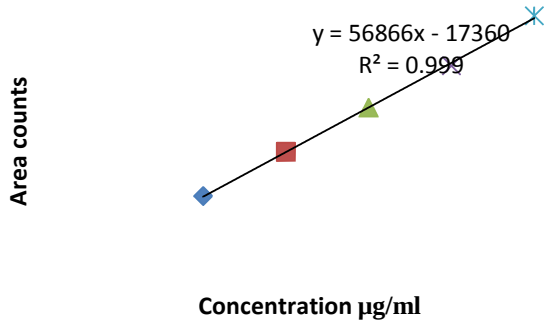

Figure 2: Linearity and Range for Amlodipine

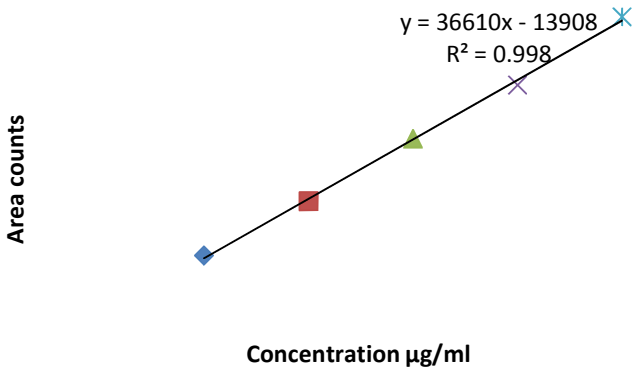

Figure 3: Linearity and Range for Atenolol

Table 7: Linearity and Range of AmlodipineandAtenolol

Amlodipine		Atenolol	
Concentration (µg/ml)	**Mean Area Counts**	**Concentration (µg/ml)**	**Mean Area Counts**
76.06	4340594.00	10.06	362352.00
114.09	6482261.00	15.09	531382.66
152.12	8621595.66	20.12	725137.50
190.15	10652428.33	25.15	891472.50
228.18	13068573.00	30.18	1103038.00
Slope	56866		36610
Intercept	-17360		-13908
Correlation Coefficient	0.999		0.998

The obtained values indicated that the response was linear over the range 76.06 to 228.18 µg/ml for Amlodipine and 10.06 to 30.18 µg/ml for Atenolol with coefficient of regression, R^2, value 0.999 for Amlodipine and 0.998 for Atenolol peak responses respectively.

Method Precision

Six sample solutions were prepared and analyzed on the same day using the HPLC method. Results of precision analysis are shown in Table 8 for Amlodipine and Atenolol.

Table 8: Method precision of Amlodipine and Atenolol

Sample Preparation	% Assay		% Deviation From Mean Assay value	
	Amlodipine	Atenolol	Amlodipine	Atenolol
1	100.75	98.95	0.99	-0.19
2	101.20	99.62	1.44	0.48
3	98.12	100.66	-1.65	1.54
4	99.53	98.43	-0.24	-0.72
5	99.81	98.91	0.05	-0.23
6	99.17	98.26	-0.59	-0.89
Mean	99.77	99.14		
± SD	1.11	0.88		
%RSD	1.11	0.89		

The % relative standard deviation for Amlodipine is 1.11% and Atenololwas 0.89% respectively. This shows that precision of the method is satisfactory as % relative standard deviation (%RSD) is not more than 2.0%.

Accuracy

Accuracy (Recovery) study was performed by spiking 30%, 50% and 70% of Amlodipine and Atenolol working standard to a pre-analyzed sample. The pre-analyzed sample was weighed in such a way that final concentration is half or 50% of the sample preparation before spiking. The percentage sum level of pre-analyzed sample and spiked amount of respective drug should be 80%, 100% and 120% of simulated dosages nominal or target concentration of sample preparation. The accuracy of the analytical method was established in duplicate across its range according to the assay procedure. The results of accuracy for Amlodipine and Atenolol are shown in Table 9and 10 respectively.

Table 9: Accuracy of Amlodipine

% Simulated Dosage Nominal	Replicates	% Sum Level	% Amt. Recovered	% Recovery
80	1	80.15	78.78	98.29
	2	80.78	81.72	101.16
	3	80.09	81.81	100.89
100	1	100.01	98.28	98.27
	2	100.62	98.72	98.11
	3	100.12	98.58	98.46
120	1	120.51	119.84	99.44
	2	120.39	119.19	99.00
	3	120.25	119.53	99.40
Mean				99.23
± Standard Deviation				1.13
% Relative Standard Deviation				1.14

Table 10: Accuracy of Atenolol

% Simulated Dosage Nominal	Replicates	% Sum Level	% Amt. Recovered	% Recovery
80	1	80.25	79.58	99.16
	2	82.00	82.40	100.49
	3	80.50	80.05	99.44
100	1	100.00	99.16	99.16
	2	100.25	99.52	99.27
	3	100.50	99.77	99.27
120	1	120.50	119.17	98.90
	2	121.25	120.72	99.56
	3	120.75	119.52	98.98
Mean				99.42
± Standard Deviation				0.56
% Relative Standard Deviation				0.57

The results indicate that the individual recovery of Amlodipine ranges from 98.11 % to 101.16 % with mean recovery of 99.23 % and % relative standard deviation of 1.14%. Whereas the individual recovery of Atenolol ranges from 98.9 % to 100.49 % with mean recovery of 99.42% and % relative standard deviation of 0.57%. The recovery of Amlodipine and Atenolol by proposed method is

satisfactory as % relative standard deviation was not more than 2 % and the mean recovery was between 98.0% - 102.0%.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The signal to noise ratios 2:1 and 10:1 were considered as LOD and LOQ respectively. The calculation is based on the standard deviation (σ) of the response and the slope of the calibration curve (S) according to equations 1 & 2.

$$LOD = 3* \sigma/S \quad (1)$$

$$LOQ = 10* \sigma/S \quad (2)$$

The LOD and LOQfor Amlodipine was found to be 6.438 µg / ml and 19.51 µg / ml respectively. Whereas, the LOD and LOQ for Atenolol was found to be 7.929 µg / ml and 24.03 µg / ml respectively.

Solution Stability

The solution stability was evaluated by injecting freshly prepared standard and sample solutions and subsequently followed by injecting the same at different time intervals.

The % deviation of Amlodipine standard and sample is -1.4 % and -1.9 % respectively for 36 hrs from initial response whereas the % deviation of Atenolol standard and sample is –0.6 % and 1.4 % respectively for 36 hrs from initial response. This indicates that Amlodipine and Atenolol are quite stable, and the % deviation is within 2.0%. The analytical solution and samples may be stored and need not be injected immediately after preparation.

Robustness and Ruggedness

Method robustness and ruggedness was determined by analyzing same sample blend at normal operating conditions and also by changing some operating analytical conditions such as the company make of the column, mobile phase composition, pH, flow rate, instrument and analyst. Table 11 summarizes theresults of robustness and ruggedness studies.

Table 11: Robustness and Ruggedness of Amlodipine and Atenolol

Chromatographic changes (Amlodipine)			Chromatographic changes (Atenolol)		
Factors	**Assay %**	**% Deviation**	**Factors**	**Assay %**	**% Deviation**
Column Make					
Thermohypersil - keystone BDS C_{18}150x4.6mm,5µ (Themohypersil, UK)	99.77	0.00	Thermohypersil - keystone BDS C_{18} 150x4.6mm,5µ (Themohypersil, UK)	99.14	0.00
Nova-pak C_{18} 150 x 3.9 mm, 4µ (Waters, USA)	98.57	1.20	Nova-pak C_{18} 150 x 3.9 mm, 4µ (Waters, USA)	98.66	0.48
Mobile Phase Composition (Buffer:Acetonitrile)					
67.5 : 32.5	99.27	0.501	67.5:32.5	99.06	0.08
70 : 30	99.77	0.00	70:30	99.14	0.00
72.5 : 27.5	99.89	-0.12	72.5:27.5	98.89	0.25
Flow Rate					
1.4 mL/min	99.53	0.24	1.4mL/min.	99.02	0.12
1.5 mL/min	99.77	0.00	1.5 mL/min.	99.14	0.00
1.6 mL/min	99.64	0.13	1.6 mL/min.	99.17	-0.03
Pump					
LC-10ATvp (Shimadzu)	99.77	0.00	LC-10ATvp (Shimadzu)	99.14	0.00
LC Module 1 Plus (Waters)	98.91	0.86	LC Module 1 Plus (Waters)	98.88	0.26
Detector					
SPD - M10Avp PDA	99.77	0.00	SPD - M10Avp PDA	99.14	0.00
UV-Visible detector	98.23	1.54	UV-Visible detector	98.11	1.03
Analyst					
Analyst-1	99.77	0.00	Analyst-1	99.14	0.00
Analyst-2	99.28	0.49	Analyst-2	99.03	0.11

The robustness and ruggedness of the method was established as the % deviation from mean assay value obtained from precision study is less than 2.0%.

CONCLUSION

The retention time of Amlodipine and Atenololwas found to be 9.73 and 12.83 minutes respectively. The method was validated according to ICH guidelines. The developed method was found to be specific as there was no interference from the mobile phase. Results indicated that the response was linear over the range of 76.06 to 228.18 µg/ml for Amlodipine and 10.06 to 30.18 µg/ml for Atenolol with coefficient of regression, R^2, value 0.999 for Amlodipine and 0.998 for Atenolol peak responses respectively. The proposed method was found to be accurate asthe individual recovery of Amlodipine ranged from 98.11 % to 101.16 % with mean recovery of 99.23 % and % relative standard deviation of 1.14 %. Whereas the individual recovery of Atenolol ranged from 98.9 % to 100.49 % with mean recovery of 99.42 % and % relative standard deviation of 0.57 %with % relative standard deviation not more than 2% and the mean recovery was between 98.0% - 102.0 %. The precision studies showed a % relative standard deviation for Amlodipine 1.11% and for Atenolol 0.89% respectively. This shows that precision of the method is satisfactory as % relative standard deviation (% RSD) is not more than 2.0 %. The method was found to be robust as no significant change in terms of % relative standard deviation was obtained.

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