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RESEARCH ARTICLE

RP-HPLC Method for the Simultaneous Estimation and Validation of Amlodipine Besylate and Atenolol in Bulk and Tablet Dosage Form in Biorelevant Dissolution Medium (Fassif)

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ABSTRACT:

Objective: A simple, rapid, and precise reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous analysis of Amlodipine Besylate and Atenolol in a tablet dosage form and in Biorelevant media has been developed and validated.

Methods:

The chromatographic separation was achieved using reverse phase C18 column; Kromasil C18 column (250 mm x 4.6 mm x 5µm). The mobile phase used was a mixture of Acetonitrile: Potassium di hydrogen phosphate solution (0.01M, pH 3.0 adjusting with Ortho phosphoric acid): Methanol (15:30:55) at isocratic mode and eluents were monitored at 254 nm using PDA detector.

Results:

By the method Amlodipine Besylate and Atenolol were eluted with retention times of 2.589 and 3.711 min, respectively. The method was continued and validated accordance with ICH guidelines. Validation revealed the method is rapid, specific, accurate, precise, reliable, and reproducible. Calibration curve plots were linear over the concentration ranges 25-125µg/mL for Amlodipine Besylate, 5-25µg/mL for Atenolol. Limits of detection (LOD) were 0.001, and 0.005µg/ml and limits of quantification (LOQ) were 0.004 and 0.015µg/mL for Amlodipine Besylate and Atenolol respectively.

Conclusion:

The statistical analysis was proves the method is suitable for the analysis of Amlodipine Besylate and Atenolol as a bulk and tablet dosage form in biorelevant dissolution media (Fasted State Simulated Intestinal Fluid-FaSSIF) without any interference from the excipients.

KEYWORDS: Amlodipine Besylate and Atenolol, RP-HPLC, Validation, Biorelevant media (FaSSIF).

INTRODUCTION:

Amlodipine Besylate is a second generation dihydro pyridine class of calcium channel blockers and is used in the treatment of both hypertension and angina pectoris. Like other calcium channel blockers, Amlodipine Besylate acts by blocking the influx of calcium ions into vascular smooth muscle and cardiac muscle cells during membrane depolarization. This action causes relaxation of vascular and arterial smooth muscle cells, resulting in arterial vasodilation and a decrease in cardiac work and oxygen consumption. Atenolol is a β-blocker seem to be equally effective as an antihypertensive, antianginal and antiarrhymthmic drug widely used as Cardiovascular drug in combination with Amlodipine Besylate. Atenolol competes with sympathomimetic neuro transmitters such as catecholamines for binding at beta (1)-adrenergic receptors in the heart and vascular smooth muscle, inhibiting sympathetic stimulation[1-4]

Amlodipine Besylate is Chemically 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-

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1, 4-dihydropyridine- 3, 5-dicarboxylate and its molecular formula $C_{20}H_{25}ClN_2O_5$ (Fig.1)[5]. It is official in IP-2007[5], USP-2010[6], and BP-2012[7].

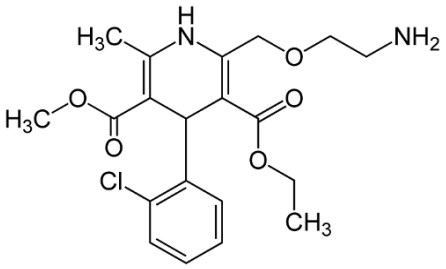

Fig.1: Chemical structure of Amlodipine Besylate

Atenolol is chemically2-(4-{2-hydroxy-3-[(propan-2yl)amino] propoxy} phenyl) acetamide and its molecular formula $C_{14}H_{22}N_2O_3$ (Fig.2) [8], It is official in IP-2007 [8] and BP-2012 [9].

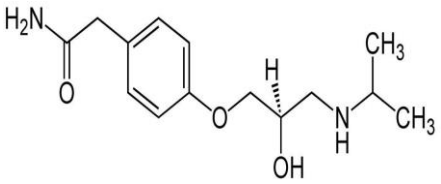

Fig.2: Chemical structure of Atenolol

In the scientific Literature survey reveals that various analytical methods have been reported for the assay of Amlodipine Besylate and Atenolol in pure form and in pharmaceutical formulations. While British Pharmacopoeia described liquid chromatography method for the assay of Amlodipine Besylate[7]. Non-aqueous titration method is specified in Indian Pharmacopoeia for the assay of Atenolol[8]. Many methods have been reported in the literature for the estimation of Amlodipine Besylate and Atenolol individually[10-12] and in other combination [13-32].

The present investigation was aimed at developing a fully validated RP-HPLC method for the simultaneous estimation of Amlodipine Besylate and Atenolol in bulk and pharmaceutical combined dosage form in biorelevant dissolution medium (FaSSIF) that is more economical, simple, precise and accurate than the previous methods.

MATERIALS AND METHODS:

1. Experimental:

1.1. Materials and Methods:

Pharmaceutical grade working standards Amlodipine Besylate and Atenolol were obtained from Hetero Labs, Jedcharla, India. All chemicals and reagents were HPLC grade and were purchased from Merck Chemicals, Mumbai, India.

1.2. Instrumentation:

The analysis was performed using SHIMADZU(UFLC-LC20) High Performance liquid chromatography, analytical balance 0.1mg Sensitivity (SHIMADZU), PDA Detector (Standard cell) and data handling system (LC-20), pH meter (lab India), Sonicator. The column used is Thermo Hypersil Keystone ODS C18 column (250x2.5mm packed with 5µm size Stationary phase) with the flow rate 1.0ml/min (isocratic).

1.3. Preparation of blank Fasted State Simulated Intestinal Fluid (FaSSIF):

Accurately weighed 1.74g of Sodium hydroxide pellets, 19.77g of Sodium dihydrogen orthophosphate, and 30.93g of Sodium chloride dissolve in 5 L of purified water and adjust the pH 6.5 exactly by using 1N Hydrochloric acid [32].

1.4. Preparation of FaSSIF[33]:

Accurately weighed 3.3g of sodium taurocholate dissolve in 500 mL blank FaSSIF solution, add 11.8 mL of a solution to 100mg/mL lecithin in methylene chloride, and forming an emulsion. The methylene chloride was eliminated under vacuum at 40°C. Then draw a vacuum for 15 minutes at 250mbar and also followed by 15 minutes at 100mbar. These results gave in a clear, micellar solution, having no perceptible odor for methylene chloride. After that, it was cool to room temperature and adjusts the volume upto 2L with blank FaSSIF [32].

1.5. Preparation of Standard Stock solution:

Accurately weighed 10 mg of Amlodipine Besylate and Atenolol working standard and separately transferred into a 10ml clean dry volumetric flasks, add about 7mL of biorelevant media (FaSSIF) to each volumetric flask and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Calibration standards at five levels were prepared by appropriately mixed and further diluted stock standard solutions in the concentration ranges from 25-125µg/mL for Amlodipine Besylate and 5-25µg/mL for Atenolol. Samples in triple injections were made for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the linearity graphs.

1.6. Preparation of Standard solution:

The above standard stock solution was containing 1000µg/mL of each Amlodipine Besylate and Atenolol in separate volumetric flasks. Then transferred the 1ml of Amlodipine Besylate and 0.1ml of Atenolol of prepared standard stock solution into a clean 10ml volumetric flask and made upto the mark with diluent. And finally the standard solution concentrations were 100µg/mL and 10µg/mL of Amlodipine Besylate and Atenolol respectively.

1.7. Preparation of Test solution:

For the analysis of a tablet dosage form, 20 tablets were weighed individually and their average mass was determined. Then, the tablets were crushed to a fine powder. The powder equivalent to 50mg of Amlodipine Besylate and 5mg of Atenolol were transferred to a 100mL volumetric flask and dissolved in 100mL of biorelevant media (FaSSIF), sonication was done for 15 min with swirling. After sonication, the solution was filtered through a membrane filter paper (#0.45µ). From the above stock solution 2mL was transferred in to 10mL volumetric flask and made volume upto the mark with diluent, the final concentrations were 100µg/mL and 10µg/mL of Amlodipine Besylate and Atenolol respectively, then injected into the chromatographic system, and analyzed quantitatively. The analysis was repeated six times and the possibility of excipient interference with the analysis was examined.

1.8. Optimization of HPLC Method:

The HPLC method was optimized and developed with a simultaneous method for Amlodipine Besylate and Atenolol. The mixed standard solution (100µg/mL of Amlodipine Besylate and 10µg/mL of Atenolol) injected in HPLC by the followed chromatographic conditions. The chromatographic separation was achieved on a Thermo Hypersil Keystone ODS C_{18} column (250x2.5mm, 5µm). The isocratic mobile phase consisting of Acetonitrile: Potassium di hydrogen phosphate solution (0.01M, pH 3.0 adjusting with Ortho phosphoric acid): Methanol (15:30:55) was used throughout the analysis. The flow rate of the mobile phase was 1.0ml/min. Detection was monitored at wavelength of 254nm. The column temperature was kept at ambient and injection volume was 20µl.

RESULTS AND DISCUSSION:

The simultaneous estimation of Amlodipine Besylate and Atenolol was done by RP-HPLC and in the optimized method the mobile phase consists of Acetonitrile: Potassium di hydrogen phosphate solution (0.01M, pH 3.0 adjusting with Ortho phosphoric acid): Methanol (15:30:55). Then finally filtered using 0.45µ membrane filter paper and degassed in sonicator for 15 minutes. The detection is carried out using PDA detector at 254nm. The solutions are following at the constant flow rate of 1.0 ml/min. The retention time for Amlodipine Besylate and Atenolol was 2.589 and 3.711minutes respectively. Linearity ranges for Amlodipine Besylate and Atenolol were 25-125µg/mL and 5-25µg/mL respectively and the results were found for in the acceptable as (R^2) = 0.999 and 0.9983 for Amlodipine Besylate and Atenolol respectively. LOD were 0.001 and 0.005µg/ml and LOQ were 0.004 and 0.015µg/mL for Amlodipine Besylate and Atenolol respectively. The all parameters value of RSD is less than 2.0% indicating the accuracy and precision of the method. The percentage recoveries were found 99.8% and 100.2% Amlodipine Besylate and Atenolol respectively.

1. Method Development and Optimization:

The HPLC procedure was optimized with a view to develop a suitable LC method for the analysis of Amlodipine Besylate and Atenolol in fixed dose for bulk and combined dosage form. It was found that Acetonitrile: Potassium di hydrogen phosphate solution (0.01M, pH 3.0 adjusting with Ortho phosphoric acid): Methanol (15:30:55) gave acceptable retention time (2.589 and 3.711min), theoretical plates, and good resolution for Amlodipine Besylate and Atenolol at the flow rate of 1.0ml/min (Table. 1; Fig. 1 & 2).

Table No. 1: Optimized Chromatographic Conditions

Parameters	Method
Stationary phase (column)	Thermo Hypersil Keystone ODS C_{18} column (250x2.5mm, 5µm)
Mobile Phase	15:30:55 v/v/v (Acetonitrile: Potassium di hydrogen phosphate solution : Methanol)
pH	2.8 ± 0.02
Flow rate (ml/min)	1.0
Run time (minutes)	6.0
Column temperature (°C)	Ambient
Volume of injection loop (µl)	20
Detection wavelength (nm)	254
Drugs Rt (min)	2.589 & 3.711

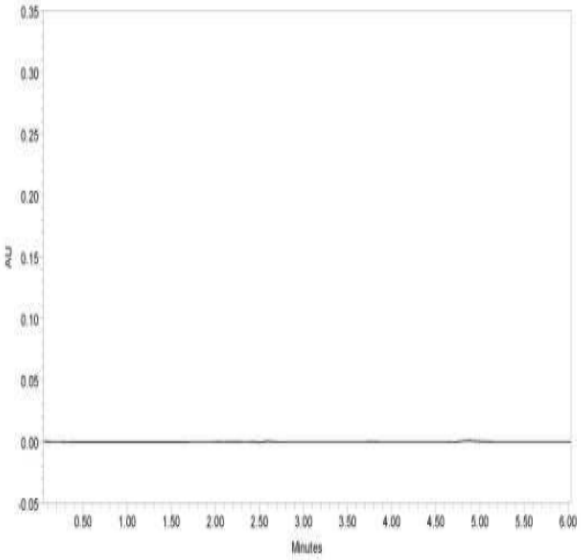

Fig.1: Blank Chromatogram

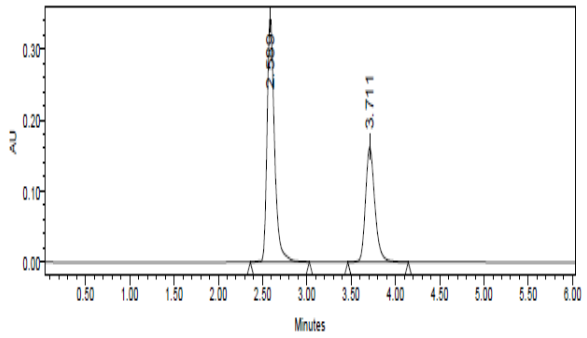

Fig.2: Chromatogram of Mixed Standared Amlodipine Besylate and Atenolol at 254nm from bulk drug

2. Assay:

Three batches of compound tablets were analyzed using the developed method. Satisfactory results were obtained that the mean percentage found for Amlodipine Besylate and Atenolol were in good agreement with the label claimed. The mean percentage found (Table-2) indicated that the proposed method could be adopted for the determination Amlodipine Besylate and Atenolol in combined tablet dosage form as shown in Fig. 3.

Table.2: Assay data for Atenolol

Tablet (AMTAS-AT)	Label Claim (mg)	Amount Estimated* (mg)	% Amount Estimated	Acceptance Range
Amlodipine Besylate	5	4.99	99.8	98%-102%
Atenolol	50	50.10	100.2	

* Mean of 3 determinations

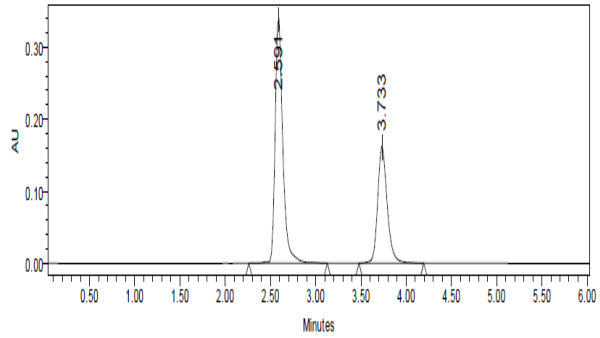

Fig.3: Chromatogram of Amlodipine Besylate and Atenolol at 254nm from pharmaceutical dosage form (AMTAS-AT)

3. Validation of Developed method[34-36]:

The proposed method was validated with the aspect of system suitability test, specificity, linearity and range, accuracy, precision, LOD, LOQ, stability and robustness according to the ICH guidelines.

3.1. System suitability:

A Standard solution of Amlodipine Besylate and Atenolol working standard was prepared as per procedure and was injected six times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from five replicate injections as shown in Table 3-4.

Table-3: Results of System suitability Test for ATENOLOL

Injection	Retention time(Rt)	Peak Area	Plate count	Tailing factor
1	3.711	1185786	6389	1.3
2	3.702	1184759	6455	1.3
3	3.698	1187496	6234	1.6
4	3.708	1190478	6478	1.3
5	3.715	1183897	6502	1.3
6	3.714	1184759	6384	1.2
Mean	-	1186196	-	-
SD	-	2433.47	-	-
% RSD	-	0.20	-	-

Table-4: Results of System suitability Test for AMLODIPINE

Injection	Retention time(Rt)	Peak Area	Plate count	Tailing Factor
1	2.589	2008408	5752	1.4
2	2.570	2008412	5758	1.3
3	2.572	2008357	5672	1.2
4	2.578	2007478	5674	1.4
5	2.582	2008475	5749	1.3
6	2.584	2008364	5843	1.4
Mean	-	2008249	-	-
SD	-	380.0	-	-
% RSD	-	0.01	-	-

3.2. Linearity:

Linearity was evaluated by analysis of working standard solutions of Amlodipine Besylate and Atenolol of five different concentrations. The range of linearity ranges from 25-125µg/ml for Amlodipine Besylate and 5-25µg/ml for Atenolol (Table. 5). The result of correlation coefficients of Amlodipine Besylate and Atenolol (R^2) = 0.999 & 0.9983 respectively (Fig. 4-5). There was an excellent correlation between peak areas and concentrations of each drug.

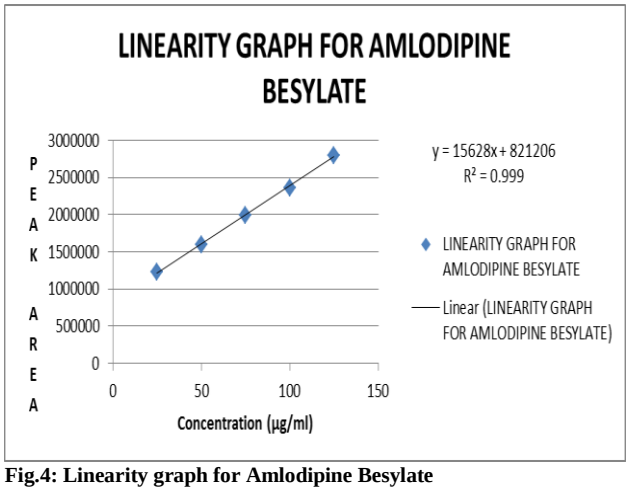

Fig.4: Linearity graph for Amlodipine Besylate

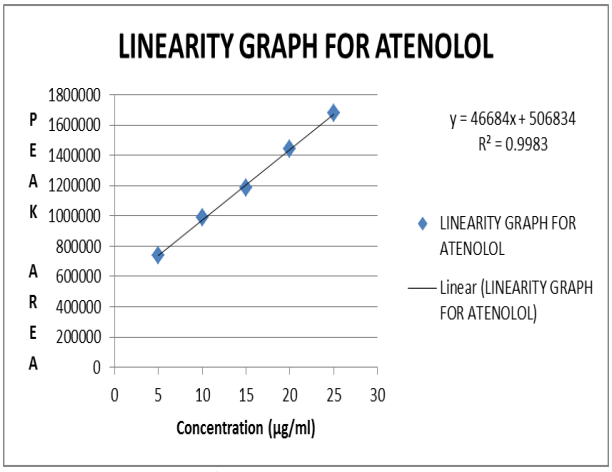

Fig.5: Linearity graph for Amlodipine Atenolol

Table No. 5: Data for Linearity

Analyte	Conc. range (µg/mL)	Correlation Coefficient (R2)	Slope	Intercept
Amlodipine Besylate	25-125	0.999	15628x	821206
Atenolol	5-25	0.9983	46684x	506834

3.3. Accuracy:

The accuracy of the method was determined by calculating the recovery studies at three levels (50%, 100% and 150%) by standard addition method. Known amounts of standard Amlodipine Besylate and Atenolol were added to the pre quantified samples and they were subjected to proposed HPLC method. The recoveries results of Amlodipine Besylate and Atenolol in pharmaceutical preparation are shown in the Table-6-7.

Table.6: Accuracy Study of Amlodipine Besylate

Sample Id	Conc found (µg/ml)	Concn Obtained (µg/ml)	% Recovery	Mean recovery	Statistical Analysis
50%	5	5.01	100.2		
50%	5	4.96	99.2	99.73	
50%	5	4.99	99.8		**%RSD= 0.505**
100%	10	9.95	99.5		
100%	10	9.87	98.7	98.8	
100%	10	9.82	98.2		**%RSD=0.66**
150%	15	14.64	97.6		
150%	15	14.76	98.4	98.8	
150%	15	15.06	100.4		**%RSD=1.45**

Table.7: Accuracy Study of Atenolol

Sample Id	Conc (µg/ml)	Concn Obtained (µg/ml)	% Recovery of drug	Mean accuracy	% RSD
50%	5	4.92	98.0		
50%	5	4.96	99.2		
50%	5	5.02	100.4	**99.2**	**1.2**
100%	10	9.95	99.5		
100%	10	9.94	99.4		
100%	10	9.98	99.8	**99.5**	**0.2**
150%	15	14.78	98.6		
150%	15	14.94	99.6	**99.0**	**0.530**
150%	15	14.83	98.8		

3.4. Precision:

Precision study was performed to find out intraday and interday variations. The intraday and interday precision study of Amlodipine Besylate and Atenolol was carried out by estimating the correspondence response 3 times on the same day and on 3 different days for 3 different concentrations of Amlodipine Besylate and Atenolol and the results were reported in terms of % relative standard deviation (% RSD). All results fall within acceptance limits (RSD < 2), as shown in Table-8.

3.5. LOD and LOQ:

The LOD and LOQ for Amlodipine Besylate and Atenolol were separately determined by based on calculating the signal-to-noise ratio. Detection limit=3.3 σ/s; quantification limit=10 σ/s; where σ is the standard deviation of y-intercept of regression line and's' is the slope of the calibration curve. Results were shown in the Table-9.

Table-8: Intra-day and Inter-day precision of Amlodipine Besylate and Atenolol Standard solutions

Drug Concentration* (µg/ml)		Intra-day		Inter-day	
		Peak Area	% RSD	Peak Area	% RSD
Amlodipine Besylate	5	2005053	0.15	2010800	0.19
	10	2007362	0.26	2002956	0.24
	15	2007473	0.26	2012800	0.26
Atenolol	5	1183951	0.22	1184689	0.20
	10	1184689	0.14	1188199	0.18
	15	1186232	0.16	1195842	0.15

* Mean of each 3 determinations

Table-9: LOD and LOQ for Amlodipine Besylate and Atenolol

Parameter	Amlodipine Besylate	Atenolol
LOD	0.001	0.005
LOQ	0.004	0.015

3.6. Robustness:

The robustness study was done by making small changes in the optimized method parameters like ±1% change in flow rate and composition of mobile phase. There was no significant impact on the retention time and tailing factor. Results were sown in Table-10 and Table-11.

Table.10: Robustness data for Amlodipine

Std. Replicate	Variation in flow rate		Variation in Mobile phase composition	
	Flow Rate 0.8ml/min	**Flow Rate1.2ml/min**	**Acetonitrile: Buffer: Methanol (15:35:50)**	**Acetonitrile: Buffer: Methanol (10:30:60)**
1	2492492	1676589	1951632	1979168
2	2495874	1675428	1954783	1967452
Mean	2494183	1676009	1953208.0	1973310
SD	2391.4	820.9	2228.0	8284.46
%RSD	0.09	0.04	0.11	0.4
Retention time	3.150	2.168	2.618	2.572
Tailing factor	1.4	1.3	1.3	1.3
Theoretical plates	5752	4207	4577	4476

Table.11: Robustness data for Atenolol

Parameter	Variation in flow rate		Variation in Mobile phase composition	
Standard	**Flow Rate 0.8ml/min**	**Flow Rate 1.2ml/min**	**Acetonitrile:Buffer: Methanol (15:35:50)**	**Acetonitrile:Buffer: Methanol (10:30:60)**
1	1500192	100524	1196996	1153397
2	1500426	100468	1198547	1154782
Mean	1500309	100496	1197772	1154090
SD	165.5	39.59	1096.2	979.34
%RSD	0.01	0.03	0.09	0.08
Retention time	4.674	3.121	4.394	3.331
Tailing factor	1.2	1.2	1.2	1.2
Theoretical plates	7187	5412	6498	6471

CONCLUSION:

The RP-HPLC method has been developed and validated for the simultaneous estimation of Amlodipine Besylate and Atenolol in tablet dosage form by using Biorelevent Dissolution Media (FaSSIF). The results show that the method is accurate, precise, linear, robust, simple and rapid. Acceptable regression values, %RSD and standard deviations which make it is versatile and valuable for simultaneous estimation of two drugs in bulk and pharmaceutical dosage forms. The run time is relatively short. The results of this developed RP-HPLC method could be conveniently adopted for quality control analysis of Amlodipine Besylate and Atenolol simultaneously from tablet dosage form in Biorelevent dissolution media.

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