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RP-HPLC method development and validation for simultaneous estimation of Atorvastatin calcium and Amlodipine besylate.

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Abstract: A novel, simole, precise and acccurate revese phase high performance liquide chromatography (RP-HPLC) method was developed and validated for the simultaneous determination of Atorvastatin calcium (ATR) and Amlodipine besylate (AML) in combined dosage form. The proposed RP-HPLC method utilizes a C18, 5µm, 250mm × 4.6mm i.d. column, mobile phase consisiting of 0.05 M ammonium acetate solution and acetonitrile in the proportion of 55:45 (v/v) with apparent pH adjusted to 6.1, and UV detection at 238 nm using UV detector L-7400. The retention times were 7.09 min and 4.20 min for ATR and AML at a flow rate of 1.2 ml/min. The discribed method was linear over a range of 5-50 µg/ml for ATR and 2.5-25 µg/ml for AML. The percentage mean recovery was found to be 100.03 for ATR and 100.09 for AML. The method ws statistically validated for its linearity, accuracy and precision. Both interday and intraday validation was found to be showing less % RSD having high grade of precision of the method.
Keywords: RP-HPLC, Atorvastatin calcium, Amlodipine beylate, UV detection, Validation.

1. Introduction

Atorvastatin calcium (ATR) is chemically described as [R - (R*, R*)] -2 - (4-Flurophenyl) -β , δ-dihydroxy- 5- (1-methylethyl)- 3- phenyl- 4 -[(phenylamino) carbonyl] -1H -pyrrole- 1-heptanoic acid : calcium salt (2:1) trihydrate [1,2] (Figure. 1). ATR is liver selective competitive inhibitor of 3-hydroxy-3-methylglutaryl Co enzyme A (HMG Co A) reductase, the rate limiting enzyme that converts 3-hydroxy-3-methylglutaryl Co enzyme A to mevalonate, a precursor of cholesterol biosynthesis. It also lowers elevated total and LDL cholesterol, apolipoprotein-B, and triglyceride levels in patients with primary hypercholesterolemia and mixed dislipidemia [3-5]. ATR is rapidly absorbed after oral administration however, due to presystemic clearance in gastro intesinal mucosa and metabolism in liver, its absolute bioavalability is approximatly 12% and low plasma concentration is achieved following administration of the drug [6-8].

Amlodipine besylate (AML) is chemically described as (R, S) 2-[(2-Aminoethoxy) methyl]-4 -(2-chlorophenyl)-1,4-dihydro-6- methyl-3,5- pyridinedi carboxylic acid 3-ethyl 5-methyl ester benzene sulphonate [1,2] (Figure. 2). AML is a dihydropyridine derivative with calcium antagonist activity. AML like other calcium channel blockers inhibits the slow channel influx of calcium into cardiac and vascular tissues. AML has peripheral vasodilatory action and also produces vasodilation in coronary vascular beds. It is used in the management of hypertension, chronic

stable angina pectoris and prinzmetal variant angina [3-5].

Survey of literature revealed that few methods have been developed for the detemination of ATR and AML individually and in combination with other drugs. Stability indicating RP-HPLC method have also been developed for the determination of both the drugs individually [9-18]. Hence an attempt has been made to develop a simple, accurate, precise and reproducible RP-HPLC method for simultaneous estimation of ATR and AML in combined dosage form with validation as per recommendation of ICH guidelines.

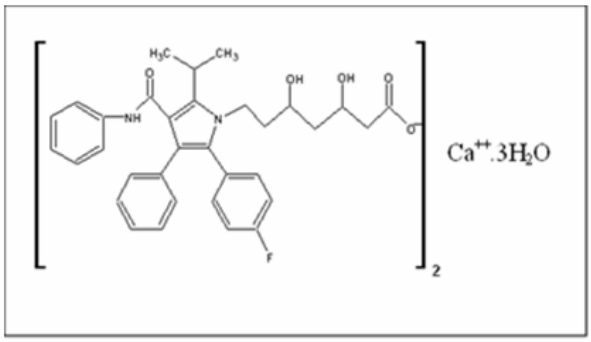

Figure 1: Structural formulae for Atorvastatin calcium (MW= 1208.42).

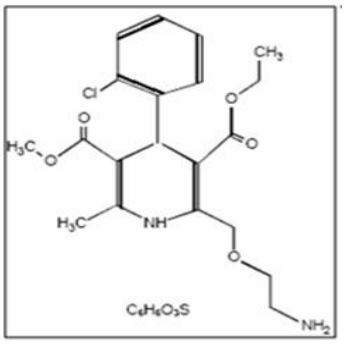

Figure 2: Structural formulae for Amlodipine besylate. (MW= 567.1).

2. Experimental

2.1. Chemicals and reagents

The working standards of ATR and AML were gifted from Torrent Pharmaceuticals Ltd. (Ahmedabad, India). The tablet formulation of ATR and AML (Label claim: Atorvastatin 10 mg, as Atorvastatin calcium and Amlodipine 5 mg, as Amlodipine besylate), Storvas tablets (Ranbaxy Laboratories Ltd. Asalali, Ahmedabad) were purchased from the local market. Acetonitrile, Ammonium acetate (HPLC grade), glacial acetic acid (AR grade) and glass double distilled water were obtained from E. Merck Ltd.

2.2. HPLC instrumentation

The liquid chromatographic system consisted of following components: Quaternary gradient HPLC system – Merck Hitachi, double receprocating pump L-7100, variable wavelength programmable UV detector L-7400, a universal injector 77251 (Rheodyne) with injection volume 20 µl. Chromatographic analysis was performed using Merck-Hitachi HSM software on a LiChrosorb^R C18 column, with 250mm × 4.6mm i.d. and 5µm particle size.

2.3. Preparation of mobile phase and standard stock solution

Ammonium acetate 0.05 M solution was prepared by dissolving accurately about 1.93 gms of CH_3COONH_4 in a 50 ml of glass double distilled water and then adding 0.25 ml of glacial acetic acid and diluting to 500 ml with glass double distilled water. Mobile phase was prepared by mixing 275ml of 0.05 M ammonium acetate solution with 225 ml of acetonitrile and its pH was found to be 6.1. This mobile phase was ultrasonicated for 20min, and then it was filtered through 0.45µm Nylon 6,6 (N_{66}) 47mm membrane filter paper.

Accurately about 10 mg of each of reference standard of ATR and AML was weighed and transferred to two separate 100ml volumetric flask. Both drugs were dissolved in 50ml of mobile phase with shaking and then volume was made upto the mark with mobile phase to get 100 µg/ml of standard stock solution of each drug. These stock solutions were filtered through 0.2µm Nylon 66 (N_{66}) membrane filter paper. The standard calibration solutions of ATR and AML having concentration range 5-50 and 2.5-25 µg/ml respectively were prepared by diluting appropriate aliquots of the standard stock solutions with the mobile phase.

2.4. Chromatographic conditions

The mobile phase consisting of acetonitrile and 0.05 M ammonium acetate solution in the ratio of 45:55 (v/v) with an apparent pH 6.1 was selected as the optimum composition of mobile phase, because it was found that this solvent system resolved both the components ideally. The mobile phase and samples were degassed by ultrasonication for 20 min and filtered through 0.45 µm Nylon 66 (N66) 47 mm membrane filter paper. The measurements were carried out with an injection volume of 20µl, flow rate was set to 1.2 ml/min and UV detection was carried out at 238 nm. All determinations were performed at constant column temperature (30°C). The chromatograms of the prepared stock solution of ATR and AML were recorded individually under the above optimized conditions (Figure. 3 and Figure. 4).

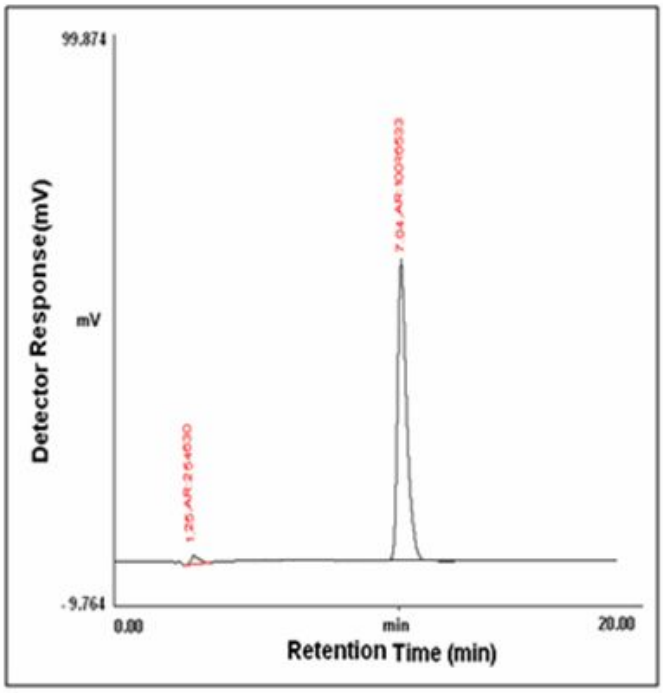

Figure 3: Structural formulae for Amlodipine besylate. (MW= 567.1).

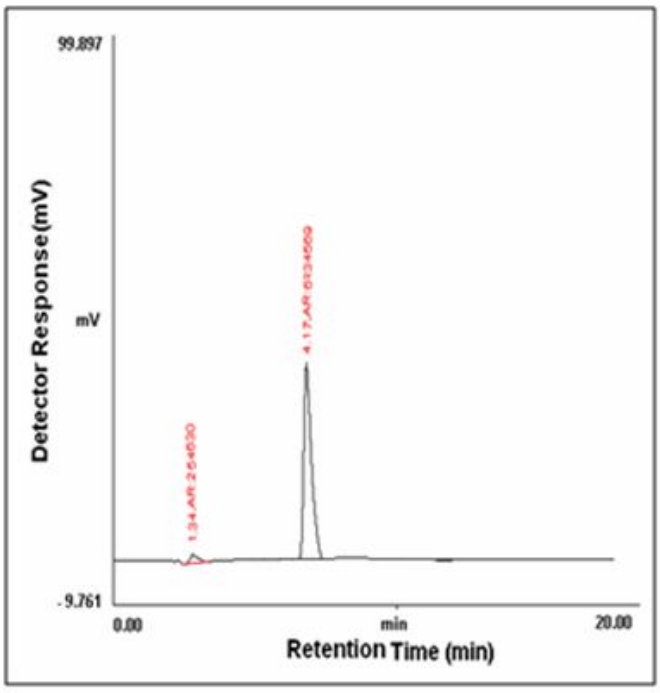

Figure 4: RP-HPLC chromatogram of a 20 µl injection containing 10 µg/ml of Amlodipine besylate.

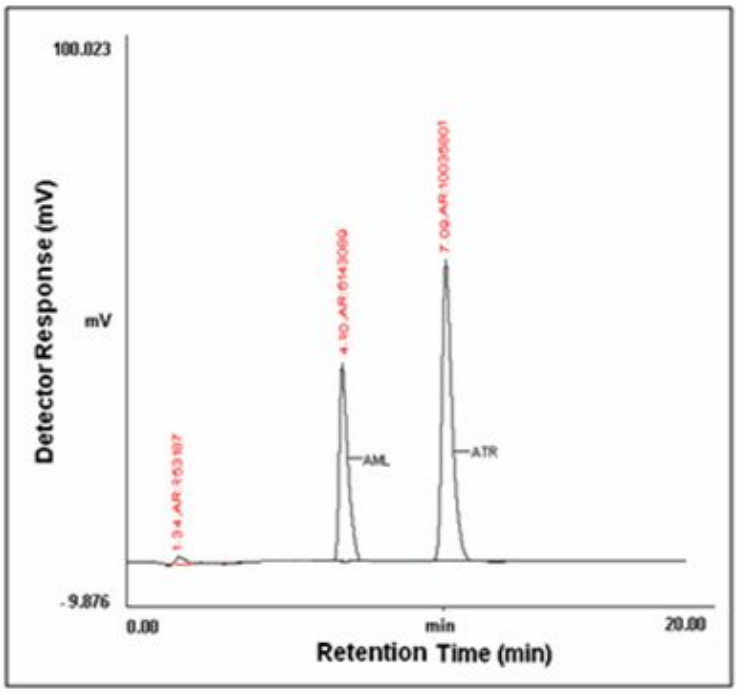

Figure 5: RP-HPLC chromatogram of a 20 µl injection containing mixture of 20 µg/ml of Atorvastatin calcium and 10 µg/ml of Amlodipine besylate in tablet formulation.

2.5. Analysis of tablet dosage form

Twenty tablets were weighed, their mean weight was determined and finally they were crushed in mortar to obtain a fine powder. An amount of powdered mass equivalent to one tablet content was transferred into a 100 ml volumetric flask and dissolved in sufficient quantity of mobile phase. The contents were ultrasonicated for 30 minutes and the final volume was made up to the mark with mobile phase. The prepared solution was then filtered through 0.2 µm Nylon 66 (N66) 47 mm membrane filter paper and was used as standard stock solution. Appropriate aliquot was pippetted out from the standard stock solution and was further diluted with the mobile phase to obtain a mixture containing 20 µg/ml of ATR and 10 µg/ml of AML. A 20 µl volume of the sample mixture was injected in to the sample injector and their chromatograms were recorded under the optimized chromatographic conditions (**Figure. 5).** The area of each peak was determined at 238 nm and the amount of drug present in the sample mixture was determined.

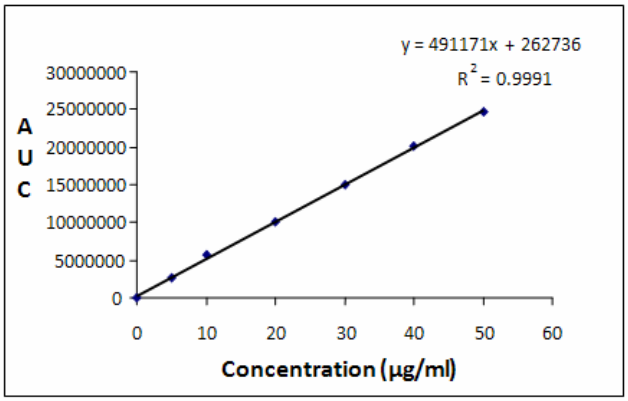

Figure 6: Calibration curve of Atorvastatin Calcium at 238 nm by RP−HPLC Method.

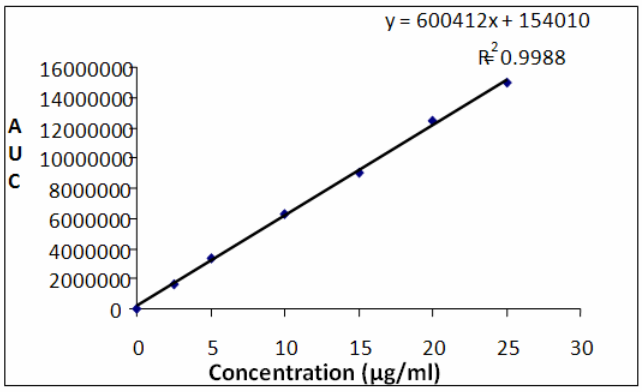

Figure 7: Calibration curve of Amlodipine besylate at 238 nm by RP−HPLC Method.

Table 1: Statistical analysis of the calibration curves of ATR and AML respectively

Parameters	ATR	AML
Range (µg/ml)	5.0 – 50.0	2.5 – 25.0
Slope*	491171	600412
Intercept*	262736	154010
Correlation coefficient (r)*	0.9991	0.9988
LOQ (µg/ml)**	0.076	0.088
LOD (µg/ml)**	0.029	0.025

Where, *n = 6 and **n = 3

3. Result and discussion

3.1. Method development and optimization

A LiChrosorb^{R} C18, 5 µm, 250mm×4.6mm i.d. column (E. Merck Ltd. India) maintained at ambient temperature (30°C) was used for the separation. Method validation was performed for the determination of ATR and AML in Storvas tablets. The composition, pH, flow rate of the mobile phase, and the column temperature were optimized to obtain good resolution. A mobile phase composition, acetonitrile and 0.05 M ammonium acetate solution in the ratio of 45:55 (v/v), pH 6.1, set at a flow rate of 1.2 ml/min was selected for the chromatographic analysis. Under above experimental conditions, all the peaks were well resolved and free from asymmetry. Methods robustness was performed to evaluate the effect of small deliberate changes in the mobile phase pH, composition, flow rate and column temperature on results.

3.2. Method validation

The developed analytical method was subjected to validation with respect to various parameters such as linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, recovery studies, specificity and reproducibility and robustness / ruggedness as per the ICH guidelines [19-22].

3.2.1. Linearity

Linearity was established by least square regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 5-50 µg/ml and 2.5-25 µg/ml for ATR and AML respectively. Peak areas of ATR and AML were plotted versus their respective concentrations and linear regression analysis was performed on the resultant curves. Correlation coefficients (n = 6) were found to be more than 0.999 for both the drugs. Typically, the regression equations were found to be: y = 491171x + 262736 (r = 0.9991) for ATR and y = 600412x + 154010 (r = 0.9993) for AML respectively.

3.2.2. LOQ and LOD

The LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. LOD was determined on the basis of signal to noise ratios and was determined using analytical response of three times the background noise [19,20]. Both LOQ and LOD were calculated on the peak area using the following equations:

$LOQ = 10 \times N/B$ $LOD = 3 \times N/B$

Where, N is the SD of the peak areas (triplicate injections) of the drug, B is the slope of the corresponding calibration curve. The limit of quantification and the limit of detection of ATR and AML were found to be 0.076 µg/ml and 0.088 µg/ml, 0.025 µg/ml and 0.029 µg/ml, respectively.

3.2.3. Accuracy

Accuracy of the method was determined by interpolation of peak areas of six replicate samples (n = 6) of tablets formulation containing 20 µg/ml of ATR and 10 µg/ml of AML, from the calibration curve that had been previously prepared. The accuracy data for the assay following the determination of each component of interest are summarized in Table 2.

Table 2: Statistical validation data for accuracy determination (n = 6)

	Interpolated concentration (mean ± SD)	RSD (%)
Atorvastatin calcium (µg/ml)		
20	19.96 ± 0.0681	0.3411
Amlodipine besylate (µg/ml)		
10	10.04 ± 0.0619	0.6165

Table 3: Statistical validation data for determination of intra-day precision (n = 6)

	Interpolated concentration (mean ± SD)	RSD (%)
Atorvastatin calcium (µg/ml)		
20	19.89 ± 0.0854	0.4273
Amlodipine besylate (µg/ml)		
10	9.93 ± 0.0804	0.8051

Table 4: Statistical validation data for determination of inter-day precision (n = 3)

	Interpolated concentration (mean ± SD)	RSD (%)
Atorvastatin calcium (µg/ml)		
20	19.90 ± 0.0693	0.3480
Amlodipine besylate (µg/ml)		
10	9.92 ± 0.0726	0.7319

3.2.4. Precision

Intra day precision was estimated by assaying the quality control sample of the tablet formulation containing 20 µg/ml of ATR and 10 µg/ml of AML, six times (results averaged for statistical evaluation) in the same analytical run [19,20]. The statistical validation data for intra day precision are summarized in Table 3.

Inter day precision was evaluated by analyzing a set of quality control samples of the tablet formulation containing 20 µg/ml of ATR and 10 µg/ml of AML, six levels analyzed on three consecutive days (results averaged for statistical evaluation) in the same analytical runs [19,20]. The statistical validation data for intra day precision are summarized in Table 4.

3.2.5. Recovery

Recovery studies were also performed to determine the accuracy and precision of the proposed RP-HPLC method. Recovery experiments was performed at three levels, in which the sample stock solutions were spiked with standard drug solution containing 80%, 100% and 120% of the labeled amount of both the drugs (10 mg ATR and 5 mg AML) in tablet formulation. Three replicate samples of each concentration levels were prepared and the percentage recovery at each level (n = 3), and mean % recovery (n = 9) were determined and summarized in Table 5. The mean (%) recovery was found to be 100.03% and 100.09% for ATR and AML respectively.

3.2.6. Specificity and reproducibility

The specificity of the RP-HPLC method was determined by complete separation of ATR and AML, with respect to various system suitability parameters like retention time (t_R), resolution (R_S), capacity factor and tailing factor (T_f) [21,22]. Here tailing factor for peaks of ATR and AML was less than 2% and resolution was more than 1%. The average retention time for ATR and AML were found to be (7.09 min and 4.20 min) respectively for six determinations. The peaks obtained for ATR and AML were sharp and have a clear baseline separation indicating high degree of specificity.

Table 5: Recovery of ATR and AML in spiked standard drug solution

Level of (%) Recovery	Amount present (mg)		Amount added (mg)		Amount found (mg)		Recovery (%)	
	ATR	AML	ATR	AML	ATR	AML	ATR	AML
Level 1	10.0	5.0	8.0	4.0	17.87	8.89	99.30	99.80
(80%)	10.0	5.0	8.0	4.0	17.95	8.96	99.70	99.60
	10.0	5.0	8.0	4.0	17.98	9.00	99.90	100.00
Level 2	10.0	5.0	10.0	5.0	20.06	9.98	100.30	99.80
(100%)	10.0	5.0	10.0	5.0	19.98	10.02	99.90	100.20
	10.0	5.0	10.0	5.0	20.10	10.02	100.50	100.20
Level 3	10.0	5.0	12.0	6.0	22.04	11.04	100.20	100.40
(120%)	10.0	5.0	12.0	6.0	21.98	11.07	99.90	100.60
	10.0	5.0	12.0	6.0	22.13	11.02	100.60	100.20

	Mean (%) recovery (n = 9)	RSD (%)
ATR	100.03	0.4091
AML	100.08	0.3176

Table 6: Summary of system suitability parameters of ATR and AML

Parameters	ATR	AML
Retention time (min)	7.09	4.20
Resolution factor	6.57	6.57
Tailing factor	1.25	1.04
Capacity factor	4.15	2.07
Separation factor	2.01	2.01

The reproducibility of proposed RP-HPLC method was evaluated by analyzing the samples by two different analysts on different days under the same chromatographic conditions [21,22]. The two sets of data obtained were subjected to t-test analysis at 95% confidence level. The result of t-test analysis was found to be 0.9981, indicating no statistically significant difference.

3.2.7. Robustness

The evaluation of robustness of the proposed RP-HPLC method was considered during the development phase. The robustness of the method was investigated by subjecting the sample analysis to small deliberate variations in the analytical conditions including pH, flow rate, composition of mobile phase and column temperature. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators had proved that the method was robust and the data are summarized in Table 7.

Table 7: Robustness evaluation of the proposed RP-HPLC method

Parameters	Modification	Retention time (min)		Tailing factor	
		ATR	AML	ATR	AML
Flow rate (ml/min)	1.0	8.14	4.92	1.13	1.29
	1.2	7.06	4.20	1.11	1.04
	1.4	6.25	3.59	1.22	1.14
(%) of acetonitrile	43	6.21	3.76	1.11	1.25
in mobile phase	45	7.06	4.20	1.11	1.04
	47	8.09	4.68	1.13	1.17
Column Temperature	28	6.95	4.14	1.25	1.18
(^{o}C)	30	7.06	4.20	1.11	1.04
	32	6.85	4.11	1.07	1.18
pH	5.9	7.72	4.57	1.13	1.30
	6.1	7.06	4.20	1.11	1.04
	6.3.	8.52	5.31	1.18	1.30

4. Conclusion

In conclusion a simple, accurate, rapid and specific reverse phase HPLC method using UV detection has been described for the determination of ATR and AML in commercial formulation. The proposed new method has been demonstrated to be suitable for use in routine analysis of the tablet formulation without any prior seperation of its components with a high grade of accuracy and precision.

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