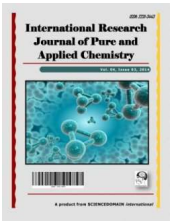

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A New RP-HPLC Method for Quantitative Analysis of Atorvastatin Calcium in Bulk and Pharmaceutical Dosage form by Using Design of Experiment Technique Optimization

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

A rapid high-performance liquid chromatography method was developed and validated for the determination of atorvastatin calcium in pharmaceutical dosage forms, and for evaluation of its stability in stress testing. Separation of atorvastatin was successfully achieved on a C-18 column utilizing -acetonitrile, Phosphate buffer pH 3.0 at the volumetric ratio of 59:41. The detection wavelength was 245 nm. The method was validated and the response was found to be linear in the drug concentration range of 6.4 µg/mL – 9.6 µg/mL. The correlation coefficient was 0.9999. The RSD values for precision as repeatability and reproducibility were 0.377 and 0.70 respectively, and the regression equation was Y= 0.9962x+0.2241. All the validation parameters were within the acceptable range. The developed method was successfully applied to estimate the amount of atorvastatin calcium in tablets.

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Keywords: DOE; validation; RP-HPLC; atorvastatin calcium.

1. INTRODUCTION

Atorvastatin Calcium is chemically described as [R-(R*, R*)] -2-(4-fluorophenyl) -β, ∂ dihydroxy-5-(1-Methylethyl) -3-phenyl-4- [(phenylamino) carbonyl] -1H-pyrrole-1-heptanoic acid calcium salt trihydrate is an anti-hyperlipoproteinemic agent act by inhibiting HMG- CoA reductase. Many analytical Methods like UV spectroscopy, HPLC were reported for determination of Atorvastatin Calcium alone and combination with other antihypertensive drugs. In the analysis of Atorvastatin, the major problem is the solubilizing of Atorvastatin in most of the solvents during analysis. Quantitative estimation of poorly Water-soluble drugs involves the use of organic solvents [1,2].

Literature survey reveals that few spectrophotometric methods and high performance liquid chromatography (HPLC) methods [3] have been reported for the estimation of atorvastatin and ezetimibe.

Several HPLC methods were reported in the literature for the quantitative determination of atorvastatin calcium in biological samples alone], and with another active drug substance. Most of the analytical techniques for atorvastatin calcium described in the literature are based on the liquid chromatographic determination of this drug alone in pharmaceutical formulations with another active drug substance. Other analytical techniques such as spectroscopy, MALDI Mass spectrometry imaging electrochemical and capillary electrophoresis has also been described. A reversed phase LC with UV detection for the quantitation of atorvastatin calcium in bulk material is described in United States Pharmacopeia [4].

Forced degradation is a degradation of new drug substance and drug product at conditions more severe than accelerated conditions. It is required to demonstrate the specificity of stability indicating a method for determination degradants of atorvastatin calcium and also provides an insight into degradation pathways and degradation products of the drug substance and helps in the elucidation of the structure of the degradation products. Forced degradation studies show the chemical behavior of the molecule which in turn helps in the development of formulation and package. In addition, the regulatory guidance is very general and does not explain the performance of forced degradation studies. Thus, this review discusses the current trends in the performance of forced degradation studies by providing a strategy for conducting studies on degradation mechanisms and also describes the analytical methods helpful for the development of stability indicating method [5]. The objection of this study was to establish degradation pathways of atorvastatin calcium active ingredient or dosage form, to understand the chemical properties of atorvastatin calcium molecules, to establish stability indicating nature of a developed method, to reveal the degradation mechanisms such as hydrolysis, oxidation, thermolysis or photolysis of atorvastatin and to generate more stable formulations.

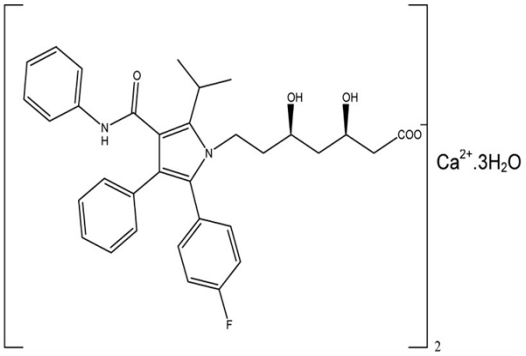

Fig. 1. Chemical structure of atorvastatin calcium

The design of experiments is part of the statistical tools which can be used to facilitate learning of the connections between processing and products, and facilitate improvement activities [4]. Quality improvement is then connected both with a deeper understanding of the product itself but also to the factors defining the process., the role of designed experiments in quality improvement by the use of sequential experimentation. One of the ideas behind sequential experimentation is that the benefits of controlling the conditions and the direction of inquiry outweigh the costs when compared to the strategy of using historical data, so the experimental design was the process of choosing how to run an experiment.

The purpose of DOE is to determine how a response Y depends on one or more input variables or predictors X so that future values of the response can be predicted from the input variables [6,7]. In statistics, a full factorial

experiment is an experiment whose design consists of two or more factors, each with discrete possible values or "levels", and whose experimental units take on all possible combinations of these levels across all such factors.

The aim of this study is to develop a simple, precise and accurate revese phase HPLC methodfor the determination of atorvastatin calcium in pharmaceutical dosafe form as per ICH guidelines [8].

2. MATERIALS SAND METHODS

All chemicals and solvents were analytical and HPLC grade, Atorvastatin calcium was kindly supplied as gift sample by GPT India, purity 99.86%, all chemicals, and reagents used of HPLC were pure from (Merck, chemical India) and tablet dosage form of atorvastatin calcium 20mg manufactured by Micro lab 92, India, excipients BP and USP from Amipharma Lab Sudan, potassium dihydrogen phosphate, methanol, and acetonitrile were obtained from seed fine-Chem Ltd, India.

2.1 Apparatus and Chromatographic Conditions

Samples were analyzed by HPLC consist of SYKAM, pump S11211, solvent delivery system Autosampler S 5200, sample injector loop reading 20 µl and detector, UV-visible S3200. The UV detector was set at 245 nm, others apparatus included pH meter MARTINI 180 Romania, oven Heraeus type VTR5022 Germany, Sartorius analytical balance CPA1245 and UV -1800 SHIMADZU spectrophotometer Japan.

An absorption maximum was detected by scanning standard solution 10 µg/ml of the drug over 200 to 400 nm wavelengths of Shimadzu model 1800 double beam UV-visible spectrometer with a pair of 10 mm matched quartz cells.

The proposed RP-HPLC method utilized a 5 µm particle Kromasil 100 column C18, (250 mm x4.6 mm) at ambient temperature. A 2^3 factorial design consisting of 3 factors at 2 levels was set up to standardize the chromatographic condition.

The mobile phase composed of 10 mM potassium dihydrogen phosphate and acetonitrile in the ratio acetonitrile: Buffer 59:41. The flow rate 1.0 ml/min, detection wavelength 245 nm Injection volume 20 µl, stationary phase column C18, the analysis of ambient temperature.

2.2 Preparation of Standard Solution

A stock solution was prepared by weighing 100 mg of atorvastatin calcium and transferred to 100 ml volumetric, the volumes were made up to the mark with methanol. The above solution was further diluted with mobile phase to obtain a concentration of 6.4, 7.2,8,8,8 and 9.6 µg/ml, respectively, for linearity, system suitability, sensitivity, precision and robustness, all samples dissolved in the mobile phase.

2.3 Preparation of Tablet Solution

Twenty tablets of Amistatin tablet containing 20 mg of atorvastatin calcium was weighed and crushed into a fine powder. The quantity of powder equivalent to the weight of one tablet was accurately weighed and dissolved in sufficient amount of mobile phase in a 100-ml volumetric flask. The solution was sonicated for 15 minutes filtered through 0.22 µm nylon membrane filter, and diluted to 100 ml with mobile phase. Further dilution was made with mobile phase to give a final concentration of atorvastatin (10 µg/ml).

2.4 Acid and Base Degradation

An accurately weighed of 42.97 mg of atorvastatin calcium was transferred into 100 volumetric flasks. 10 ml of methanol was added and sonicated for 15 minutes with intermittent shaking, 5 ml of 0.1 M of HCl or of 0.1 M NaOH was added separately. The sample was heated in boiling water bath for 45 minutes, cool to room temperature and diluted to volume with diluent. The sample was neutralized to pH 7 by adding 0.1 M HCl or 0.1 M NaOH, mixed well. The acidic degradation and the alkaline forced degradation was performed in the dark in order to exclude the possible degradation effect of light. This solution was filtered through the 0.45 um filter, 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to the volume with the mobile phase and injected into the HPLC system.

2.5 Oxidative Degradation

An accurately weighed of 42.97 mg of atorvastatin calcium was transferred into 100 volumetric flasks. 10 ml of methanol was added

and sonicated for 15 minutes with intermittent shaking. 5 ml of 3% H2O2 was added. The sample was heated on a boiling water bath for 45 minutes, cool to room temperature and diluted to volume with the diluent, mixed well. This solution was filtered through the 0.45 um filter, 5 ml of the filtrate was transferred to 25 ml volumetric flask, diluted to the volume with the mobile phase and injected into the HPLC system.

2.6 Thermal Degradation

An Accurately weighed of 42.97 mg of atorvastatin calcium was transferred into 100 volumetric flasks. 10 ml of methanol was added and sonicated for 15 minutes with intermittent shaking. The sample was heated on a boiling water bath for 45 minutes, cool to room temperature and diluted to volume with the diluent, mixed well. This solution was filtered through the 0.45 um filter, 5 ml of the filtrate was transferred to 25 ml volumetric flask, diluted to the volume with the mobile phase and injected into the HPLC system.

3. RESULTS AND DISCUSSION

In this work HPLC method for determination of Atorvastatin calcium in tablets and their degradation products were developed and validated. The optimization of the method was done by selected of suitable solvents such as methanol and acetonitrile, different columns C8, C18, detection wavelength and analyte concentration. The detection wavelength of 245nm was selected after scanning the standard solution over range 200-400 nm by using UV detector.

The traditional approach to HPLC optimization is to perform an experiment by trial and error or by change one control variable at the time; such method can frequently require a very large number of experiments to identify the optimal condition. Recently computer assessed to HPLC separation has addressed the problem using factorial design strategies.

Three factors with two level were applied to predict the retention behavior of Atorvastatin calcium and optimize their isocratic elution using acetonitrile as organic modifier and buffer as mobile phase.

The process of choosing how to run an experiment and measuring the change by manipulation independent factor in X -axis with respect to response in Y-axis this is factorial design.

An eight- run, 3^2 factorial design of three factors at two level was set up to standardize the spectrographic condition which is likely to be employed. Percentage of acetonitrile in the organic phase (X1), a proportion of phosphate buffer pH 3% (X2) and flow rate (X3) as per 3^2 factorial design are represented in the Table1: Factors and their corresponding levels as per 3^2 factorial design. The optimization of the method was done by selected of suitable solvents such as methanol and acetonitrile, different columns C8, C18, detection wavelength and analyte concentration. The detection wavelength of 245 nm was selected after scanning the standard solution over range 200-400 nm by using UV detector.

3.1 Optimization of the Method

3.1.1 Experimental design

In an experiment, we deliberately change one or more process variables (or factors) in order to observe the effect the changes have on one or more response variables. The (statistical) design of experiments (*DOE*) is an efficient procedure for planning experiments so that the data obtained can be analyzed to yield valid and objective.

Table 1. The level factors as per 2^3 factorial design

Factor	Level	
	-1	+1
Acetonitrile %	65	70
Buffer V/V	35	40
Flow rate ml/min	1.0	1.5

-1 denote to the lower value of acetonitrile, buffer and flow rate

+1 denote to the higher value of acetonitrile, buffer and flow rate

3.2 Method Validation [9,10]

3.2.1 Range of linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample. Linearity of detector response for atorvastatin was established by analyzing serial dilutions of a stock solution of the working standard. Six

concentrations such as 6.4, 7.2, 8.0, 8.8 and 9.6 µg/ml for atorvastatin were prepared and analyzed. The linearity graph was plotted.

By applying the statistical software minitab 17, the statistical model for analyzing the data Table 2. The R squire between (x) independent factor and (retention time) dependent factor; was 0. 973. The linear regression equation for retention time at p =0.05 significant level, 95% confidence level indicated the model was fit. From the linear equation, the retention time for experiment was 7.35 min and the predicative value was 7.49 with the error about 1.86%. All the factors which are manipulated showed highly significant as 0.00,0.003 and 0.007 respectively Fig. 2.

Table 2. Chromatographic conditions employed as per 2^3 factorial design

Run	X1	X2	X3	Result			
				Y1	Y2	Y3	Y4
1	65	45	1.3	5.483	1.38	224.51	7403
2	65	40	0.8	7.333	1.3	353.67	10725
3	65	40	1.3	4.583	1.29	223.05	6546
4	65	45	0.8	8.866	1.27	365.00	12958
5	55	45	1.3	6.916	1.2	215.19	9541
6	55	45	0.8	11.100	1.31	354.70	12537
7	55	40	1.3	5.700	1.22	215.78	6480
8	55	40	0.8	9.216	1.17	347.78	11765

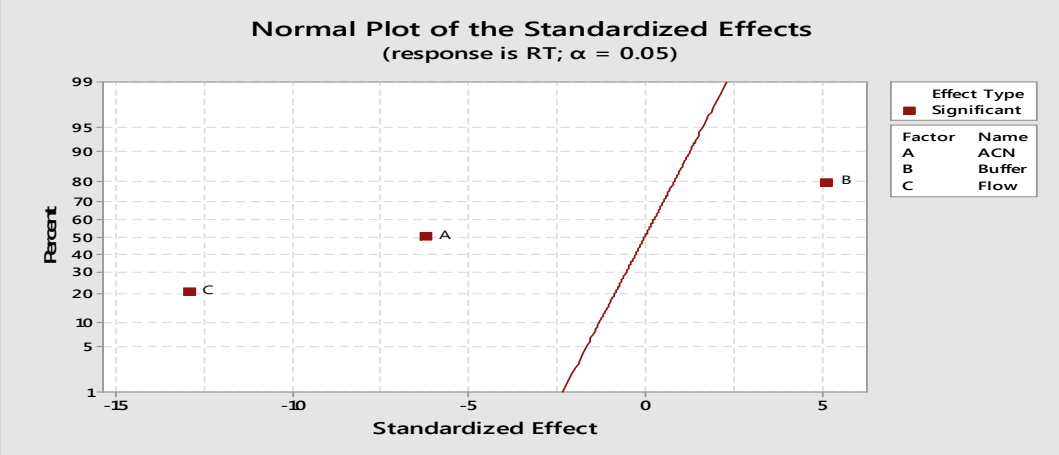

Fig. 2. Response of retention time at p-value 0.05

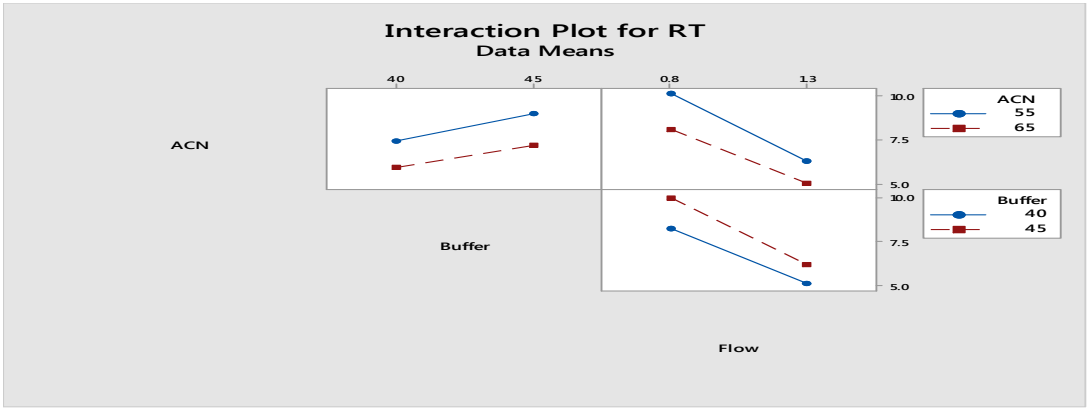

Fig. 3. The interaction of factors at p-value 0.05

Table 3. Linearity of the method at range of 80% to 120%

Method level %	Peak area at 245 nm	Amount found	RSD%
80	181.70	80.57	
80	181.86	80.64	0.65
80	179.70	79.70	
90	203.56	90.26	
90	202.85	89.95	0.40
90	204.46	90.67	
100	225.79	99.99	
100	225.50	99.88	0.12
100	225.25	99.99	
110	248.10	110.00	
110	247.24	109.64	0.46
110	245.26	109.00	
120	268.30	119.00	
120	271.65	120.47	0.83
120	267.41	118.60	

3.3 Accuracy

The accuracy of the method was determined by recovery experiments, known concentrations of working standard was added to the fixed concentration of the pre-analyzed tablet sample. Percent recovery was calculated by comparing the area of the sample, recovery was performed in triplicate.

3.4 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under prescribed conditions. Repeatability of the method was checked by injecting replicate injections of 50 µg/ml of the solution for 6 times on the same day as intra-day precision study of atorvastatin and the chromatogram was recorded. The mean area and % relative standard deviation (RSD) was calculated. From the data obtained.

3.5 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It was observed that the variations like flow rate of the mobile phase, column temperature, ratio of organic content in the mobile phase, etc. Does not have any significant effect on the method performance, which demonstrated that the developed RP-HPLC method is robust.

3.6 Specificity

Spectral purities of atorvastatin chromatography peaks were evaluated for the interference of the tablet excipients, degradation components or due to the presence of impurities as per the methodology. In the work, a solution containing a mixture of the tablet excipients was prepared using the sample preparation procedure to evaluate possible interference peak. The representative chromatogram did not show any other peak, which confirms the specificity of the method.

Atorvastatin calcium was highly stable towards thermal degradation on the other hand when treated with peroxide it showed significant degradation compared to the standard solution of atorvastatin calcium Fig. 6.

Table 4. Results of the recovery test for atorvastatin calcium

Method level %	Actual amount	Recovery%	Bias≤5. 0%	CL≤5. 0%	RSD≤2%
80	80.57	99.30			
80	80.64	99.20	+0.38	99.62±0.58	0.65
80	79.70	100.37			
90	90.26	99.71			
90	89.95	100.05	+0.39	99.67±0.33	0.40
90	90.67	99.26			
100	99.99	100.01			
100	99.88	100.12	-0.04	100.04±0.055	0.12
100	99.99	100.01			
110	110.00	100.00			
110	109.64	100.90	- 0.43	100.43±0.45	0.46
110	109.00	100.40			
120	119.00	100.84			
120	120.47	99.61	-0.54	100.54±0.79	0.83
120	118.6	101.19			

Table 5. Repeatability of the validated method

Sample no	Response peak area at 245 nm	Retention time
1	241.9610	7.383
2	239.1005	7.316
3	241.4260	7.330
4	239.6900	7.350
5	243.4875	7.366
6	243.5025	7.383
Mean	241.530	7.354
RSD	0.760%	0.377%
Limit	NMT2%	NMT1%

Specificity experiment shows that there is no interference of excipients with the main peaks, which confirmed the specificity of the method. Atorvastatin calcium acceptable to acid hydrolysis Fig. 8, but more stable in alkaline medium Fig. 7.

Table 6. Reproducibility of the validated method

Run	Retention time	Peak area at 245 nm
1	7.400	247.6545
2	7.416	245.7960
3	7.416	247.4660
4	7.400	244.440
5	7.383	243.833
6	7.366	243.9330
Mean	7.3968	245.520
RSD	0.263%	0.70%
Limit	NMT1%	NMT2%

Table 7. Intermediate precision analyst to analyst of the validated method

Analyst	Peak area response	%Recovery	RSD (≤2.0%)	% Bias (≤5.0%)
Analyst X	231.6170	99.51		-0.49
	229.8520	100.28	0.42	+0.28
	230.0190	100.20		[+0.20
Analyst Y	232.1620	100.11		+0.11
	231.8825	100.23	0.30	+0.23
	233.2250	99.66		-0.34
Analyst Z	228.4560	99.91		-0.09
	227.5820	100.30	0.26	+0.30
	228.7485	99.80		-0.20

Table 8. Robustness change of flow rate

Flow rate (ml/min)			Peak area	RSD%	tR	% RSD	Bias%
Original	Used	Level					
			257.2365		7.733		
	0.9	-0.1	257.2280	0.239	7.816	0.615	-0.14
			258.300		7.816		
1.0			222.9185		6.950		
	1.0	0	223.8030	1.33	7.00	0.364	0.00
			218.2730		6.983		
			211.7200		6.350		
	1.10	+0.1	211.6500	0.160	6.333	0.268	-0.53
			211.0940		6.316		

Table 9. Robustness change of mobile phase composition

Change of pH of mobil phase			Mean peak area	RSD%	Mean tR	% RSD	% Bias
Original	Used	Level					
	2.8	-0.2	199.53	1.06	6.666	0.75	-0.048
PH 3.0	3.0	0.0	227.355	0.33	6.860	0.56	-0.073
	3.2	+0.2	225.610	0.24	7.155	0.26	0.08

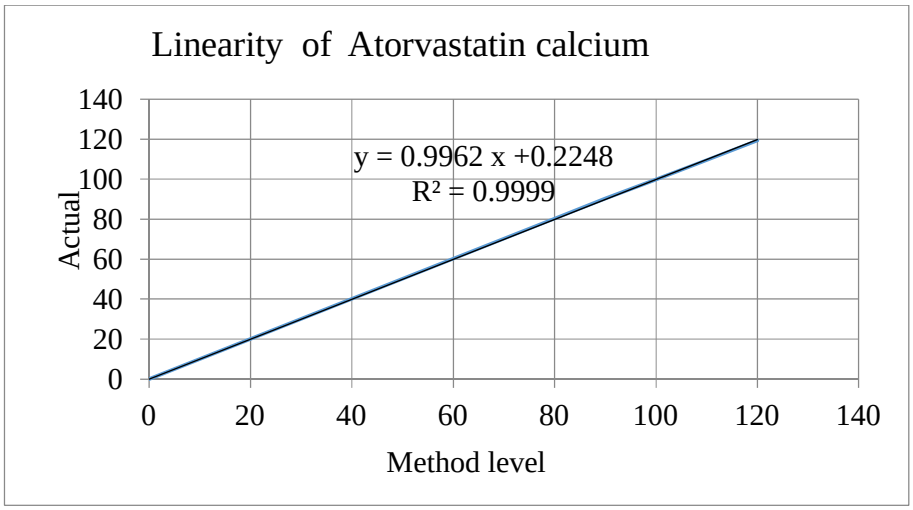

Fig. 4. Calibration curve of atorvastatin calcium

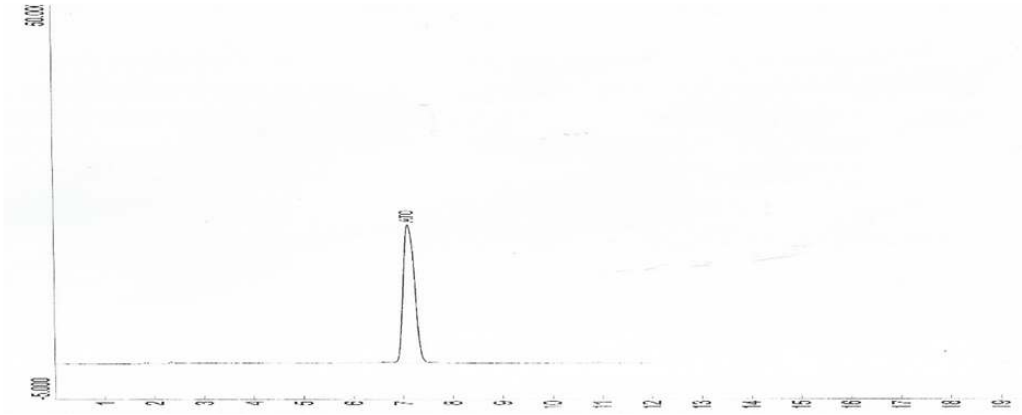

Fig. 5. HPLC Chromatogram of atorvastatin standard

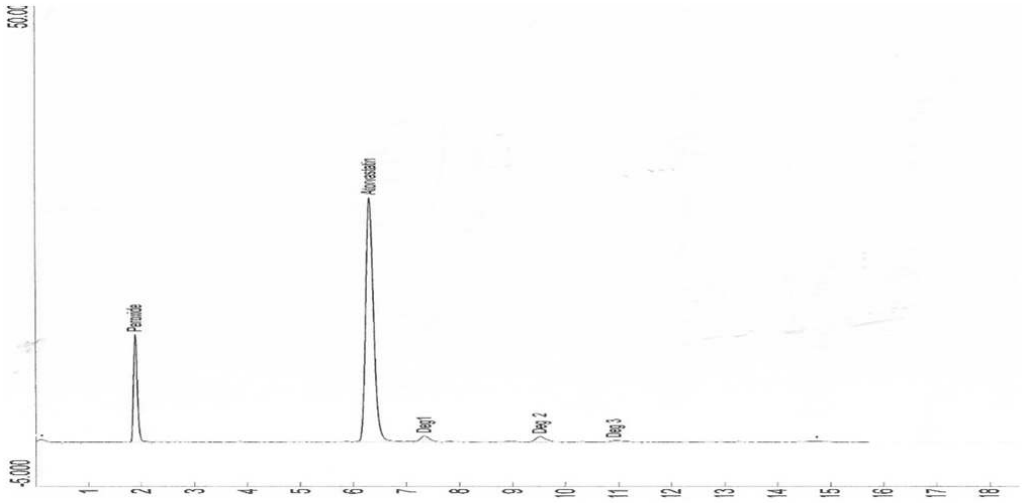

Fig. 6. HPLC Chromatogram of peroxide effect on atorvastatin calcium

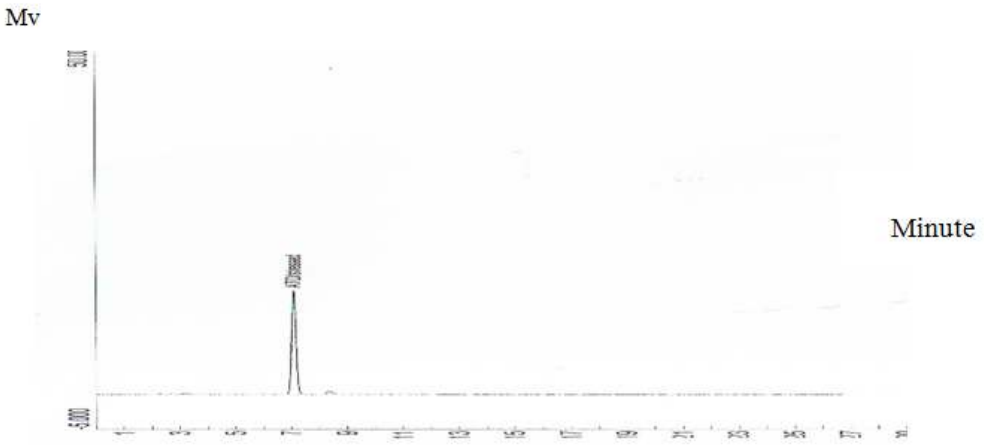

Fig. 7. Chromatogram of hydrolysis of atorvastatin calcium in 0.1 M Sodium hydroxide

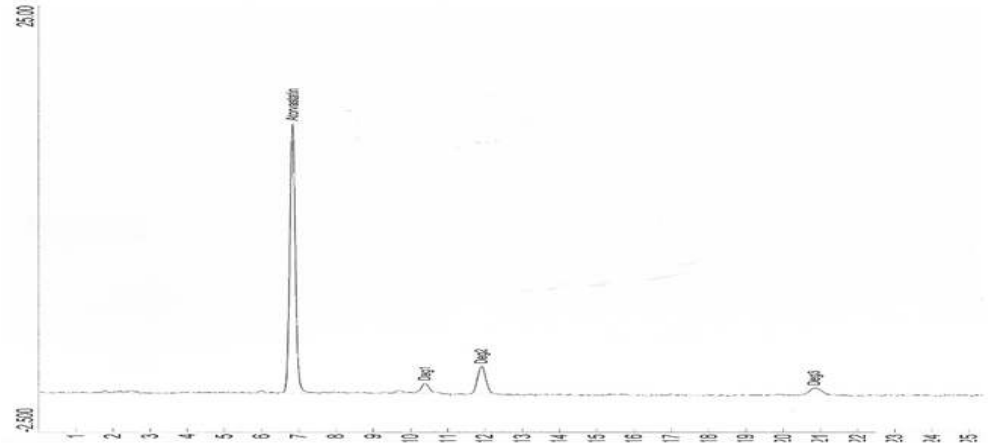

Fig. 8. HPLC Chromatogram of acid hydrolysis of atorvastatin calcium

Table 10. Assay of atorvastatin calcium tablet 20 mg

Drug	n	Amount claimed (mg/tablet)	The amount found (mg/tablet)	Recovery	RSD
Atorvastatin	3	20	19.98	99.64	0.56

3.7 Detection and Quantitation Limit

The limit of detection (LOD) and limit of quantitation (LOQ) of atorvastatin was determined by using the signal to noise ratio approach as defined in ICH guidelines. According to the determined signal to noise ratio, the LOD and LOQ for atorvastatin was 0.399 µg/ml and 1.21 µg/ml, respectively.

The lowest values of LOD and LOQ, which obtained by the method, indicate the sensitivity of the method.

3.8 Assay of Tablet

The proposed method which used to determination atorvastatin calcium in tablet dosage form was reproducible, reliable and are in good agreement with a label claim of the drug, thus this method can be useful for routine work to determine of atorvastatin calcium in a dosage form.

Three different batches atorvastatin calcium 20 mg was analyzed using the validated method for the analysis, three replicate of each batch were assayed. The mean peak area of the drug was

calculated, and the drug content in the tablet was quantified. The result found was comparable with corresponding labelled amounts Table 10.

The proposed new method was precise and accurate, easy and can be used for stability indicating method to determine the degradation products. Stress testing of the assay results of the pharmaceutical formulation of this method has highly improved that atorvastatin acceptable to acid medium so that in formulation we should use alkaline excipient and the pH of the dosage form should be greater than 6.

4. CONCLUSION

The developed HPLC was conducted by implementing factorial design 2^3, this technique is precise, specific, accurate and stability indicating. Statistical analysis proves that the method is reproducible and selective for the analysis of atorvastatin calcium in pharmaceutical dosage form. The method can be used to estimation the purity of the drug available from various sources. The proposed method potentially separate the drug from its degradation products, it can be employed as stability indicating method.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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