International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 6, 2016

Original Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC FOR DETERMINATION OF ATORVASTATIN CALCIUM and EZETIMIBE IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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Received: 26 Nov 2015 Revised and Accepted: 21 Apr 2016

ABSTRACT

Objective: A simple, specific, sensitive, accurate and precise stability indicating methods were described for the quantitative determination of the lipid-lowering agent drug Atorvastatin calcium and Ezetimibe.

Methods: The method was high performance liquid chromatographic with the use of a reversed phase Grace C-18 column (250 mm x 4.66 mm, i.d. 5 µm) and a mobile phase of buffer: acetonitrile (60:40 v/v) at a flow rate of 1.0 ml/min.

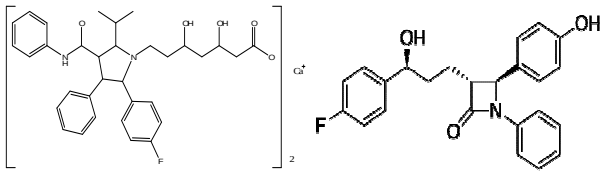
Results: The retention time of drug was found to be 6.91 min. and 10.31 min, respectively. Quantification of the drug was achieved with UV detection at 240 nm. Linear calibration curve was obtained in concentration range 2–12 µg/ml for both drugs, with r^2value of 0.9992 and 0.9990. The limit of detection and limit of quantification were found to be 0.81 µg/ml and 2.47 µg/ml respectively for Atorvastatin calcium and 0.76 µg/ml and 2.31 µg/ml respectively for Ezetimibe.

Conclusion: The developed methods were successfully validated as per International Conference on Harmonization guideline (ICH). Atorvastatin calcium and Ezetimibe was subjected to different stress conditions. Stress samples were successfully assayed by developed high performance liquid chromatographic and high-performance thin layer liquid chromatographic method. Statistically, analysis proves that there were no statistically significant differences between two developed methods.

Keywords: RP-HPLC, Atorvastatin calcium, Ezetimibe, Stability indicating, Validation

INTRODUCTION

Atorvastatin calcium (ATO) chemically is Atorvastatin calcium ([*R*-(*R**,*R**)]-(2-(4-fluoro-phenyl)-β,δ-dihydro-xy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1*H*-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate) is a synthetic lipid-lowering agent. It is a selective competitive inhibitor of the enzyme HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to mevalonate, an important rate-limiting step in cholesterol biosynthesis. It lowers serum cholesterol in patients with homozygous familial hyper-cholesterolemia [1]. Ezetimibe (EZE) (1-(4-fluorophenyl)-3(*R*)-[3-(4-fluorophenyl)-3(*S*)-hydroxylpropyl]-4(*S*)-(4 hydroxyphenyl)-2-azetidinone) is another lipid-lowering agent [1].



(a) (b)

Fig. 1: Chemical structure of atorvastatin calcium (a) and Ezetimibe (b)

After extensive literature survey, several methods have been found for determination of Atorvastatin calcium and Ezetimibe including determination in pure form or in combination with other drugs. Determinations of Atorvastatin calcium with Telmisartan by spectrophotometric method, several chromatographic methods have been reported including HPLC [2-5]. The electrochemical methods have been reported for the determination of Atorvastatin calcium based on square-wave voltammetry [6-9]. As per reported literature no stability indicating HPLC were available for determination of atorvastatin calcium and Ezetimibe, so it was thought worthwhile to develop a stability indicating HPLC for determination of Atorvastatin Calcium and Ezetimibe from bulk. Stability testing involves forced degradation or stress studies indicating hydrolysis, oxidation, photolytic and thermal degradation, etc. Stability indicating methods are developed to supervise the stability of drug substance and pharmaceutical dosage forms for the duration of the early phase of medicine development, and once the medicine is entered to the marketed, for the continuing product stability studies which must be performed as per ICH or regulatory guidelines.

The reason of stability studies testing is to give evidence on how the quality of drug differs with moment under the influence of a multiplicity of ecological factors such as humidity, temperature, and light, enables suggested storage conditions, re-analysis intervals and shelf life to be recognized. Once the analytical method was developed it is to be validated according to the regulatory guideline. In the present study both the developed methods were validated as per ICH guideline and were used for estimation of Atorvastatin Calcium and Ezetimibe under stressed conditions. Both the developed methods successfully quantify the Atorvastatin Calcium and Ezetimibe in the presence of degradant product without any interference.

MATERIALS AND METHODS

Reagents and instruments

Atorvastatin calcium and EZETIMIBE was obtained from Gen international pvt. Ltd. as a gift sample. All chemicals and reagents such as methanol, ammonium dihydrogen phosphate, hydrochloric acid, sodium hydroxide, and hydrogen peroxide solution used were of HPLC grade and were purchased from Merck Chemicals, India.

HPLC instrumentation and chromatographic conditions

The method was developed using Shimadzu HPLC-2010 instrument

equipped with UV detector. The Grace C-18 column (250 mm x 4.66 mm, i.d. 5 µm) was used as stationary phase. The mobile phase consisted of Buffer: Acetonitrile (60:40 v/v) pH 5.0 with acetic acid and was pumped at a flow rate of 1.0 ml/min. The mobile phase was filtered through a membrane filter of 0.45µ membrane filter. The elution was monitored at 240 nm and the injection volume was 20 µl.

Preparation of atorvastatin calcium (100µg/ml) and ezetimibe (100µg/ml) standard stock solution

Accurately weighed 10 mg Atorvastatin Calcium was transferred to 100 ml volumetric flask dissolved in methanol and add distilled water and sonicate up to 10 minutes. The volume was adjusted to the mark to give final strength i.e. 100µg/ml.

Accurately weighed 10 mg Ezetimibe was transferred to 100 ml volumetric flask dissolved in methanol and add distilled water and sonicate up to 10 minutes. The volume was adjusted to the mark to give final strength i. e 100µg/ml. A standard typical chromatogram of Atorvastatin Calcium and Ezetimibe (fig. 2).

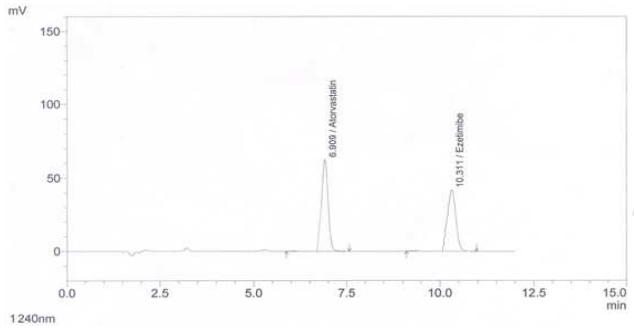


Fig. 2: A typical standard chromatogram of atorvastatin calcium and ezetimibe

Method validation [10]

Precision

Repeatability, intra-day and inter-day precision studies were carried out by estimating corresponding responses three times on the same day and three times on different days for one concentration of Atorvastatin Calcium and Ezetimibe (10 µg/ml for HPLC) and results are reported in terms of % relative standard deviation. table 1 and 2.

Table 1: Data of precision of atorvastatin calcium by HPLC

Precision	Concentration (µg/ml)	S. D	% RSD
Repeatability	10	0.05753	0.5819
Intermediate	10	0.05785	0.5860
Reproducibility	10	0.05753	0.5860

SD-Standard deviation RSD-Relative standard deviation

Table 2: Data of precision of ezetimibe by HPLC

Precision	Concentration (µg/ml)	S. D	% RSD
Repeatability	10	0.04226	0.4282
Intermediate	10	0.05244	0.5299
Reproducibility	10	0.04326	0.4382

SD-Standard deviation RSD-Relative standard deviation

Accuracy

The accuracy of HPLC methods was determined by recovery study carried out at three different concentrations (80%, 100%, and 120% test solution concentration). For each concentration, three sets were prepared, and % recovery was calculated table 3.

Linearity

Calibration curve was constructed by plotting the peak area v/s concentration of Atorvastatin Calcium and Ezetimibe and regression equations were calculated for both the methods. The linearity of HPLC and the linearity for HPLC were found to be 2–12 µg/ml table 4.

Limit of detection and limit of quantification

LOD and LOQ for both the developed methods were calculated using following Eq. (1) as per ICH guideline

LOD= 3.3 X S. D/S and LOQ = 10 X S. D/S (1)

Where, SD the standard deviation of the precision and S is the slope of the calibration curve.

Table 3: Data of recovery study of atorvastatin calcium and Ezetimibe by HPLC

Recovery level (%)	Conc. of test sol. (µg/ml)		Conc. of std. sol. (µg/ml)		% Recovery		% RSD	
	ATO	EZE	ATO	EZE	ATO	EZE	ATO	EZE
80	10	10	8	8	98.26	98.49	0.7607	0.7216
100	10	10	10	10	100.54	100.69	0.8520	0.7326
120	10	10	12	12	99.26	98.55	0.7490	0.7357

ATO-Atorvastatin calcium EZE-ezetimibe

Table 4: Linearity data of atorvastatin calcium and ezetimibe by HPLC

S. No.	Concentration for HPLC (µg/ml)		Area for HPLC	
	ATO	EZE	ATO	EZE
1	2	2	10224	11062
2	4	4	21131	20132
3	6	6	30123	31082
4	8	8	41243	39216
5	10	10	50143	50164
6	12	12	61042	59234

ATO-Atorvastatin calcium EZE-Ezetimibe

Robustness

The robustness of HPLC method was studied by changing mobile phase composition (±1%), pH of mobile phase (±1), flow rate (±0.2 ml/min) and working wavelength (±2 nm). The robustness of HPTLC was studied by changing mobile phase composition (±1%), saturation time (±5 min) and wavelength (±2 nm). Robustness of both the developed methods was calculated in terms of % RSD table 5.

Table 5: Robustness parameter for HPLC method

Parameter	Normal condition		Variable 1		Variable 2	
Wavelength	**240 nm**		**238 nm**		**242 nm**	
	ATO	**EZE**	**ATO**	**EZE**	**ATO**	**EZE**
Area	926821	834564	937402	833561	934248	834648
	938786	841153	928201	824240	934851	824860
	917742	824551	926402	822130	921213	842130
Average	938786	833422	929768	826643	930104	833879
SD	40956.0	8359.6	4366.3	6082.7	7705.7	8660.6
%RSD	1.36	1.00	0.46	0.73	0.83	1.04
Flow rate	1.0 ml/min.		0.8 ml/min.		1.2 ml/min.	
	ATO	EZE	ATO	EZE	ATO	EZE
Area	921545	838551	924140	826450	938082	824551
	938554	843655	933148	838552	921810	834564
	919852	826451	933842	843655	921416	841153
Average	926650	833422	930376	833422	927102.7	833422
SD	10343.5	8359.6	5412.2	8359.6	9510.4	8359.4
%RSD	1.11	1.00	0.58	1.00	1.02	1.00
pH	5.0		4.0		6.0	
	ATO	EZE	ATO	EZE	ATO	EZE
Area	927072	826742	921545	841254	932350	836471
	924640	838421	919852	831820	913242	847363
	923542	833440	938554	834224	901106	848252
Average	925084	832867	926650	835766	915566	844028
SD	1806.5	5860.4	10343.5	4902.38	15751.1	6560.2
%RSD	0.19	0.70	1.11	0.58	1.72	0.77

ATO-Atorvastatin calcium EZE-Ezetimibe

Specificity

Specificity of the developed methods was checked by recording chromatogram of placebo and was compared with chromate gram of Atorvastatin Calcium and Ezetimibe. Specificity of both the developed methods was further studied by conducting the forced degradation studies, including acid hydrolysis, alkaline hydrolysis, photodegradation and thermal degradation. In all tested conditions, interference of degradation product was determined.

System suitability test

Analytical system performance before and/or during the analysis was evaluated by system suitability test. System suitability tests are an integral part of method development and are performed to evaluate the behavior of the chromatographic system such as capacity factor (k), plate number (N) and tailing factor (T).

Solution stability

Stability of sample solution was studied at ambient temperature for 24 h.

Forced degradation studies [11]

Acid degradation studies

10 mg of Atorvastatin Calcium and Ezetimibe was dissolved in 10 ml of 0.1 N HCl and kept for 6 h at room temperature. From the above solution, 1 ml was withdrawn and neutralized with 0.1 N NaOH and diluted to 10 ml methanol. The solution was analyzed by proposed HPLC and HPTLC method fig. 3(a).

Alkali degradation studies

10 mg of Atorvastatin Calcium and Ezetimibe was separately dissolved in 10 ml of 0.1 N NaOH and kept for 6 h at room temperature. From the above solution, 1 ml was withdrawn and neutralized with 0.1 N HCl and diluted to 10 ml methanol. The prepared solution was analyzed by HPLC and HPTLC method fig. 3(b).

Oxidative degradation studies

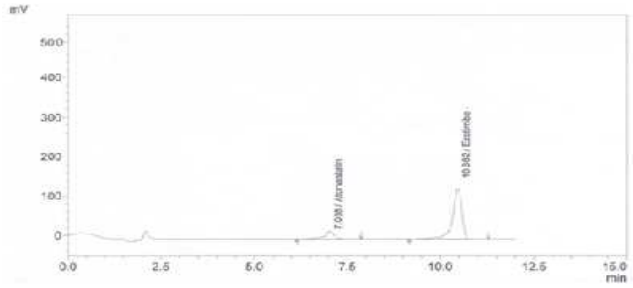
10 mg of each drug was dissolved in 10 ml of 10% H_2O_2 in 10 ml volumetric flask. This solution was kept for 6 h at room temperature. From the above solution, 1 ml was withdrawn and makeup with methanol up to 10 ml. The prepared solution was analyzed by HPLC and HPTLC method fig. 3(c).

Thermal degradation studies

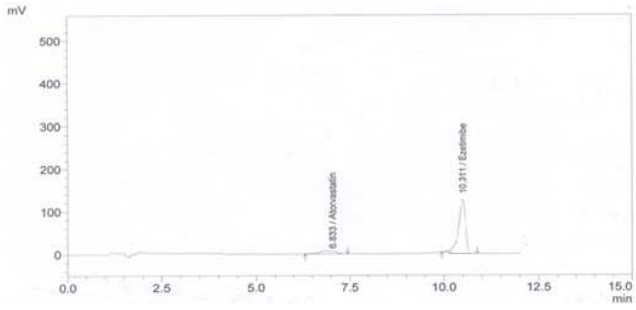
For thermal decomposition drug powder was kept at 60 °C for 6 h. From that powder solution having a concentration of 100 µg/ml was prepared and analyzed for thermal degradation study. The prepared solutions were analyzed by HPLC and HPTLC method fig. 3(d).

Photo degradation studies

A sample of the drug was exposed to a near ultraviolet lamp in a UV Chamber. The drug was kept in a petri dish for 24 h and solution having a concentration of 100 µg/ml was prepared and analyzed for photolytic (UV Light) degradation study. The prepared solution was analyzed by HPLC and HPTLC method fig. 3(e).



[A]



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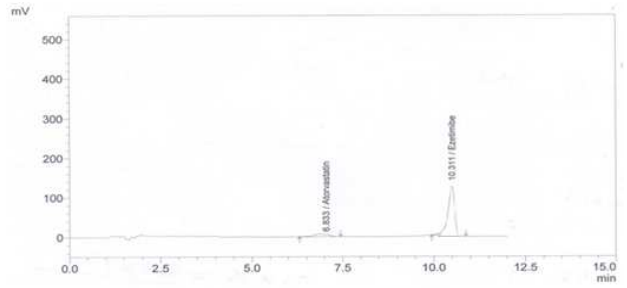

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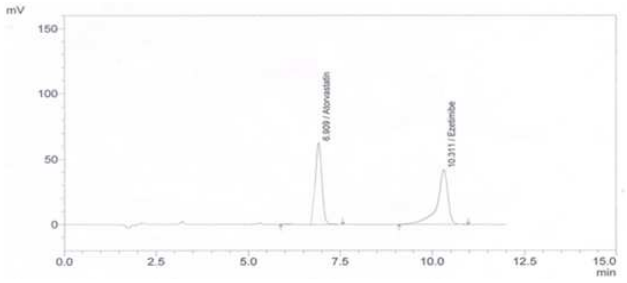

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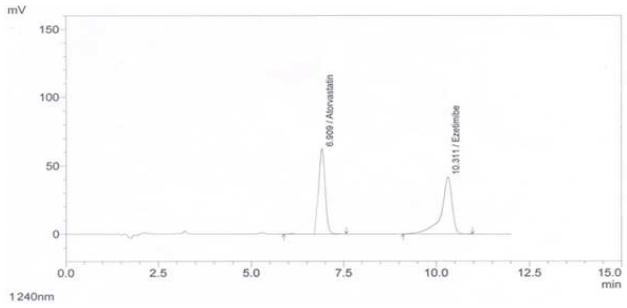

[E]

Fig. 3: Representative chromatograms of Acid (A), Base (B), Peroxide (C), Thermal (D), Photolytic (E) degradation of atorvastatin calcium and ezetimibe

RESULT AND DISCUSSION

Method development and optimization

Prime objective of development and validation of HPLC method for determination of Atorvastatin Calcium and Ezetimibe was run and should be accurate, precise, reproducible, robust and stability indicating. All degradation products from stress conditions should be well separated from each other and method should be simple to useful for routine analytical work. Stability-indicating methods demonstrate the capability of the method for the accurate determination of active ingredients without interference from possible degradation products, process impurities, excipients or other potential impurities.

For the developed methods mobile phase selected with a view to best sensitivity and selectivity along with short elution time. In HPLC method the combination of Buffer: Acetonitrile (60:40 v/v) resulted in a high sensitivity, short analysis time and good peak symmetry. Among the different columns Grace C-18 column (250 mm x 4.66 mm, i.d. 5 µm) analytical column was selected, as it provided the best chromatographic separation and good peak characteristics. The detection was carried out with UV detector at 240 nm, indicated the good resolution of Atorvastatin Calcium and Ezetimibe from its degradant. Detection was carried out at 240 nm. The retention factor was observed 0.24 and 0.36. Both the developed methods were validated according to ICH guideline.

Method validation

Specificity of the analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of sample matrix. In the present study, the ability of the methods to separate the drug from its degradation products without the interference of other sample components indicated the specificity of the developed methods. Values of the peak purity index were found to be higher than 0.9999 indicated that the proposed methods are specific.

The precision, for both the developed methods, was evaluated as repeatability and calculating the % RSD. The RSD values of repeatability study were found to be<2%, indicated that the proposed methods are repeatable. The between-analysts precisions of developed methods were determined by calculating the % RSD for the analysis of one sample of the NIC by two different analysts. % RSD was found to be 1.075% for HPLC. The % RSD of intermediate precision for both the developed methods was found to be<2%, which indicated that the proposed methods are reproducible (table 1and 2). The accuracy of HPLC was assessed by the standard addition method. Three replicate determinations were performed at three different levels. The recoveries were obtained in a range of 100.00–101.56% and 99.36–100.41% for HPLC. (Tables 5) The high values indicated that the proposed HPLC method was accurate. (table 3).

The linearity of a method reveals the linear relationship of response against the selected concentration of the analyte. For HPLC method, a linear correlation was obtained between peak areas and concentrations of Atorvastatin Calcium in the range of 2-12µg/ml. The following regression equation was found by plotting the peak area (y) versus the NIC concentration (x) expressed in µg/ml: y = 0.020x+0.005. The correlation coefficient (r^2: 1) obtained for the regression line demonstrates the excellent relationship between peak area and concentration of Atorvastatin Calcium. (table 4).

The LOD and LOQ were determined from slopes of linear regression curves. LOD and LOQ for HPLC method were found to be 0.81 µg/ml and 2.47 µg/ml and for Atorvastatin Calcium and 0.76 µg/ml and 2.31 µg/ml for Ezetimibe respectively.

Stability indicating properties of HPLC method was performed by forced degradations study. The results of stress testing indicated that the developed methods were highly specific in nature. The study data revealed that selected drug was unstable in the acidic, basic and oxidative medium. In HPLC method, acidic stress led to 65.85% for Atorvastatin Calcium and 16.11% for Ezetimibe degradation. Alkaline stress led to 80.15% for Atorvastatin Calcium and 10.65% for Ezetimibe degradation. Peroxide stress led to 31.46 for Atorvastatin Calcium and 11.88% for degradation. The forced degradation studies by thermal found 3.69% for Atorvastatin Calcium and 2.37% for Ezetimibe. Photolytic degradation found 5.81 for Atorvastatin Calcium and 1.10% for Ezetimibe. (table 5). There were no significant changes were observed in the chromatographic pattern when the modifications were made in the experimental conditions, indicated that method was robust.

Table 5: Degradation atorvastatin calcium and Ezetimibe at different stress conditions at 6 hr by HPLC

Stress condition	Standard conc.		% Area	
	ATO (µg/ml)	EZE(µg/ml)	ATO	EZE
0.1N HCL	100	100	65.85	59.89
0.1N NaOH	100	100	31.49	81.58
6% H2O2	100	100	3.69	35.08
Thermal	100	100	3.21	2.40
UV(240 nm)	100	100	5.81	3.64

ATO-Atorvastatin calcium EZE-Ezetimibe

CONCLUSION

This study presents simple and validated stability indicating RP-HPLC method for estimation of Atorvastatin Calcium and Ezetimibe in the presence of degradation products. The developed methods are sensitive, specific, rapid, robust, precise and accurate. All the degradation products were well separated from the analyte peak demonstrating that the developed methods were specific and stability indicating. Statistically, analysis proves that there were no statistical significant differences in between two developed methods. Developed methods can be used as a quality-control tool for routine quantitative analysis of atorvastatin calcium and Ezetimibe.

CONFLICT OF INTERESTS

Declared none

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