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Simultaneous Determination of Atorvastatin Calcium and Ezetimibe in Pharmaceutical Formulations by Liquid Chromatography

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ABSTRACT

A simple, precise and sensitive reverse-phase high performance liquid chromatographic (RP-HPLC) method has been developed for the quantitation of atorvastatin calcium simultaneously with ezetimibe in pharmaceutical formulations. Chromatographic separation was achieved on a 250 × 4.6 mm, 5μ Hypersil® phenyl-2 column. Eluent was monitored by absorbance at 242 nm using a mixture of 0.1 M ammonium acetate (pH 6.5) and acetonitrile in the ratio of 28:72 (v/v). Calibration plots were linear in the concentration range of 12-52 μg mL^{-1} for both atorvastatin calcium and ezetimibe with correlation coefficient (R2) between 0.9966 and 0.9993. The total run time is less than 5 min. The proposed method was validated by testing its linearity, recovery, selectivity, repeatability and LOD/LOQ values and it was successfully employed for the determination of atorvastatin calcium and ezetimibe in pharmaceutical tablet formulations.

Key words: HPLC, acetonitrile, isocratic, atorvastatin calcium, ezetimibe

INTRODUCTION

Atorvastatin calcium (Figure 1) is the calcium salt (2:1) trihydrate of [R-(R*,R*)]-2-(4-fluorophenyl)-b,d-dihydroxy-5-(1-methylethyl)-3-phenyl-4[(phenylamino)carbonyl]-lH-pyrrole-1-heptanoic acid. It is a synthetic liquid-reducing agent and an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. Atorvastatin is the most efficacious of the currently available HMG-CoA reductase inhibitors in terms of lowering plasma cholesterol levels by suppressing the hepatic production of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol[(1)]. A number of methods have been developed for the analysis of this drug, which include liquid chromatography tandem mass spectrometry[(2-4)], HPLC with gradient elution[(5)] and isocratic HPLC methods for the detection of the drug in biofluids[(6-7)].

Ezetimibe (Figure 2), a selective inhibitor of intestinal cholesterol and related phytosterol absorption, is designated as 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone. Ezetimibe selectively prevents the absorption of cholesterol from dietary and biliary sources by blocking the transport of cholesterol through the intestinal wall. This reduces the overall delivery of cholesterol to the liver, thereby promoting the synthesis of LDL receptors and the subsequent reduction in serum LDL-C[(8-9)]. Few HPLC methods for the determination of ezetimibe were reported in literatures[(10-11)]. Although the combinational use of atorvastatin and ezetimibe is continuously increas-

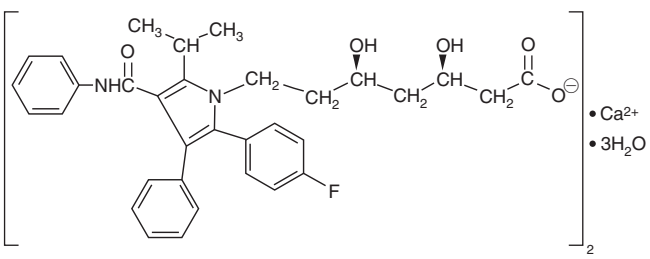

Figure 1. Chemical structure of atorvastatin calcium.

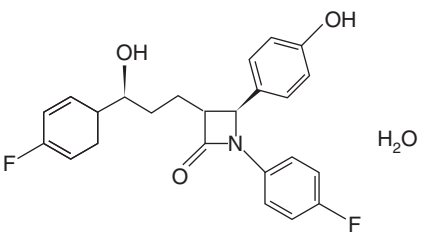

Figure 2. Chemical structure of ezetimibe.

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ing, simultaneous analysis of these two components in their pharmaceutical preparation is not reported in literature. There is an urgent need to develop validated analytical methods for the simultaneous analysis of atorvastatin calcium and ezetimibe in pharmaceutical dosage forms.

We describe herein a simple, sensitive and validated HPLC method utilizing isocratic mobile phase with short retention time for the simultaneous determination of these two components in tablets. The developed method can be successfully applied to quality control and other analytical purposes.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Standard atorvastatin calcium and ezetimibe were acquired from Schazoo Laboratories, Lahore (Pakistan), while pharmaceuticals containing atorvastatin calcium and ezetimibe, Zetab Plus, from the same laboratory were used in the experiments. Zetab Plus tablets were claimed to contain 10 mg each of atorvastatin (base) and ezetimibe. Ammonium acetate and acetonitrile were of HPLC grade (Merck), glacial acetic acid was of reagent grade and de-ionized water was used.

II. *Apparatus and Chromatographic Conditions*

HPLC analysis were performed on Varian Prostar system equipped with a model Prostar 210 solvent delivery module, a Varian prostar 320 variable wavelength detector with class Varian prostar software and a Rheodyne injection valve with a loop of 20 µL. Separations were carried on a Hypersil phenyl-2 column (250 × 4.6 mm, i.d., 5 µm particle size) using isocratic elution. The flow rate was 0.5 mL min^{-1}. UV detection was performed at 242 nm. Peak identity was confirmed by retention time comparison and the HPLC was operated at room temperature.

III. *Preparation of Mobile Phase*

The mobile phase is composed of a mixture of 0.1 M-ammonium acetate (pH 6.5) and acetonitrile in the ratio of 28:72 (v/v). The pH of the mixture was adjusted to 6.5 with 1% glacial acetic acid, filtered through a 0.45 µm nylon filter (Millipore, USA) and degassed by sonication prior to use.

IV. *Preparation of Standard Solution*

The standard stock solution of atorvastatin calcium and ezetimibe (0.2 mg mL^{-1} each) was prepared in methanol since both drugs are soluble in this solvent. The working standard solution (32 µg mL^{-1} for both) was prepared by diluting the stock solution in mobile phase solution.

V. *Preparation of Sample Solution*

Twenty tablets were weighed to get the average weight and then ground. An amount of powder equivalent to 20 mg each of atorvastatin calcium and ezetimibe was transferred to a 100-mL volumetric flask with 70-mL methanol and shaken for 5 min, followed by making up to volume with methanol to obtain a solution containing 0.2 mg mL^{-1} atorvastatin calcium and 0.2 mg mL^{-1} ezetimibe. The solution was filtered manually using Whatmann No. 41 filter paper and a glass funnel. After filtration, the solutions were diluted with mobile phase to give a final concentration of 32 µg mL^{-1}each.

VI. *Linearity*

Linearity of the proposed method was verified by analyzing five solutions in the range of 12-52 µg mL^{-1} for both atorvastatin calcium and ezetimibe (12, 22, 32, 42 and 52 µg mL^{-1}). Each concentration was made in triplicate.

VII. *Accuracy*

The accuracy of the method was performed by adding known amounts of atorvastatin calcium and ezetimibe to the sample solution. The actual and measured concentrations were then compared. Sample solution of atorvastatin calcium and ezetimibe (0.2 mg mL^{-1}each) was prepared as described above and aliquots of 3 mL were transferred to 50-mL volumetric flasks containing 1.0, 2.0, 3.0 and 4.0 mL of atorvastatin calcium and ezetimibe standard solution (0.2 mg mL^{-1} each). Mobile phase was then added to make up the volume, giving final concentration of 16.0, 20.0, 24.0 and 28.0 µg mL^{-1}. Each level was made in triplicate.

VIII. *Selectivity*

Twenty milligrams each of atorvastatin calcium and ezetimibe and 30 mg each of starch, lactose, magnesium stearate and avicel, that represent interfering substances and may be present with atorvastatin calcium and ezetimibe in their pharmaceutical formulations, were accurately weighed and transferred into a 100-mL volumetric flask. The mixture was dissolved well by shaking with 70-mL methanol, the volume was completed with methanol and the mixture was filtered. Four milliliter of this filtrate was transferred into a 25-mL volumetric flask and the mobile phase was added to volume to give a final concentration of 32 µg mL^{-1} each.

RESULTS AND DISCUSSION

I. *Method Optimization*

In this work, an isocratic, simple, accurate and

sensitive HPLC method suitable for the simultaneous determination of atorvastatin calcium and ezetimibe in tablets has been developed. Initially various mobile phases and stationery phases were tested in an attempt to obtain the best resolution for atorvastatin calcium and ezetimibe. The mobile phase consisting of 0.1 M ammonium acetate (pH 6.5) and acetonitrile in the ratio of 28:72 (v/v) was found to be the most appropriate allowing adequate separation of both the components using a Hypersil Phenyl-2 column at a flow rate of 0.5 mL min^{-1}. Using the chromatographic conditions mentioned above, resolved sharp peaks corresponding to atorvastatin calcium and ezetimibe can be obtained at retention time of 3.06 and 4.46 min respectively, as shown in Figures 3 and 4.

Method development began with less polar mobile phase (50% acetonitrile); however, no peak could be obtained. The polarity of the mobile phase was increased by the addition of 0.1 M ammonium acetate. A ratio of 28:72 (v/v) for ammonium acetate and acetonitrile affected good separation and symmetric peaks. The optimum mobile phase composition was found to be 0.1 M ammonium acetate (pH 6.5) and acetonitrile in the ratio of 28:72 (v/v).

II. *Method Validation*

The proposed HPLC assay for simultaneous determination of atorvastatin calcium and ezetimibe was validated for the linearity, limit of detection/quantitation, selectivity, accuracy and repeatability.

(I) *Linearity*

The linearity of the method was evaluated by processing five-point calibration curves. Good linearity was observed over the concentration range of 12-52 μg mL^{-1} for both atorvastatin calcium and ezetimibe. The peak areas versus concentrations of drugs were plotted and a linear least-square regression analysis was conducted to determine the slope, intercept and coefficient of determination (R^2) to demonstrate the linearity of the method. The goodness of fit (R^2) in all cases was found to be > 0.9966 indicating functional linear relationship between concentrations of analyte and the areas under peak. The linear regression analysis data are summarized in Table 1.

(II) *Limit of Detection and Limit of Quantitation*

Two types of solutions, i.e. blank and spiked with known concentrations of each analyte, were prepared and analysed. The limit of detection (LOD) and quantification (LOQ) were then established by evaluating the signal to noise ratio of 3:1 and 10:1 respectively. The LOD and LOQ values are given in Table 1.

(III) *Accuracy*

To study the accuracy of the proposed method, synthetic mixtures containing various amounts of atorvastatin calcium and ezetimibe (16.0, 20.0, 24.0 and 28.0 μg mL^{-1} each) were prepared and analyzed by the

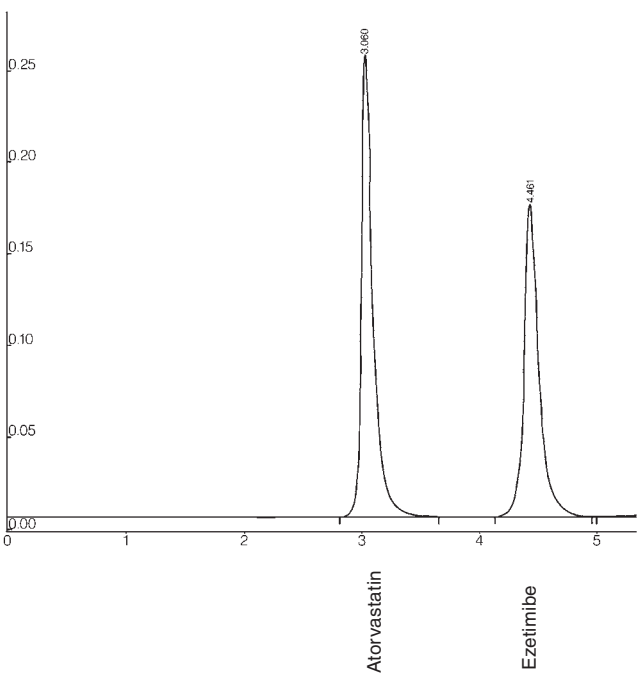

Figure 3. Chromatograms of atorvastatin calcium and ezetimibe reference substance.

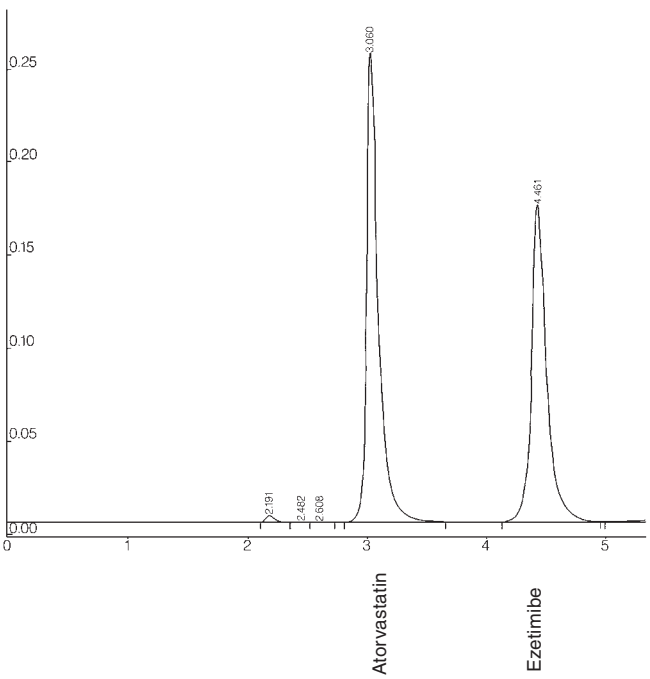

Figure 4. Chromatograms of atorvastatin calcium and ezetimibe Tablets.

method. The mean percentage recovery ± SD and the %CV are calculated from recovery experiments and presented in Table 2. Mean percent recoveries were in the range of 98.25 to 101.75 % with %CV less than 2%.

(IV) *Precision*

The precision of the proposed method, expressed as %CV were determined by the analysis of three different concentrations. The intra-day precision was assessed from the results of five replicate analyses of quality control samples on a single day. The inter-day precision was determined from the same quality control samples analyzed on five consecutive days. The results of intra-day and inter-day precision are illustrated in Table 3.

(V) *Selectivity*

To test the selectivity of the proposed method, the percentage recovery of each component is determined in mixture of them with possible interfering materials such as starch, lactose, magnesium stearate and avicel. The results exhibited no interference as shown in Table 4.

(VI) *Stability*

Stability of each component in solution was assessed by determining the %CV of replicate injections of the same solution over a period of 72 hr. The results presented in Table 5 indicate adequate stability for each drug in solution and during the analysis time.

III. *Application of the Method in Tablets*

The proposed HPLC method was applied for the determination of atorvastatin calcium and ezetimibe in their combined pharmaceutical formulations and the results are shown in Table 6. The high percentage recov-

Table 1. Linear regression analysis of calibration curves

Drug	Linearity range*	Intercept		Slope		R^2	LOD	LOQ
	(μg mL^{-1})	Mean	SD	Mean	SD		(μg mL^{-1})	(μg mL^{-1})
Atorvastatin calcium	12-52	0.0238	0.0001	0.0154	0.0004	0.9966	0.11	0.25
Ezetimibe	12-52	0.0665	0.0002	0.0448	0.0008	0.9993	0.07	0.18

*Five standard concentrations were used for the construction of the calibration curves (n = 5).

Table 2. Recovery experiments of the proposed HPLC method (n = 3)

Drug	Concentration (μg mL^{-1})	Amount recovered (μg mL^{-1})	SD (%)	Recovery (%)	SD (%)	CV (%)
Atorvastatin calcium	16.0	16.16	0.17	101.0	1.06	1.05
	20.0	20.28	0.06	101.40	0.29	0.29
	24.0	23.68	0.10	98.67	0.41	0.42
	28.0	28.12	0.35	100.43	1.24	1.24
Ezetimibe	16.0	15.88	0.09	99.25	0.57	0.57
	20.0	19.65	0.17	98.25	0.84	0.86
	24.0	24.42	0.41	101.75	1.71	1.68
	28.0	27.82	0.03	99.36	0.11	0.11

Table 3. Intra-day and inter-day precision of the proposed HPLC method

Compound	Conc.	n	Intra-day precision			Intra-day precision		
	(μg mL^{-1})		Mean	SD (±)	CV (%)	Mean	SD (±)	CV (%)
Atorvastatin calcium	16.0	5	16.28	0.18	1.11	16.36	0.13	1.59
	32.0	5	32.15	0.33	1.03	32.48	`0.49	1.51
	48.0	5	47.72	0.41	0.86	48.61	0.61	1.25
Ezetimibe	16.0	5	15.70	0.11	0.70	16.33	0.22	1.35
	32.0	5	32.52	0.27	0.83	31.58	0.28	0.89
	8.0	5	48.82	0.19	0.39	48.02	0.53	1.10

Table 4. Selectivity of the proposed HPLC method

Atorvastatin calcium			Ezetimibe		
Added ($\mu g\ mL^{-1}$)	Recovered ($\mu g\ mL^{-1}$)	Recovery (%)	Added ($\mu g\ mL^{-1}$)	Recovered ($\mu g\ mL^{-1}$)	Recovery (%)
32	32.18	100.56	32	32.51	101.59
32	31.62	98.81	32	31.86	99.56
32	31.78	99.31	32	31.58	98.69
32	32.52	101.62	32	32.24	100.75
Mean % recovery = 100.08%			Mean % recovery = 100.15%		
SD = ±1.26			SD = ±1.28		
CV = 1.26%			CV = 1.28%		

Table 5. Stability study of atorvastatin calcium and ezetimibe in solution over 72 hr

Concentration ($\mu g\ mL^{-1}$)	Recovered concentration				
	After 24 hr	After 48 hr	After 72 hr	SD (±)	CV (%)
1-Atorvastatin calcium					
16.0	15.73	15.82	16.15	0.22	1.38
32.0	31.48	31.68	31.50	0.11	0.35
48.0	48.18	47.98	48.80	0.43	0.89
2-Ezetimibe					
16.0	16.32	16.11	16.30	0.12	0.74
32.0	32.81	32.42	32.18	0.32	0.94
48.0	47.72	48.48	48.20	0.55	1.14

Table 6. Analysis of atorvastatin calcium and ezetimibe in tablets by the proposed HPLC method

Atorvastatin calcium			Ezetimibe		
Added ($\mu g\ mL^{-1}$)	Recovered ($\mu g\ mL^{-1}$)	Recovery (%)	Added ($\mu g\ mL^{-1}$)	Recovered ($\mu g\ mL^{-1}$)	Recovery (%)
32	32.62	101.94	32	32.56	101.75
32	32.15	100.47	32	32.56	100.56
32	31.68	99.00	32	32.48	102.03
Mean recovery = 100.47%			Mean recovery = 101.45%		
SD = ± 1.47			SD = ± 0.47		
CV = 1.46%			CV = 0.48%		

eries (99.00-102.03) and low %CV (0.48-1.46) values confirm the suitability of the proposed method for the routine determination of these components in multi-component preparations.

CONCLUSIONS

The proposed HPLC method is simple and the total run time for the two components is less than 5 min. The quantitation of each component was not affected by any of the possible interfering substances included during tablet manufacturing. The method is accurate and precise as indicated from the recovery study and the low %CV. It can be concluded that the proposed HPLC method has great promise for the simultaneous determination of two components in pharmaceutical formulations.

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