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Research Article

REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) METHOD FOR SIMULTANEOUS DETERMINATION OF ATORVASTATIN, EZETIMIBE AND FENOFIBRATE IN COMMERCIAL TABLETS

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ABSTRACT

A simple, rapid, precise and accurate gradient reverse-phase HPLC method was developed and validated for the simultaneous determination of Atorvastatin (AT), Ezetimibe (EZ) and Fenofibrate (FE) in commercial tablets. The method has shown adequate separation for AT, EZ and FE. Separation was achieved on Purospher® C18, 5µm, 250mm X 4.6mm analytical column using gradient elution with acetonitrile –water system at room temperature and the detection was carried out at 254nm using photodiode array (PDA) detector. The linearity of the proposed method was investigated in the range of 2-10µg/ml (r=0.991), 2-10µg/ml (r=0.990), 32-160µg/ml (r=0.994) for AT, EZ and FE respectively. The limit of detection (LOD) was 0.1577, 0.1266 and 1.9544 for AT, EZ and FE respectively. The limit of quantification (LOQ) was 0.4780, 0.3838 and 3.6225 for AT, EZ and FE respectively. The relative standard deviation (RSD) of six replicates is less than 2%. This HPLC method is applied successfully to the simultaneous quantitative analysis of AT, EZ and FE in commercial tablets.

Keywords: Atorvastatin Calcium; Ezetimibe; Fenofibrate; Gradient; Reverse phase HPLC; Photodiode array (PDA).

INTRODUCTION

Atorvastatin (AT) calcium, chemically [R-(R*,R*)]-2-(4-fluorophenyl)- β, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate, is a synthetic lipid-lowering agent. AT is an inhibitor of 3-hydroxy-3- methylglutaryl-coenzyme A (HMG-CoA) reducates, the enzyme which catalyzes the conversion of HMG-CoA to mevalonate an early and rate-limiting step in cholesterol biosynthesis[1,2]. AT is indicated to reduce the risk of myocardial infarction, stroke and to reduce the risk for revascularization procedures and angina. AT is also prescribed for the stabilization of plaque[3,4]. AT is official in Indian Pharmacopoeia[5] and British Pharmacopoeia[6]. Bioanalytical, HPLC, HPTLC, UPLC and FT- Raman Spectroscopy methods are reported for its individual determination and in combination with other drugs[7-14]. Ezetimibe (EZ), chemically (1-(4- flurophenyl)-3(R) - [3(S)-(4- flurophenyl) –3-hydroxy propyl] –4(S) (4 – hydroxyphenyl) azetidin- 2- one), belongs to a group of selective and very effective 2-azetidione cholesterol absorption inhibitors, acts at the level of cholesterol entry into enterocytes[15]. Co-administration of ezetimibe with statins could provide an additional reduction in LDL cholesterol as well as total cholesterol[16]. Bioanalytical, HPLC and stability indicating HPLC methods are reported for its individual determination and in combination with other drugs[17-23]. Fenofibrate (FE), chemically 2-[4-(4-chlorobenzoyl) phenoxy] - 2-methyl-propanoic acid 1-methylethyl ester, is a lipid regulating agent. It is a white solid and is insoluble in water. FE is official in United States Pharmacopoeia[24] and British Pharmacopoeia[6]. Stability indicating UPLC in combination with AT[13] and HPLC methods for assay and purity and an NMR method for purity[25], spectroscopy and LC method for its determination with vinpocetine in formulations[26] are reported.

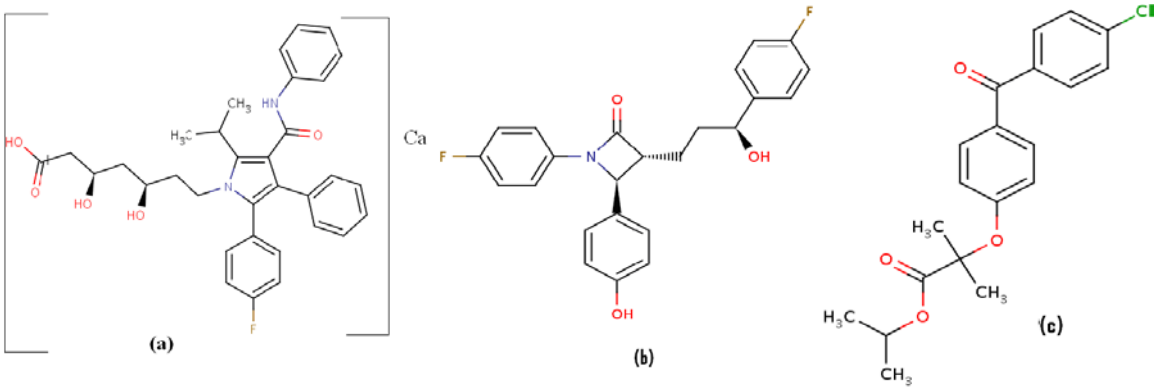

Fig. 1: **The chemical structure of (a) AT, (b) EZ and (c) FE**

Combination of FE and AT has additive beneficial effect in the treatment of mixed dyslipidemia[27]. The effects of combined therapy of FE and AT on plasma adiponectin levels and insulin sensitivity were significantly greater than those of AT alone and FE alone[27]. The co administration of EZ with FE offers a well tolerated, lipid management strategy for patients with mixed dyslipidemia. The combined use of these agents provides a therapy with complementary effects to improve the atherogenic lipid profile observed for these patients[28].

The present manuscript first time describes a reverse phase HPLC method which is simple, rapid, accurate and precise method for the simultaneous determination of AT, EZ and FE in the commercial tablets.

MATERIAL AND METHODS

Chemical and Reagents

AT calcium, EZ and FE were supplied by Torrent Research Centre, Astron Research Centre, Zydus Research Centre, Ahmedabad, India, respectively. TriTonact® (Lot: IL258011, Lupin Ltd., Mumbai) was purchased from Indian market. HPLC grade Acetonitrile (ACN), water, and Methanol were purchased from Ranchem (A division of Ranbaxy) laboratory ltd.

HPLC instrumentation & conditions

The HPLC system consisted of a Young Lin 9101 vaccum degasser, a Young Lin 9001 quaternary pump and a Young Lin 9160 PDA detector (Seoul, South Korea). An YL-clarity chromatography data system was used to record and evaluate the data collected during and following chromatographic analysis. The chromatographic separation was achieved on a Purospher® 5µm, 250mm X 4.6mm i.d. column using a mobile phase consisting of ACN- water with gradient elution. The eluent was monitored using PDA detector at a wavelength of 254nm. The column was maintained at room temperature and injection volume of 20µl was used. The mobile phase was filtered through 0.45µm Chrom Tech Nylon-66 filter for use.

Preparation of stock and standard solutions

Stock solution of AT calcium (equivalent to 100µg/ml), EZ (equivalent to 100µg/ml) and FE (equivalent to 1600µg/ml) were prepared in methanol. The stock solution were protected from light using aluminium foil and stored for three weeks at 4^{0}C with no evidence of decomposition. Aliquots of standard stock solution of AT, EZ and FE were transferred using A-grade bulb pipettes into 100ml volumetric flasks and the solution were made up to volume with methanol to yield final concentration of 2, 4 6, 8 and 10 µg/ml for AT; 2, 4, 6, 8 and 10 µg/ml for EZ and 32, 64, 96, 128 and 160 µg/ml for FE.

Preparation of tablets for assay

Twenty tablets were weighed, crushed and mixed in a mortar and pestle for 20 minutes. A portion of powder equivalent to the weight of half tablet was accurately weighed into each of nine, 100ml A-grade volumetric flasks and 100ml of methanol was added to each flask. The volumetric flasks were sonicated for 20 minutes to prepare complete solution of the AT, EZ and FE. Aliquot of the solution were filtered through a 0.45 µm nylon filter and 1ml of the filtered solution was transferred to a 100ml A-grade volumetric flask and made up to volume with methanol, to yield concentrations of each of the volume of the three drugs in the range of linearity previously described.

RESULT AND DISCUSSION

HPLC method development and optimization

Purospher® RP 5µm, 250mm x 4.6mm i.d. column (Merck, Germany) maintained at ambient temperature (25^{0}C) was used for the separation and the method validated for the determination of AT, EZ and FE in TriTonact® tablets. The composition, pH and flow rate of the mobile phase were changed to optimize the separation conditions using main substances of the three compounds of interest. A mobile phase consisting of ACN-water with gradient elution (Table -1) was selected for use for further studies after several preliminary investigatory chromatographic runs. Under the described experimental conditions, all peaks were well defined and free from tailing.

Table 1: Gradient flow condition

Time [min]	Water %	ACN %	Flow Rate [ml/min]
0.0	48	52	1
8.0	48	52	1
9.0	30	70	2
22	30	70	2
23	48	52	1

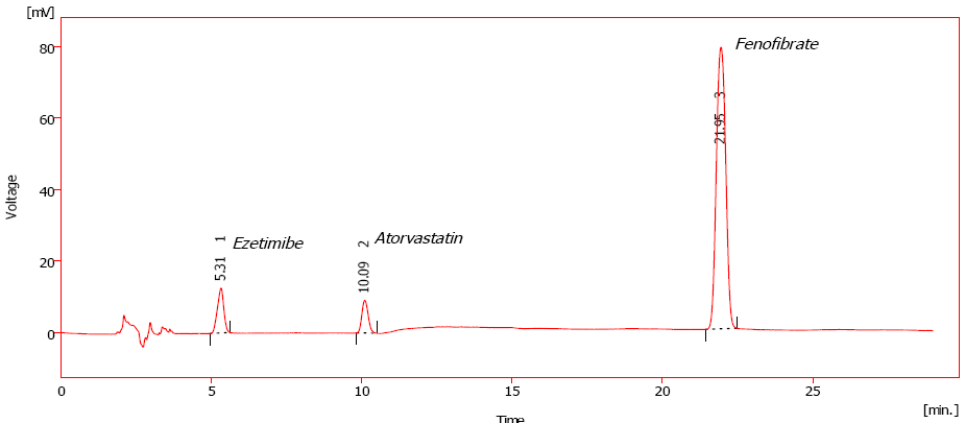

Fig. 2: Representative chromatogram obtained for standard solution corresponding 4, 4 and 64 µg/ml of AT, EZ and FE, respectively

Validation of the method

The analytical method was validated with respect to parameters such as linearity, limit of detection (LOD), precision, accuracy, selectivity, and recovery[29].

Linearity

Linearity was established by least squares linear regression analysis of the calibration curve [29]. The constructed calibration curves were linear over the concentration range of 2-10µg/ml, 2-10µg/ml and 32-160µg/ml for AT, EZ and FE, respectively. Peak areas of AT or EZ or FE were plotted versus their respective concentrations and linear regression analysis was performed on the resultant curves. Typically, the regression equations were: y = 80.25x – 155.6 (R = 0.9915), y = 109.3x –213.2 (R = 0.9903), y=53.97x –1395.9 (R = 0.9942) for AT, EZ and FE, respectively.

LOQ and LOD

LOD and LOQ were performed on samples containing concentrations of analytes, based on calibration curve method. Standard Solution of AT, EZ and FE were injected in six replicate. Average peak area of six

analytes was plotted against concentration. LOD and LOQ were calculated by using following equations

$$LOD = (3.3 \times \sigma)/S \quad LOQ = (10.0 \times \sigma)/S$$

Where σ = the standard deviation of y-intercepts of regression lines of the calibration curve, S = the slope of the calibration curve.

The LOD and LOQ value were found to be 0.1577µg/ml, 0.1266µg/ml, 1.9544µg/ml and 0.4780µg/ml, 0.3838µg/ml, 3.6225µg/ml, for AT, EZ and FE, respectively (Table-2).

Precision

The intra and inter-day variability or precision data are summarized in table-3. They were assessed by using standard solutions prepared to produce solutions of three different concentrations of each drug. AT, EZ and FE were used in the same solution for the purpose of these studies. Intra-day precision were investigated by injecting three replicate samples of each of the samples of three different concentrations. Inter-day precision were assessed by injecting the same three samples over three consecutive days.

Repeatability was investigated by injecting six replicate samples of each of the samples of five different concentrations. %RSD values ranging from 0.7508-1.7540, 0.8878-1.7754 and 0.5163-1.4090 was found for AT, EZ and FE, respectively across the concentration ranges studies.

Recovery

A known amount of each standard powder (0%, 50%, 100% and 150%) was added to samples of tablet powders, which was then mixed, extracted and subsequently diluted to yield a starting concentration of 4µg/ml, 4µg/ml and 64µg/ml for AT, EZ and FE, respectively. The observed percentage recovery of AT, EZ and FE were ranging from 99.58-101.31%, 99.18-100.30% and 98.35-100.23%, respectively.

Assay

The validated method was applied to the determination of AT, EZ and FE in commercially available TriTonact® tablets. The result of assays (n=3) undertaken yielded 100.11% (% RSD = 1.6181 %), 100.88% (% RSD = 1.8163 %) and 100.54% (% RSD = 1.7063 %) of label claim for AT, EZ and FE, respectively. The observed concentrations of AT, EZ and FE were found to be 5.004 ± 0.081µg/ml (mean ± SD), 5.033 ± 0.091µg/ml and 80.324 ± 1.372µg/ml, respectively (Table-4).

Table 2: Statistical data of standard curve of AT, EZ and FE

Parameters	AT	EZ	FE
Linear Range (µg/ml)	2-10mcg/ml	2-10mcg/ml	32-160mcg
Slope	80.25117	109.2833	53.9716
Intercept	155.667	213.1667	1395.667
Standard deviation of slope	0.7	1.0778	0.2979
Standard deviation of intercept	3.8364	4.1955	19.551
Limit of Detection (µg/ml)	0.1577	0.1266	1.9544
Limit of Quantification (µg/ml)	0.4780	0.3838	3.6225
Linear equation	Y=80.25x-155.6	Y=109.3x-213.2	Y=53.97x-1395.9
R^2 value	0.9915	0.9903	0.9942

Table 3: Summary of validation parameter

Parameters	AT	EZ	FE
Recovery (%)	99.58-101.31	99.18-100.30	98.35-100.23
Repeatability (RSD, n=6)	0.75088 – 1.7540	0.8878 – 1.7754	0.5163 – 1.4090
Precision Range (CV)			
Intra-day (n=3)	0.7329 – 0.9936	0.5299 - 0.9015	0.1024 – 0.6282
Inter-day (n=3)	1.2868 – 1.5967	1.064 – 1.7378	0.3172 – 1.5933
Specificity	specific	specific	specific

Table 4: Assay Results of Market formulation

Formulation	Actual concentration (µg/ml)			Found concentration (µg/ml)		
	AT	EZ	FE	AT	EZ	FE
TriTonact®	5	5	80	5.004 ± 0.081	5.033 ± 0.091	80.324 ± 1.372

CONCLUSION

A simple, rapid, accurate and precise HPLC analytical method has been developed and validated for the routine analysis of AT, EZ and FE in API and tablet dosage forms. The proposed method has the ability to separate these drugs from excipients found in tablet dosage forms.

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