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Simultaneous Estimation of Atorvastatin, Ezetimibe and Fenofibrate in Pharmaceutical Formulation by RP-LC-PDA

Vishnu P. Choudhari[1] and Anna Pratima Nikalje[2]*

[1]Maharashtra Institute of Pharmacy, MIT Campus, Paud Road, Kothrud, Pune, 411038, MS, India
[2]Y.B. Chavan College of Pharmacy, Dr. Rafiq Zakaria Campus, Rauza Bagh, Aurangabad, 431001, MS, India

Abstract

A simple, rapid and precise reversed-phase liquid chromatographic method is developed for simultaneous determination of Atorvastatin, Ezetimibe and Fenofibrate in their ternary mixture of commercial pharmaceutical preparations. This method, reported first time for a ternary mixture, uses a Kromasil C18, 250 × 4.6 mm, 5µm analytical column. Analytes were estimated by gradient elution with methanol/water at flow rate of 0.9 mL/min; the column temperature is 40°C and detector wavelength is 240 nm. The sample concentrations are measured on weight basis to avoid the internal standard. The method is validated and shown to be linear. The correlation coefficients for Atorvastatin, Ezetimibe and Fenofibrate are 0.9995, 0.9993 and 0.9996, respectively. The recovery values for Atorvastatin, Ezetimibe and Fenofibrate ranged from 99.7–101.1%, 99.8–101.3% and 99.7–101.7%, respectively. The relative standard deviation for six replicates is always less than 2%. This HPLC method is successfully applied to the simultaneous quantitative analysis of the title drugs in tablets.

Keywords: Atorvastatin calcium; Ezetimibe; Fenofibrate; Gradient; RP-HPLC-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) reductase, the enzyme 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 reduce the risk for revascularization procedures and angina [3,4]. Bioanalytical, HPLC, HPTLC, UPLC and FT- Raman Spectroscopy methods are reported for its individual determination and in combination with other drugs [5-12]. Ezetimibe (EZ); chemically (1-(4- flurophenyl)-3(R)-[3(S)-(4- flurophenyl) –3-hydroxy propyl] –4(S) (4 – hydroxyphenyl) azetidin- 2- one), which belongs to a group of selective and very effective 2-azetidione cholesterol absorption inhibitors acts at the level of cholesterol entry into enterocytes [13]. Co-administration of ezetimibe with statins could provide an additional reduction in LDL cholesterol as well as total cholesterol [14]. Bioanalytical, HPLC and stability indicating HPLC methods are reported for its individual determination and in combination with other drugs [15-21].

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 [22]. FEN is official in USP [23] and BP [24]. Stability indicating UPLC in combination with AT [11] and HPLC methods for assay and purity and an NMR method for purity [25], spectroscopy and LC method for its determination with vanpocitin in formulations [26] are reported. Structures of AT, EZ and FE are given in Figure 1.

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

The combination of AT + EZ + FN is not official in any pharmacopoeia. So far, no HPLC method is reported for this combination. The present manuscript first time describes a LC method which is simple, rapid, precise, sensitive, selective and accurate gradient reversed phase HPLC–PDA method for the simultaneous determination of AT, EZ and FN in the tablet dosage form. During present study efforts were directed towards use of mobile phase without salt to increase column life.

Material and Methods

Chemicals

AT (purity, 99.83%) was gifted by Litaka Pharmaceuticals, Pune (MS). EZ (purity, 99.78%) and FN (purity, 99.8%) were obtained

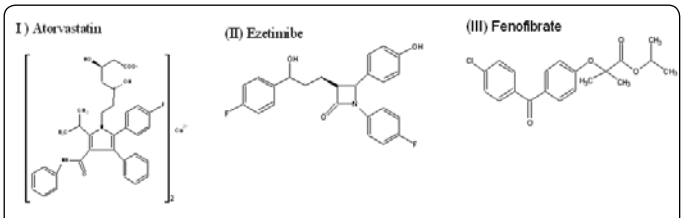

Figure 1: Representative chromatograms obtained for the mobile phase (A), placebo (B) and standard solution (C) corresponding to the 16, 16 and 256µg/mL of AT, EZ and FE, respectively.

***Corresponding author:** Anna Pratima Nikalje, Y.B. Chavan College of Pharmacy, Dr. Rafiq Zakaria Campus, Rauza Bagh, Aurangabad, 431001, MS, India, E-Mail: ana@k.st

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from Ranbaxy Labs Ltd., Dewas (MP), Methanol (HPLC grade) was purchased from E. Merck (India) Ltd, Worli, Mumbai, India. Double distilled water was used throughout the experiment. Tablets were purchased from Indian market, containing FN 160 mg, AT Calcium equivalent to AT and EZ 10mg each per tablet. (Tablet Fibator EZ, Lot: 6K70712A, Sun Pharmaceuticals Ltd., Mumbai and Tablet TriTonact, Lot: LL258001, Lupin Ltd., Mumbai).

Instrumentation and chromatographic conditions

The HPLC system consisted of a binary pump (model Waters 515 HPLC pump), auto sampler (model 717 plus Auto sampler), column heater and PDA detector (Waters 2998). Data collection and analysis were performed using Empower- version 2 software. Separation was achieved on Symmetry C–18 (250 mm × 4.6 mm, 5.0µ) and Kromasil C–18 (250 mm × 4.6 mm, 5.0µ) columns maintained at 40°C using column oven. Gradient elution with methanol/water mobile phase at the flow rate of 0.7 mL/min was carried out. The column was supported with waters symmetry C- 18, (3.9×20mm, 5.0µ) guard column. The detection was monitored at 240 nm and injection volume was 20µL. The peak purity was checked with the photodiode array detector.

Preparation of standard and sample solutions and calibration graphs

Standard stock solution AT, EZ and FE (1000µg/mL) were separately prepared in methanol. To study the linearity range of each component, serial dilutions of AT and EZ each were made from 0.5 to 32µg/mL and 8 to 512µg/mL of FE and injected on to column. Calibration curves were plotted as concentration of drugs versus peak area response. From the standard stock solutions, a mixed standard solution was prepared containing the analytes in the given ratio. The system suitability test was performed from six replicate injections of mixed standard solution (Table 1). Sample solution was prepared by using portion of tablet powder equivalent to one tablet. Tablet powder was transferred into 100 mL flasks and 80 mL methanol was added and sonicated for 3 min, filtered, filter paper was washed by adding washings to flask and volume was made up with methanol. The solution was filtered through 0.45µ nylon filter. The aliquot portions of the filtrate were diluted to get final concentration of 16µg/mL each of AT, EZ and 256µg/mL of FE.

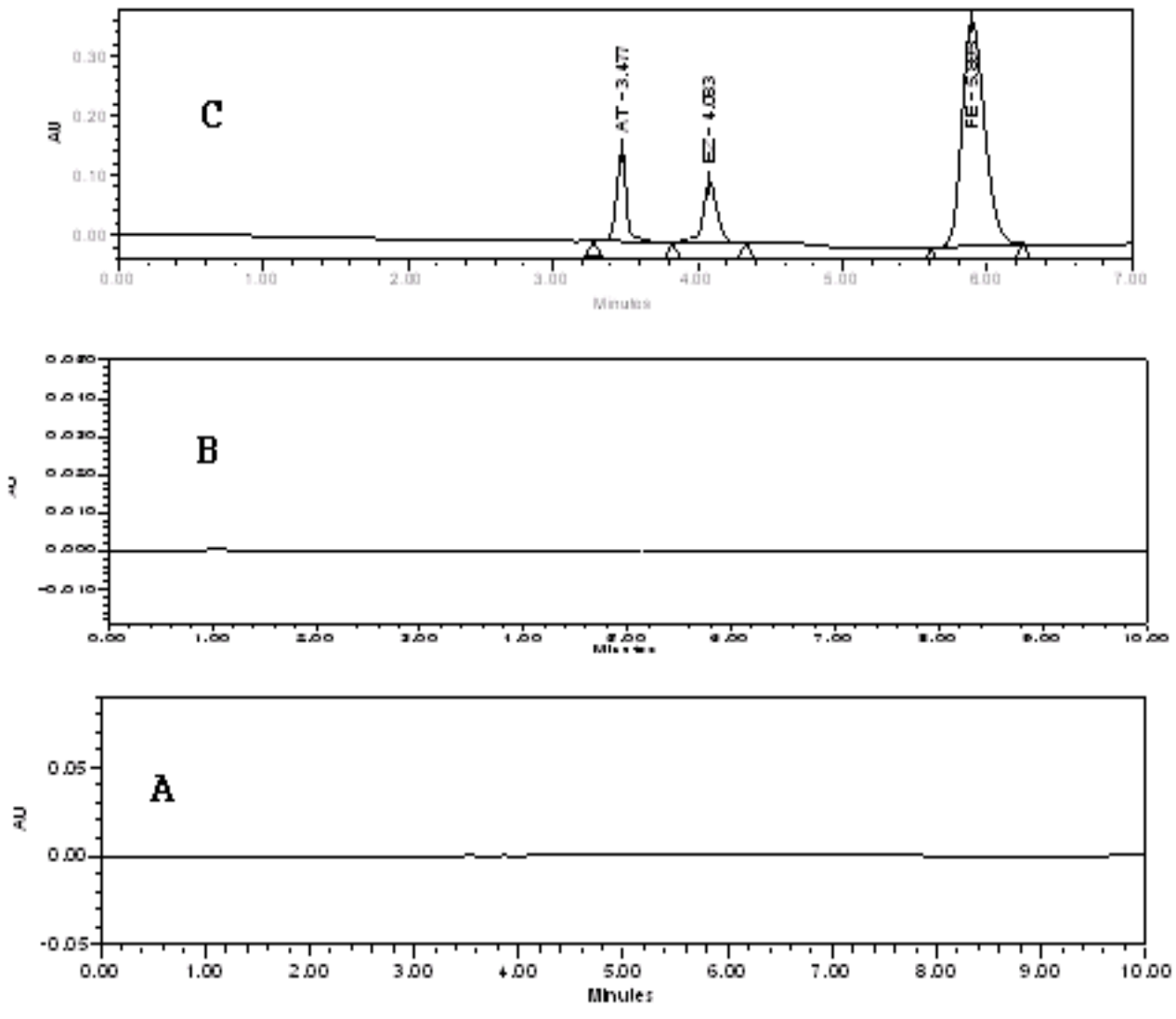

Figure 2: Representative chromatograms obtained for the mobile phase (A), placebo (B) and standard solution (C) corresponding to the 16, 16 and 256µg/mL of AT, EZ and FE, respectively.

Drug Name	System Suitability (n=6)		Precision of the Method [b](n=3)		
	Parameter	Value	Actual Conc. (µg/mL)	Measured conc. (µg/mL), % R.S.D	
				Intra-day	Inter-day
AT	Plates Count	15595	8	8.02, 1.08	8.03, 1.85
	Symmetry[a]	1.06	16	16.08, 1.21	16.06, 1.68
	% R.S.D.	0.87	24	24.17, 0.91	24.13, 1.23
EZ	Plates Count	15875	8	8.06, 0.95	7.90, 0.76
	Resolution[a]	5.2	16	16.05, 1.80	16.12, 1.62
	Symmetry[a]	1.10	24	24.19, 1.30	24.08, 1.05
	% R.S.D.	1.42			
FN	Plates Count	10304	128	128.80, 0.59	128.50,1.31
	Resolution[a]	9.9	256	257.10, 1.32	256.94, 1.73
	Symmetry[a]	1.10	384	384.67, 0.79	384.98, 0.88
	% R.S.D.	0.70			

[a]USP-NF 29 section 621, pp. 2135. [b]Data expressed as mean for "measured concentration" Values

Table 1: System suitability parameters and results of precision.

Drug Name	System Suitability (n=6)		Precision of the Method [b](n=3)		
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	Resolution[a]	9.9	256	257.10, 1.32	256.94, 1.73
	Symmetry[a]	1.10	384	384.67, 0.79	384.98, 0.88
	% R.S.D.	0.70			

Table 2: Results of formulation analysis and accuracy studies.

Factor	Level	Mean % assay(n=3), % RSD of results		
		Atorvastatin	Ezetimibe	Fenofibrate
Flow rate (mL/min)	0.8	100.6, 0.79	100.4, 0.49	100.2, 0.32
	1.0	98.6, 0.40	98.6, 1.25	99.6, 0.83
Column oven temperature (°C)	45	99.1, 0.82	99.0, 0.51	99.5, 1.33
	35	99.8, 1.53	100.6, 1.21	99.9, 1.18
Separation Column	Column I[a]	100.9, 0.69	99.6, 1.10	99.4, 0.58
	Column II[b]	99.8, 1.53	101.2, 0.28	100.2, 1.16
Measurement wavelength (nm)	239	99.35, 1.53	99.6, 1.50	99.9, 0.76
	241	99.91, 1.07	98.4, 0.48	99.8, 0.59

Table 3: Result of robustness study.

Method validation

The HPLC method was validated in terms of precision, accuracy and linearity according to ICH guidelines [29]. Assay method precision was determined using nine-independent test solutions. The intermediate precision of the assay method was also evaluated. Assay method was evaluated with the recovery of the standards from excipients. Three different quantities (low, medium and high) of the authentic standards were added to pre analyzed tablet powder. The mixtures were extracted as described in Section 2.3 and were analyzed using the developed HPLC method. Linearity test solutions were prepared as described in Section 2.3. The LOD and LOQ for analytes were estimated by injecting a series of dilute solutions with known concentration. To determine the robustness of the method, the final experimental conditions were purposely altered and the results were examined. The flow rate was varied by (±) 0.1 mL/min. Column temperature was varied by (±) 2^0C and effect of column from different suppliers was studied. Measurement wavelength was varied by (±) 1nm. The stability of the drug solution was determined using the samples for short-term stability by keeping at room temperature for 12 h and then analyzing. The long-term stability was determined by storing at 4°C for 30 days. Auto-sampler stability was determined by storing the samples for 24 h in the auto-sampler [30].

Results and Discussion

Optimization of the chromatographic conditions

Different mobile phase compositions of methanol with water and acetonitrile with water were tried. Most of the time AT and EZ eluted with very small value of retention factor (k) associated with long retention time (t_R) for FE. With regards to the mobile phase acetonitrile was used as composition of the mobile phase used for determination of AT with its impurities, AT with amlodipine, AT with ramipril and aspirin, AT + FE, EZ, AT+EZ, EZ with simvastatin, FE and EFNO with vinpocetine [7-10,15-17,25,26]. During method development preference was given to methanol as solvent of choice. Methanol in various proportions was tired and ultimately methanol: water 80:20 resolved AT and EZ with resolution factor of 1.7 and giving suitable k vale for AT which was eluted first but with peak tailing. This separation was associated with long t_R for FN (18 min). Temperature was increased to facilitate mass exchange with the corresponding decrease of peak broadening and increase in sensibility. Various column oven temperature were tried for improving the resolution. With temp. resolution was further improved but there was slight AT peak tailing. Ultimate choice of temp. and mobile phase was 45°C and 73:27, methanol : water, gave satisfactory AT and EZ resolution. The optimum wavelength for detection was 240 nm, as shown in Figure 2. Acquisition of PDA spectra showed no indigenous interfering components eluted at the retention times of the drugs. With regard to the mobile phase, an HPLC methods for AT with its impurities, AT in combinations with amlodipine, AT with ramipril and aspirin, AT + FN and AT + EZ, EZ with simvastatin [7-10,16,17] salts are described as composition of the mobile phase. The use of salts in the mobile phase, however, can affect the lifespan of the column. In order to avoid this drawback, a mobile phase containing various ratios of water and methanol was initially used. Water, however, produced a high t_R for FN and therefore gradient was applied near about the elution time of the AT for faster elution of FN. Gradient used with respect time in minute and ratio of aqueous to organic phase was at 0.0 27/73, 3.0 27/73, 3.5 05/95, 6.0 05/95, 6.5 27/73, 7.0 27/73 was applied. The flow rate kept was 0.9 mL/min to achieve adequate retention time of first two peaks and to achieve early elution of FN.

Validation of method

Specificity: The specificity of the HPLC method is illustrated in (Figure 2), where complete separation of AT, EZ and FN was noticed in presence of tablet placebo. In addition there was no any interference at the retention time of AT, EZ and FN in the chromatogram of tablet solution. In peak purity analysis with photo diode array detector, purity angle was always less than purity threshold for all the analytes. This shows that the peak of analytes was pure and excipients in the formulation did not interfere the analytes.

Precision and accuracy: The precision of the method was determined by performing five replicate analyses of the same working solution. The relative standard deviation (R.S.D.) obtained for AT, EZ and FN were 1.43, 1.03 and 0.72 %, respectively. Intra-day precision of the developed LC method was determined by preparing the tablet samples of the same batch in nine determinations with three concentrations and three replicate each. The R.S.D. of the assay results, expressed as a percentage of the label claim, was used to evaluate the method precision. The inter-day precision was also determined by assaying the tablets in triplicate per day for consecutive 3 days. The results indicated the good precision of the developed method (Table 1). Accuracy of the method was calculated

by recovery studies at three levels by standard addition method. The mean percentage recoveries obtained for AT, EZ and FN was 100.57, 100.77 and 100.36%, respectively (Table 2).

Linearity and Range: For the construction of calibration curves, seven calibration standard solutions were prepared over the concentration range. Linearity was determined for AT and EZ in the range of 0.50-32µg/mL; and for FN, 8–512µg/mL. The correlation coefficient ('r') values were >0.999(n = 6). Typically, the regression equations for the calibration curve was found to be y =292770X +34689 for AT, y = 3285597X +31684 for EZ and y = 119274X +24756 for FN.

Sensitivity: LOD and LOQ for the procedure were performed on samples containing very low concentrations of analytes based on calibration curve method. Solutions of AT, EZ and FN were prepared in the range of 0.1 to 5µg/ml and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using following equations.

$$LOD = (3.3 \times Syx)/b \quad LOQ = (10.0 \times Syx)/b$$

Where Syx is residual variance due to regression; b is slope. The LOD and LOQ values were found to be 0.097, 0.13, 0.15µg/mL and 0.3, 0.4, 0.45µg/mL for AT, EZ and FN, respectively.

Solution Stability: Solution stability as described in method validation under experimental section was studied. Result of short-term, long-term and the auto sampler stability of the AT, EZ and FN solutions were calculated form nominal concentrations and found concentration. Results of the stability studies were within the acceptable limit (98–102%).

Application of the method to dosage forms: Two marketed formulations as described in section 2.1 were evaluated for the amount of the drugs present in the formulation. The amount of AT, EZ and FN estimated in 'Fibator EZ' and 'TriTonact' was in the range of 98.26 -101.78, 97.84 – 100.98 and 98.67- 101.89 % respectively. None of the tablet ingredients interfered with the analyte peak (Table 2 & Figure 2).

Robustness: Robustness of the method was investigated under a variety of conditions including changes of flow rate, column oven temperature, column form different suppliers and wavelength of measurement. The mixed standard solution is injected in five replicates and sample solution of 100% concentration is prepared and injected in triplicate for every condition and % R.S.D. of assay was calculated for each condition. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters has proven that the method is robust (Table 3).

Conclusions

A simple, specific, linear, precise and accurate RP-HPLC-PDA method has been developed and validated for quantitative determination of AT, EZ and FN form two tablet formulations; this is the first report of simultaneous estimation of this combination of ternary mixture. All the parameters for the three titled drugs met the criteria of ICH guidelines for method validation. The method is very simple and specific as all peaks are well separated and there is no interference by excipients peaks with total runtime of 7 min, which makes it especially suitable for routine quality control analysis work. The method can be used for individual analysis of the titled drugs or their ternary and binary combinations.

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