



Journal home page:
<http://www.iajpr.com/index.php/en/>

**INDO AMERICAN
JOURNAL OF
PHARMACEUTICAL
RESEARCH**

RP-HPLC and chemometric assisted UV-spectrophotometric methods for simultaneous *in vitro* analysis of atorvastatin calcium, ezetimibe and fenofibrate in their pharmaceutical formulation

Abhishek Pathak¹, Sadhana J Rajput^{*1}, Rahul S Gamit²

^{1,2} Pharmaceutical Quality Assurance Laboratory, Centre of Relevance and Excellence in Novel Drug Delivery System, Shri G H Patel Pharmacy Building, Pharmacy Department, The Maharaja Sayajirao University of Baroda, Fatehgunj, Vadodara-390 002, Gujarat, INDIA.

ARTICLE INFO

Article history

Received 25 July 2012
 Available online 30 July 2012

Keywords

Atorvastatin calcium,
 chemometric,
 Ezetimibe,
 Fenofibrate, Reversed
 phase-high performance
 liquid chromatography
 (RP-HPLC)

ABSTRACT

One RP-HPLC and two chemometric assisted UV spectrophotometric methods were developed and validated for the simultaneous *in vitro* analysis of Atorvastatin calcium (ATV), Ezetimibe (EZET) and Fenofibrate (FEN) in their pharmaceutical preparation and ternary mixtures. The chromatographic separation was achieved on a reversed-phase, Hypersil BDS C8 column (250X4.6 mm i.d, 5 μ particle size) with a mobile phase consisting of methanol and 0.05M phosphate buffer (pH-6.3 adjusted with sodium hydroxide) in the ratio of (85:15)% v/v. The total run time was 7 min. Quantitation was achieved with UV detection at 248nm based on peak area. Linearity was observed over concentration range of 5 - 12 μ g mL⁻¹ for ATV and EZET and 80 - 192 μ g mL⁻¹ for FEN. The two chemometric methods applied were inverse least square (ILS) and classical least square (CLS). These approaches were successfully applied to quantify each drug in their mixture using the information included in the UV absorption spectra of appropriate solutions in the wavelength range 220-310nm with the intervals of 5nm ($\Delta\lambda = 5$ nm) at 19 wavelength points. For the chemometric calibration, 18 ternary solutions were prepared as training set and 10 ternary solutions were prepared as validation set. The developed methods were successfully applied for laboratory prepared mixtures as well as commercial tablet formulation for ATV, EZET and FEN concentration. The results obtained for pharmaceutical formulation by ILS and CLS methods were compared with isocratic HPLC method and a good agreement was found.

Corresponding author

Dr. (Mrs.) Sadhana J Rajput

Prof. of Pharmaceutical Quality Assurance, Centre of Relevance and Excellence in Novel Drug Delivery System, Shri G H Patel Pharmacy Building Pharmacy Department, The Maharaja Sayajirao University of Baroda, Fatehgunj, Vadodara – 390 002, Gujarat, INDIA, Phone No. +091-265-2794051, Fax No. +091-265-2750605, Email ID: sjrajput@gmail.com

Please cite this article in press as Abhishek Pathak et.al. RP-HPLC and chemometric assisted UV-spectrophotometric methods for simultaneous *in vitro* analysis of atorvastatin calcium, ezetimibe and fenofibrate in their pharmaceutical formulation. Indo American Journal of Pharm Research.2012;2(9).

1. Introduction

Atorvastatin Calcium (ATV); is chemically known as (βR , $8R$)-2-(4-fluorophenyl)- α,δ -dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]1H-pyrrole-1-heptanoic acid trihydrate. It is a synthetic lipid-lowering agent. ATV 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^[1-3]. ATV stabilizes plaque and prevents strokes through anti-inflammatory and other mechanisms^[4]. Several analytical methods that have been reported for determination of ATV individually and in combination with other drugs are HPTLC^[5-10], HPLC^[10-17], UPLC^[18], LCMS^[19-25], UV-Visible spectrophotometry^[26-28] and FT-Raman Spectroscopy^[29-30]. Ezetimibe (EZET); chemically (1-(4-fluorophenyl)-3(R)-[3(S)-(4-fluorophenyl)-3-hydroxy propyl]-4(S)-(4-hydroxyphenyl) azetidin-2-one), which is another lipid-lowering agent. EZET belongs to a group of selective and very effective 2-azetidine cholesterol absorption inhibitors acts at the level of cholesterol entry into enterocytes^[31]. EZET has no inhibitory effect on absorption of lipid soluble vitamins, triglycerides or bile acids, as do statins. This distinct mechanism of action results in a synergistic cholesterol lowering effect when used together with statins that inhibits cholesterol synthesis by liver^[32]. Literature review reveals that methods have been reported for analysis of EZET by HPTLC^[6-7], HPLC^[33-36], LCMS^[37-38] and UV-Visible spectrophotometry^[39-40] for its individual determination and in combination with other drugs.

Fenofibrate (FEN); chemically 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl-propanoic acid 1-methylethyl ester, is a lipid regulating agent. FEN is a white colored solid which is stable under ordinary conditions. It is insoluble in water^[41]. FEN is official in USP^[42] and BP^[43]. There have been several papers on the determination of fenofibrate individually and in combination with other drugs using techniques such as TLC^[44], HPTLC^[45], NMR method for purity^[46], HPLC^[46-52], UPLC^[18,53], LCMS^[54], UV-Visible spectrophotometry^[48,54-55] and capillary electrophoresis^[56]. Chemical structures of ATV, EZET and FEN are given in Figure 1.

Literature revealed that for the treatment of combined hyperlipidemia and type 2 diabetes, the combined ATV/FEN therapy is safe and has beneficial additive effects than either treatment alone^[57]. In addition, the co administration of EZET with FEN offers a well tolerated, lipid management strategy for the patients suffering from mixed hyperlipidemia. The combined use of these agents provides a therapy with complementary effects to improve the atherogenic lipid profile observed for these patients^[58].

The combination of ATV, EZET and FEN is not official in any pharmacopoeia. One TLC^[59] and two HPLC^[60-61] methods are reported for this combination but no chemometric assisted UV-spectrophotometric method is reported till date for the same. The objective of this report is to study the ability of ILS and CLS methods to estimate the mixture of ATV, EZET and FEN, whereas the zero order UV absorption spectra of ATV, EZET and FEN are critically interfere with each other. In addition, an economical, simple, rapid, precise, sensitive, selective and accurate isocratic HPLC-UV method was developed for the simultaneous determination of ATV, EZET and FEN in the tablet dosage form. The optimized methods were successfully applied for the determination of ATV, EZET and FEN in marketed preparation. The proposed HPLC method was found to be easy and simpler than the other reported methods for the analysis of studied drugs in their combination. The results obtained for the estimation of ATV, EZET and FEN in marketed preparation by ILS and CLS methods were compared with isocratic HPLC method and a good agreement was found.

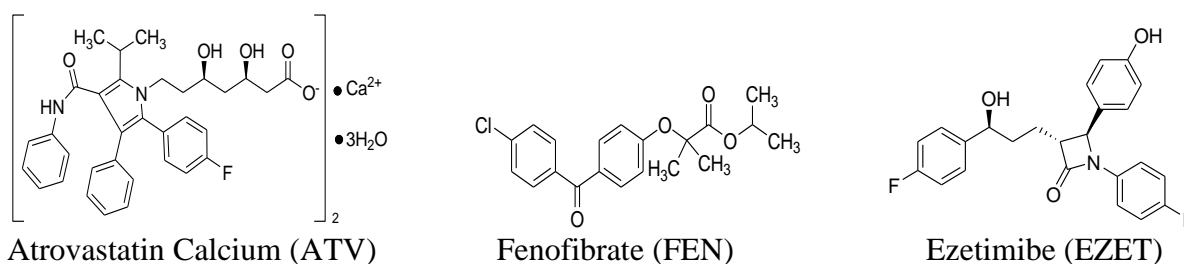


Figure 1: Chemical structures of Atrovastatin Calcium (ATV), Fenofibrate (FEN) and Ezetimibe (EZET).

2. Experimental

2.1. Instrumentation

2.1.1. For HPLC

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV absorbance detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 μ l. Separation and quantitation were made on a reversed-phase, Hypersil BDS C8 column (250X4.6 mm i.d, 5 μ particle size) with a mobile phase consisting of methanol and 0.05M phosphate buffer (pH-6.3 adjusted with sodium hydroxide) in the ratio of (85:15)% v/v. Detector was set at 248nm. Data acquisition and integration was performed using Spinchrome software (Spincho Biotech, Vadodara).

2.1.2. For chemometric spectrophotometry

A double-beam Shimadzu UV-1700 spectrophotometer (Kyoto, Japan) connected to a computer loaded with Shimadzu UV Probe 2.10 software was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were carried out in 1cm quartz cells over the range of 200-360 nm. The numerical calculations for ILS and CLS methods were performed by using MATLAB R2007a Software and Excel.

2.2. Materials and Reagents

Pure drug samples of ATV and FEN was kindly gifted by Sun Pharmaceutical, Vadodara and EZET was kindly gifted by ALEMBIC Pharmaceutical Ltd, Vadodara. The gift samples were used as standard without further purification. Double distilled water filtered through nylon filter paper 0.2 μ m pore size and 47 mm diameter (Pall Lifesciences, Mumbai, India), was used throughout the analysis. Potassium dihydrogen orthophosphate and sodium hydroxide were analytical grade. Methanol used was HPLC grade and analytical grade (Qualigens fine chemicals, Mumbai) for HPLC and spectrophotometry, respectively. Marketed formulation Lorlip-EZ tablets (Unichem Laboratories Limited, India) were used for the analysis. Each tablet was labeled to contain 10 mg ATV, 10 mg EZET and 160 mg FEN.

2.3. Experimental conditions

2.3.1. For HPLC

The mobile phase was prepared by mixing methanol and 0.05M phosphate buffer (pH-6.3 adjusted with sodium hydroxide and filtered through 0.20 μ m nylon filter paper) in a ratio of (85:15)% v/v and degassed. The flow rate was maintained at 0.8 ml min⁻¹. All determinations were performed at ambient temperature. Quantitation based on peak area was achieved with UV detection at 248nm. The injection volume was 20 μ l.

2.3.2. For chemometric spectrophotometry

The UV absorption spectra of appropriate solutions were recorded in the wavelength range 220-310nm with the intervals of 5nm ($\Delta\lambda = 5$ nm) at 19 wavelength points. The scanning range selected was 200-360nm.

2.4. Experimental design for ILS and CLS spectrophotometric methods

Chemometric method or multivariate analysis was carried out using ILS and CLS methods. The methods are carried out in two steps. In the first step, an empirical mathematical model was built representing the relationship between the absorbance and concentration data generated from a set of standard samples (calibration set). The second step is the prediction step in which the calibration model is used to determine the concentration of the components (validation set) from their spectral data. The accuracy and precision of predictive ability of the model can be defined as root mean square error of prediction (RMSEP). A mathematical model is accepted if the value of RMSEP units is found to be less than three. Inverse least square (ILS) is the application of multiple linear regression (MLR) to the inverse expression of the Beer-Lambert's law of spectroscopy. The mathematical expression is given as,

$$C = P \times A \dots \dots \dots (1)$$

Where, C is the concentration matrix, A is the absorbance matrix and P is the calibration constant. The above equation can be written as a linear equation system as follows, $C_1 = P_{11} \cdot A_1 + P_{12} \cdot A_2 + \dots + P_{1w} \cdot A_w$, $C_2 = P_{21} \cdot A_1 + P_{22} \cdot A_2 + \dots + P_{2w} \cdot A_w$ or $C_c = P_{c1} \cdot A_1 + P_{c2} \cdot A_2 + \dots + P_{cw} \cdot A_w$, where A_w is the absorbance at w^{th} wavelength, P_{cw} is the calibration coefficient for the c^{th} component at w^{th} wavelength and C_w is the concentration of c^{th} component.

The classical least square (CLS) calibration technique, based on linear CLS algorithm, involves the calculation of K, calibration coefficient using the absorbance data from the calibration set. The linear CLS algorithm has the following steps,

$$A = K \times C \dots \dots \dots (2)$$

In this equation, A is the absorbance matrix, C is the concentration matrix and K is the calibration constant. The linear form of the equation can be written as, $A_1 = K_{11} \cdot C_1 + K_{12} \cdot C_2 + \dots + K_{1c} \cdot C_c$, $A_2 = K_{21} \cdot C_1 + K_{22} \cdot C_2 + \dots + K_{2c} \cdot C_c$, Or $A_w = K_{w1} \cdot C_1 + K_{w2} \cdot C_2 + \dots + K_{cw} \cdot C_c$, where A_w is the absorbance at w^{th} wavelength, K_{cw} is the calibration coefficient for c^{th} component at w^{th} wavelength and C_c is the concentration of c^{th} component.

The calibration equations (1) and (2) are used for the estimation of the amounts of components in the samples. The ability and efficiency of calibration was studied by estimating the standard variation of chemometric calibrations in case of investigated mixtures. The root mean square error of prediction (RMSEP) was calculated from the following formula,

$$RMSEP = \sqrt{\frac{\sum_{i=1}^N (C_i^{added} - C_i^{found})^2}{N}}$$

Where, C_i^{added} = added concentration of the drug and C_i^{found} = predicted concentration of the drug.

2.5. Standard solutions and calibrations

Stock standard solutions of ATV, EZET and FEN were prepared separately by dissolving 25 mg of each drug in 25 ml methanol. Working standard solutions of ATV, EZET and FEN were prepared separately by transferring 1.0 ml from stock standard solution of each drug into separate 10 ml volumetric flasks and diluted to the mark with methanol. Standard solutions for HPLC analysis and spectrophotometric analysis were prepared separately using methanol HPLC grade and methanol analytical grade respectively.

2.5.1. For HPLC

The solutions for calibration were prepared by further dilutions of different aliquots of the working standard solutions with mobile phase to reach the concentration range of 5-12 $\mu\text{g ml}^{-1}$ for ATV and EZET and 80-192 $\mu\text{g ml}^{-1}$ for FEN. Triplicate 20 μl injections were made for each concentration and chromatogram was obtained under the specified chromatographic conditions described previously. The calibration graph was constructed by plotting peak area versus concentration of each drug and the regression equation was calculated. Linear relationships were obtained.

2.5.2. For chemometric spectrophotometry

A training set (calibration set) of 18 synthetic ternary mixture solutions (Table 1) and a validation set containing 10 synthetic ternary mixture solutions (Table 2) in the possible combinations containing 2-5 $\mu\text{g ml}^{-1}$ of ATV and EZET and 8-32 $\mu\text{g ml}^{-1}$ of FEN were prepared with methanol analytical grade using the above working standard solutions to develop the chemometric calibrations. The UV absorption spectra of appropriate solutions were recorded in the wavelength range 220-310nm with the intervals of 5nm ($\Delta\lambda = 5\text{nm}$) at 19 wavelength points. The scanning range selected was 200-360nm. The numerical calculations for ILS and CLS analyses were performed by using MATLAB R2007a Software and Excel.

2.6. Sample preparation

Twenty tablets were accurately weighed and finely powdered. The portions of powder equivalent to 160 mg of FEN for HPLC and equivalent to 32mg of FEN for spectrophotometric analysis were accurately weighed and transferred separately into two 100ml volumetric flasks. Then 60 mL of methanol was added to each flask and sonicated for 15 min to solubilize the solid content. The volume was made up to the mark with methanol and mixed well. The sample solution for HPLC analysis and the sample solution for spectrophotometric analysis were filtered through 0.45 μm membrane filter and whatman filter paper no.41 respectively. Final test solution for HPLC was obtained by diluting the 1.0 ml of filtrate to 10 ml with mobile phase. And the final test solution for spectrophotometry was obtained by diluting the 1.0 ml of filtrate to 10 ml with methanol. Final test solution for HPLC contained 112 μg of FEN and 7 μg of ATV and EZET per ml of final solution. And the final test solution for spectrophotometry contained 32 μg of FEN and 2 μg of ATV and EZET per ml of final solution.

Table 1: Construction of Calibration set

S. No.	ATV ($\mu\text{g ml}^{-1}$)	EZET ($\mu\text{g ml}^{-1}$)	FEN ($\mu\text{g ml}^{-1}$)
1	4	5	8
2	4	5	16
3	4	5	24
4	4	5	32
5	3	2	16
6	3	3	16
7	3	4	16
8	3	5	16
9	2	4	32
10	4	4	32
11	5	4	32
12	5	4	0
13	5	0	32
14	0	4	32
15	0	0	32
16	0	4	0
17	5	0	0
18	2	2	32

2.7. Procedure for determination of ATV, EZET & FEN

2.7.1. For HPLC

Triplicate 20 μl injections of the appropriate solutions were injected and chromatogram was recorded under the specified HPLC conditions previously described in 2.3.1. The peak area values were determined and the concentration of each compound was calculated.

2.7.2. For chemometric spectrophotometry

The UV absorption spectra of appropriate solutions were recorded over the wavelength range of 220-310 nm with the intervals of 5nm ($\Delta\lambda = 5\text{nm}$) at 19 wavelength points. The scanning range selected was 200-360nm. The numerical calculations for ILS and CLS analyses were performed by using MATLAB R2007a Software and Excel. Concentration of each compound was calculated using each model.

Table 2: Construction of validation set

S. No.	ATV ($\mu\text{g ml}^{-1}$)	EZET ($\mu\text{g ml}^{-1}$)	FEN ($\mu\text{g ml}^{-1}$)
1	2	2	32
2	3	2	32
3	4	2	32
4	5	2	32
5	4	3	16
6	4	4	16
7	4	5	16
8	5	3	8
9	5	3	12
10	5	3	24

3. Results and discussion

3.1. For HPLC

The developed HPLC method has been applied for the simultaneous determination of ATV, EZET and FEN. The chromatographic method depends on reversed phase separation using Hypersil BDS C8 column (250X4.6 mm i.d, 5 μ , particle size). The method has been optimized after studying the effect of different parameters on the separation. The mobile phase was chosen after several trials with methanol and buffer solutions in various proportions and at different pH. The studies suggested that a mobile phase consisting of methanol and 0.05M phosphate buffer (pH-6.3 adjusted with sodium hydroxide) in the ratio of (85:15)% v/v was found to give good peak shape, optimum retention and resolution of ATV, EZET and FEN on above said column at ambient temperature. Depending on the observations of various trials, flow rate was fixed at 0.8 ml min⁻¹. Quantitation was achieved with UV detection at 248nm based on peak area. The specificity of the HPLC method is illustrated in Figure:2 where complete separation of the three drugs was noticed. The average retention time \pm standard deviation (S.D.) for the ATV, EZET and FEN were found to be 3.18 ± 0.04 , 4.21 ± 0.05 and 5.34 ± 0.07 min, respectively, for 10 replicates.

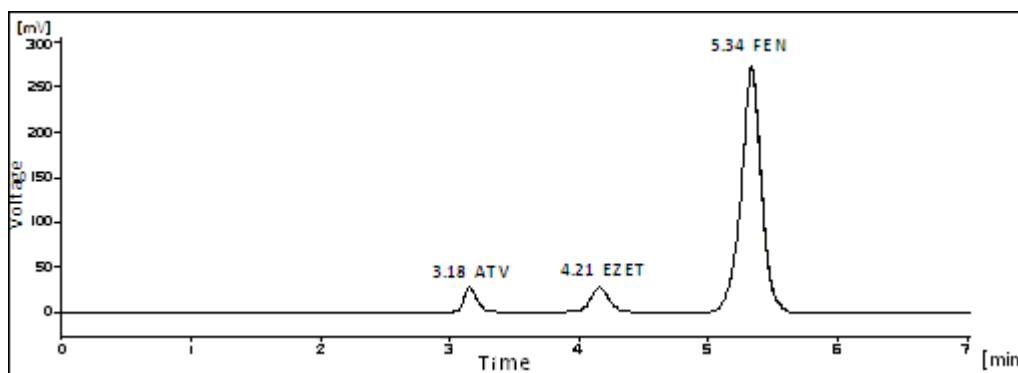


Figure 2: HPLC chromatogram of 20 μl injection of tablet sample containing $112 \mu\text{g ml}^{-1}$ of FEN and $7 \mu\text{g ml}^{-1}$ of ATV and EZET.

3.1.1. Method validation

3.1.1.1. Linearity and range

Linearity study of HPLC detector response for determination of ATV, EZET and FEN was evaluated by analyzing a series of standard solutions of five different concentrations of each compound. Calibration curves constructed were linear over the concentration range of 5 –12 $\mu\text{g mL}^{-1}$ for ATV and EZET and 80-192 $\mu\text{g mL}^{-1}$

for FEN. Regression analysis has been carried out with correlation coefficient, (0.9996, 0.9994 and 0.9999), intercept, (- 23.29, -21.20 and 144.30) and slope (30.35, 37.61 and 21.54) for ATV, EZET and FEN respectively. (Table 3)

3.1.1.2. Precision

Precision was estimated by the determination of the repeatability of the method. Repeatability was assessed using three determinations at each of three different test concentrations (covering the specified range of the method), in a day for Intra-day precision and on three different days for Inter-day precision. Ternary mixtures containing different concentrations of ATV, EZET and FEN were prepared with mobile phase. 20 μ L solutions were injected and chromatograms were recorded. The average % RSD of intra-day and inter-day measurements for determination of ATV was found to be 0.578 and 0.751 respectively. For EZET % RSD of intra-day and inter-day measurements was found to be 0.539 and 0.658 respectively. For FEN % RSD of intra-day and inter-day measurements was found to be 1.031 and 0.759 respectively. The values confirm the precision of the method. (Table 3)

3.1.1.3. Accuracy

Accuracy of the method was confirmed by recovery study from prepared Laboratory sample at 3 level of standard addition (80%, 100%, and 120%) of label claim. No interference could be observed with the proposed methods. The mean percentage recoveries were found to be 100.31 ± 0.30 %, 100.09 ± 0.13 % and 100.27 ± 0.36 % for ATV, EZET and FEN, respectively. The excellent recoveries of standard addition method with low SD justified the high accuracy of the proposed method. (Table 3)

3.1.1.4. Detection and Quantitation limits

Calibration curve was repeated for 3 times and the standard deviation (SD) of the intercepts was calculated. According to ICH recommendations (1996) ^[62], the approach based on the S.D. of the response and the slope was used for determining the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were measured as follows.

$LOD = 3.3 * SD / \text{slope of calibration curve}$

$LOQ = 10 * SD / \text{slope of calibration curve}$

SD = Standard deviation of intercepts

The theoretical values were assessed practically. The detection limits were found to be 0.54, 0.56 and 5.08 $\mu\text{g mL}^{-1}$ for ATV, EZET and FEN, respectively. The quantitation limits were found to be 1.79, 1.74 and 15.38 $\mu\text{g mL}^{-1}$ for ATV, EZET and FEN, respectively. (Table 3)

3.1.1.5. Robustness

To evaluate robustness of the method few parameters were deliberately varied. The parameters included variation of flow rate by ± 0.1 , change in pH of buffer by ± 0.2 , two different instruments and methanol of two different manufacturers. The average value of % RSD for determination of ATV, EZET and FEN less than 2 % revealed the robustness of the method.

3.1.1.6. Stability

The standard and sample solutions of ATV, EZET and FEN in methanol and mobile phase were stored at room temperature for 24hrs and at 5 °C in refrigerator for 4 days. These stored solutions were injected in to LC system. No additional peak found in chromatogram indicating the stability of ATV, EZET and FEN in the standard and sample solutions.

3.1.1.7. Specificity

According to ICH document for specificity (1996) ^[62], the method is specific when the results are unaffected by the presence of the dosage form excipients, so the above results demonstrated the specificity of the method. Furthermore, the specificity of the proposed HPLC method was confirmed by comparing the chromatograms of

standards and sample solutions. The average retention times \pm standard deviation for ATV, EZET and FEN in the tablets were found to be 3.18 ± 0.04 min [3.19 ± 0.06 min for standard ATV], and 4.21 ± 0.05 min [4.22 ± 0.04 min for standard EZET] and 5.34 ± 0.07 min [5.35 ± 0.03 min for standard FEN] for ATV, EZET and FEN respectively.

Table 3: Summary of validation parameters for the proposed HPLC method for the determination of ATV, EZET and FEN

No.	Parameters	HPLC		
		ATV	EZET	FEN
1.	Linearity range ($\mu\text{g mL}^{-1}$)	5-12	5-12	80-192
2.	Slope	30.35	37.61	21.54
	\pm SD	0.03	0.09	0.09
	% RSD	0.11	0.25	0.43
	Standard error	1.36×10^{-2}	3.84×10^{-2}	3.78×10^{-2}
3.	Intercept (% RSD)	-23.29	-21.20	144.30
	\pm SD	0.08	0.11	0.85
	% RSD	0.34	0.51	0.59
	Standard error	3.23×10^{-2}	4.41×10^{-2}	34.76×10^{-2}
4.	Correlation coefficient	0.9996	0.9994	0.9999
	\pm SD	8.4×10^{-4}	5.0×10^{-4}	7.0×10^{-4}
	%RSD	0.08	0.05	0.07
	Standard error	3.4×10^{-4}	2.0×10^{-4}	2.7×10^{-4}
5.	LOD ($\mu\text{g mL}^{-1}$)	0.54	0.56	5.08
6.	LOQ ($\mu\text{g mL}^{-1}$)	1.79	1.74	15.38
7.	Accuracy (%)	100.31	100.09	100.27
	\pm SD	0.30	0.13	0.36
	%RSD	0.30	0.13	0.36
	Standard error	0.12	0.05	0.15
8.	% RSD of Intra-day precision*	0.578	0.539	1.031
9.	% RSD of Inter-day precision *	0.751	0.658	0.759

*mean value of precision of six determinations

3.1.1.8. System suitability

Theoretical plates per meter \pm RSD, Theoretical plates per column \pm RSD, capacity factor, selectivity (α), symmetry factor and resolution for ATV, EZET and FEN in the sample solution were calculated for system suitability of HPLC method. Satisfactory results were obtained. (Table 4)

Table 4: Results of system suitability parameters for the proposed HPLC method for the determination of ATV, EZET and FEN

No.	Parameters	Data obtained		
		ATV	EZET	FEN
1.	Theoretical plates per meter	92794	85084	118137
	% RSD	0.45	0.85	0.14
2.	Theoretical plates per column	4640	4254	5907
	% RSD	0.64	0.24	0.65
3.	Capacity factor	2.85	1.12	1.65
4.	Selectivity(α)	2.544	1.272	2.152
5.	Symmetry factor/Tailing factor	1.257	1.122	0.873
6.	Resolution		4.417	4.431

3.2. For chemometric spectrophotometry

Figure 3 shows the overlain zero-order absorption spectra of ATV, EZET and FEN and their ternary mixture solution in methanol in the 200-400 nm absorption region.

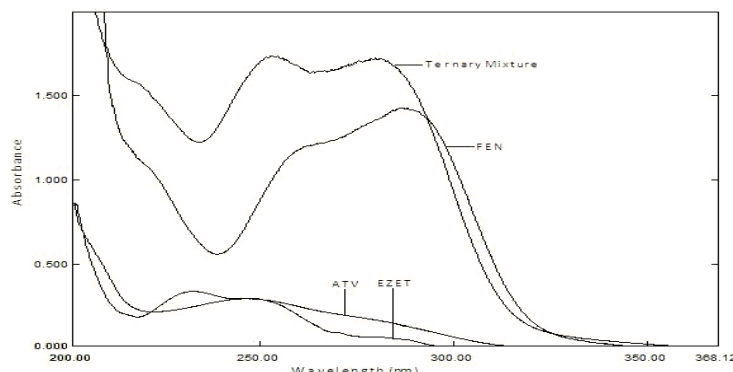


Figure 3: overlain zero-order absorption spectra of $2 \mu\text{g ml}^{-1}$ ATV, $2 \mu\text{g ml}^{-1}$ EZET and $32 \mu\text{g ml}^{-1}$ FEN and their ternary mixture (containing $2 \mu\text{g ml}^{-1}$ ATV, $2 \mu\text{g ml}^{-1}$ EZET and $32 \mu\text{g ml}^{-1}$ FEN) solution in methanol.

The zero-order absorption spectra (Figure 3) of ATV, EZET and FEN completely overlap with each other that makes impossible to use the conventional spectrophotometric methods to analyze the compounds in presence of each other. For example, the zero-crossing technique is not suitable for the resolution of ternary mixture. There are some methods to obviate this kind of problem in ternary mixtures like simultaneous application of division and derivation of the absorption spectra, application of double divisor method and the concept of mathematical modeling based on multivariate calibration [63-66]. Thus the ILS and CLS chemometric spectrophotometric methods were found to be appropriate for determination of ATV, EZET and FEN in ternary mixture. A training set (calibration set) of 18 synthetic ternary mixture solutions (Table 1) and a validation set containing 10 synthetic ternary mixture solutions (Table 2) in the possible combinations with methanol analytical grade were prepared. The UV absorption spectra of appropriate solutions were recorded in scanning range of 200-360nm and the wavelength range selected was 220-310nm with the intervals of 5nm ($\Delta\lambda = 5\text{nm}$) at 19 wavelength points.

3.2.1. ILS method

In this method, the coefficient matrix (P) was obtained from the linear equation system using the absorbance data and the calibration set. Introducing (P) into the linear equation system we obtain the calibration for ILS as:

$$\begin{bmatrix} C_{\text{ATV}} \\ C_{\text{EZET}} \\ C_{\text{FEN}} \end{bmatrix} = \begin{bmatrix} 0.027 & 0.027 & 0.029 & 0.032 & 0.035 & 0.035 & 0.035 & 0.033 & 0.030 & 0.026 & 0.024 & 0.022 & 0.019 & 0.016 & 0.013 & 0.010 & 0.007 & 0.004 & 0.002 \\ 0.023 & 0.031 & 0.039 & 0.039 & 0.036 & 0.034 & 0.029 & 0.022 & 0.013 & 0.009 & 0.007 & 0.006 & 0.005 & 0.003 & -0.0002 & -0.0006 & -0.0006 & -0.0003 & -0.0002 \\ 0.035 & 0.031 & 0.025 & 0.020 & 0.009 & 0.022 & 0.028 & 0.034 & 0.038 & 0.039 & 0.040 & 0.042 & 0.043 & 0.045 & 0.045 & 0.042 & 0.035 & 0.026 & 0.017 \end{bmatrix} \begin{bmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \\ A_6 \\ A_7 \\ A_8 \\ A_9 \\ A_{10} \\ A_{11} \\ A_{12} \\ A_{13} \\ A_{14} \\ A_{15} \\ A_{16} \\ A_{17} \\ A_{18} \\ A_{19} \end{bmatrix}$$

In this calibration, C_{ATV} , C_{EZET} and C_{FEN} are the concentration of ATV, EZET and FEN, respectively. The observed absorbance values of the compounds in the ternary mixture solutions, at the 19 wavelength points in

the spectral region from 200nm to 360nm, were replaced in the above equation and the amount of ATV, EZET and FEN in the synthetic mixtures and tablets were calculated.

3.2.2. CLS method

In this method, the coefficient matrix (K) was calculated by using the linear equation system between the absorbance data and training set. Replacing the coefficient matrix (K) into the linear equation system, the calibration of CLS can be written as:

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \\ A_6 \\ A_7 \\ A_8 \\ A_9 \\ A_{10} \\ A_{11} \\ A_{12} \\ A_{13} \\ A_{14} \\ A_{15} \\ A_{16} \\ A_{17} \\ A_{18} \\ A_{19} \end{bmatrix} = \begin{bmatrix} 0.0274 & 0.0227 & 0.0349 \\ 0.0267 & 0.0308 & 0.0309 \\ 0.0287 & 0.0386 & 0.0249 \\ 0.0316 & 0.0393 & 0.0203 \\ 0.0346 & 0.0356 & 0.0187 \\ 0.0359 & 0.0346 & 0.0222 \\ 0.0351 & 0.0337 & 0.0284 \\ 0.0328 & 0.0294 & 0.0343 \\ 0.0296 & 0.0215 & 0.0379 \\ 0.0264 & 0.0134 & 0.0390 \\ 0.0240 & 0.0091 & 0.0399 \\ 0.0217 & 0.0065 & 0.0415 \\ 0.0189 & 0.0061 & 0.0432 \\ 0.0161 & 0.0048 & 0.0450 \\ 0.0129 & 0.0027 & 0.0447 \\ 0.0097 & -0.0002 & 0.0419 \\ 0.0067 & -0.0006 & 0.0351 \\ 0.0042 & -0.0003 & 0.0261 \\ 0.0021 & -0.0002 & 0.0174 \end{bmatrix} \begin{bmatrix} C_{ATV} \\ C_{EZET} \\ C_{FEN} \end{bmatrix}$$

Where, C_{ATV} , C_{EZET} and C_{FEN} are the concentrations of ATV, EZET and FEN, respectively. The observed absorbance values of the compounds in the ternary mixture solutions, at the 19 wavelength points with the interval of 5nm ($\Delta\lambda = 5\text{nm}$) in the spectral region from 200nm to 360nm, were replaced in the above equation and the amount of ATV, EZET and FEN in the synthetic mixtures were calculated.

The tested mixtures for ILS and CLS were subjected to recovery studies. The results obtained were good. These results, % recoveries and standard deviation are shown in (Table 5). The ILS and CLS models were then applied to estimate ATV, EZET and FEN in commercial tablet preparations. The results are shown in (Table 7). The numerical values of all statistical parameters (Table 6) indicated that the proposed techniques are suitable for the determination of these drugs in the tablet formulation as excipients do not interfere.

3.3. Analysis of commercial tablet preparation

The proposed multivariate ILS and CLS spectrophotometric methods and HPLC method were applied to the simultaneous determination of ATV, EZET and FEN in commercial Lorlip-EZ tablet preparations. Satisfactory results were obtained for each compound which were in good agreement with label claim. The assay results of the proposed ILS and CLS spectrophotometric methods were compared to those of the proposed HPLC method. The results obtained were statistically analyzed and compared using paired t-test and F-test at 95% confidence level. The results are shown in Table 7. The calculated values did not exceed the theoretical ones; indicating that there was no significant difference between the methods compared.

Table 5: Percentage recovery results of ATV, EZET and FEN in synthetic mixtures (validation set) by the proposed ILS and CLS chemometric methods

Mixture composition				% recovery					
				ILS			CLS		
N	A	EZ	FE	AT	EZE	FEN	AT	EZE	FEN
1.	2	2	32	101.	100.	99.9	99.6	100.	100.
2.	3	2	32	100.	99.6	100.	99.4	99.6	100.
3.	4	2	32	99.9	100.	99.8	100.	100.	99.9
4.	5	2	32	100.	99.9	100.	101.	100.	99.1
5.	4	3	16	100.	99.8	99.9	99.9	99.9	99.9
6.	4	4	16	99.9	100.	100.	101.	100.	99.9
7.	4	5	16	101.	99.9	99.8	101.	100.	100.
8.	5	3	8	100.	101.	101.	99.7	99.9	101.
9.	5	3	12	99.7	99.9	99.9	100.	100.	99.9
10	5	3	24	100.	100.	100.	101.	99.5	100.
Mean				100.	100.	100.	100.	100.	100.
±SD				0.45	0.48	0.37	0.69	0.39	0.50
Variance (SD)				0.21	0.23	0.13	0.48	0.15	0.25
% RSD				0.45	0.48	0.37	0.69	0.39	0.50
Standard error				0.18	0.19	0.15	0.28	0.16	0.20

Table 6: Statistical parameters of ATV, EZET and FEN in synthetic mixtures (validation set) using the proposed ILS and CLS chemometric methods

No	Statistical parameters	ILS			CLS		
		ATV	EZET	FEN	ATV	EZET	FEN
1.	Range ($\mu\text{g ml}^{-1}$)	2-5	2-5	8-32	2-5	2-5	8-32
2.	Slope	1.000	1.004	0.999	0.998	1.023	0.999
	±SD	6.5×10^{-3}	1.9×10^{-3}	2.1×10^{-3}	2×10^{-3}	0.98×10^{-3}	1.7×10^{-3}
	%RSD	0.65	0.19	0.21	0.20	0.09	0.17
	Standard error	2.7×10^{-3}	8.0×10^{-4}	8.7×10^{-4}	8.2×10^{-4}	4.0×10^{-4}	6.8×10^{-4}
3.	Intercept	0.03	0.04	0.043	0.029	0.042	0.03
	±SD	1.9×10^{-4}	1.9×10^{-4}	1.0×10^{-4}	1.5×10^{-4}	2.1×10^{-4}	0.7×10^{-4}
	%RSD	0.63	0.47	0.23	0.57	0.50	0.23
	Standard error	7.7×10^{-5}	7.8×10^{-5}	4.1×10^{-5}	6.1×10^{-5}	8.6×10^{-5}	2.9×10^{-5}
4.	Correlation	0.999	0.999	1.000	0.999	1.000	0.999
	±SD	1.1×10^{-3}	1.4×10^{-3}	0.9×10^{-3}	1.4×10^{-3}	1.3×10^{-3}	1.4×10^{-3}
	%RSD	0.11	0.14	0.09	0.14	0.13	0.14
	Standard error	4.5×10^{-4}	5.8×10^{-4}	3.6×10^{-4}	5.8×10^{-4}	5.1×10^{-4}	5.8×10^{-4}
5.	Accuracy (%)	100.21	100.26	100.16	100.45	100.04	100.31
	±SD	0.45	0.48	0.37	0.74	0.39	0.45
	%RSD	0.45	0.48	0.37	0.74	0.39	0.45
	Standard error	0.08	0.08	0.06	0.12	0.06	0.08
6.	RMSEP	0.034	0.024	0.045	0.035	0.043	0.059

Table 7: Assay results of the commercial tablet preparation (Lorlip-EZ) by the proposed ILS, CLS chemometric methods and HPLC method

Formulation	Lorlip-EZ								
Method	ILS			CLS			HPLC		
Drugs	ATV	EZET	FEN	ATV	EZET	FEN	ATV	EZET	FEN
Label claim	10	10	160	10	10	160	10	10	160
Amount found*	10.029	10.017	159.712	10.056	9.985	159.996	10.025	9.985	160.067
% Assay	100.29	100.17	99.82	100.56	99.85	99.99	100.25	99.95	100.04
± SD	0.32	0.27	0.19	0.53	0.16	0.25	0.15	0.18	0.09
% RSD	0.32	0.27	0.19	0.53	0.16	0.25	0.15	0.18	0.09
Standard Error	0.13	0.11	0.08	0.22	0.07	0.10	0.06	0.07	0.04
t-value	0.959	0.737	0.624	0.683	0.884	0.882	(2.571) [#]	(2.571) [#]	(2.571) [#]
F-value	0.360	0.332	0.068	0.015	0.597	0.173	(5.050) [†]	(5.050) [†]	(5.050) [†]

* Mean amount found of six determinations

Theoretical t- value at p=0.05 and 95% confidence level

† Theoretical F- value at p=0.05 and 95% confidence level

4. Conclusion

RP-HPLC techniques are generally used for separation and determination of components in final pharmaceutical preparations and are superior with regard to identification and specificity. However, the chemometric methods are less expensive by comparison and do not require sophisticated instrumentation nor any prior separation step. The proposed chemometric-assisted spectrophotometric methods are applicable, prompt, and specific for the simultaneous determination of ATV, EZET and FEN in their synthetic mixtures and commercial pharmaceutical tablets. The results obtained were compared with the proposed RP-HPLC method and good coincidence in the means of recovery was observed as there was no significant difference between the methods compared. The three proposed methods were accurate, precise with good reproducibility and sensitivity; hence can be used for the routine analysis of ATV, EZET and FEN in their combined pharmaceutical formulations.

References

1. Martindale. The complete drug reference. Pharmaceutical Press, 1999;1:1268.
2. Remington. The science and practice of pharmacy. Lippincott Williams & Wilkins, 2006; 1368.
3. Indian Pharmacopoeia. Published by the Government of India, Ministry of Health and Family Welfare, the Indian Pharmacopoeia commission, New Delhi, 2010; Volume II,849.
4. Sever PS, Poulter NR, Dahlof B, Wedel H. Different Time Course for Prevention of Coronary and Stroke Events by Atorvastatin in the Anglo-Scandinavian Cardiac Outcomes Trial–Lipid-Lowering Arm (ASCOT-LLA). *Am J Cardiol.* 2005;96:39-44.
5. Dhaneshwar SR, Yadav S, Mhaske A, Kadam S. HPTLC method for determination of content uniformity of atorvastatin calcium tablets. *Indian J Pharm Sci.* 2005;67:182-186.
6. Chaudhari BG, Patel NM, Shah PB, Modi KP. Development and Validation of a HPTLC method for the simultaneous estimation of Atorvastatin Calcium and Ezetimibe, *Indian J Pharm Sci.* 2006;68(6):793-796.
7. Dhaneshwar SS, Dhaneshwar SR, Deshpande P, Patil M. Development and validation of a method for simultaneous densitometric estimation of atorvastatin calcium and ezetimibe as the bulk drug and in tablet dosage forms, *Acta Chromatographica.* 2007;19:141-148.
8. Jamshidi A, Nateghi AR. HPTLC Determination of Atorvastatin in Plasma. *Chromatographia.* 2007;65:763-766.
9. Shirkhedkar AA, Surana SJ. Development and validation of a reversed-phase high-performance thin-layer chromatography-densitometric method for determination of atorvastatin calcium in bulk drug and tablets. *J AOAC Int.* 2010;93(3):798-803.
10. Farahani H, Norouzi P, Beheshti A, Sobhi HR, Dinarvand R, Ganjali MR. Quantitation of atorvastatin in human plasma using directly suspended acceptor droplet in liquid–liquid–liquid microextraction and high-performance liquid chromatography-ultraviolet detection. *Talanta* 2009;80:1001–1006.
11. Panchal HJ, Suhagia BN. Simultaneous determination of atorvastatin calcium and ramipril in capsule dosage forms by high-performance liquid chromatography and high-performance thin layer chromatography. *J AOAC Int.* 2010;93(5):1450-1457.
12. Bahrami G, Mohammadi B, Mirzaeei S, Kiani A. Determination of atorvastatin in human serum by reversed-phase high-performance liquid chromatography with UV detection. *J Chromatogr B.* 2005;826:41-45.
13. Khedr A. Stability-indicating high-performance liquid chromatographic assay of atorvastatin with fluorescence detection. *J AOAC Int.* 2007;90(6):1547-1553.
14. Panchal HJ, Suhagia BN, Patel NJ, Rathod IS, Patel BH. Simultaneous Estimation of Atorvastatin Calcium, Ramipril and Aspirin in Capsule Dosage Form by RP-LC. *Chromatographia.* 2009;69:91-95.
15. Shah Y, Iqbal Z, Ahmad L, Khan A, Khan MI, Nazir S, Nasir F. Simultaneous determination of rosuvastatin and atorvastatin in human serum using RP-HPLC/UV detection: method development, validation and optimization of various experimental parameters. *J Chromatogr B.* 2011;879:557–563.
16. Londhe SV, Deshmukh RS, Mulgund SV, Jain KS. Development and Validation of a Reversed-phase HPLC Method for Simultaneous Determination of Aspirin, Atorvastatin Calcium and Clopidogrel Bisulphate in Capsules. *Indian J Pharm Sci.* 2011;73(1):23-29.
17. Gupta LK. Spectroscopic characterization and quantitative determination of atorvastatin calcium impurities by novel HPLC method. *Spectrochim Acta A Mol Biomol Spectrosc.* 2012;97:495–501.
18. Kadav A, Vora DN. Stability indicating UPLC method for simultaneous determination of atorvastatin, fenofibrate and their degradation products in tablets. *J Pharm Biomed Anal.* 2008;48(1):120-126.
19. Ravi VB, Mullangi R, Inamadugu JK, Pilli NR, Gajula R, Ponneri V. Simultaneous determination of atorvastatin and niacin in human plasma by LC-MS/MS and its application to a human pharmacokinetic study. *Biomed Chromatogr.* 2012;14.
20. Macwan JS, Ionita IA, Dostalek M, Akhlaghi F. Development and validation of a sensitive, simple, and rapid method for simultaneous quantitation of atorvastatin and its acid and lactone metabolites

- by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Anal Bioanal Chem.* 2011;400(2):423-433.
21. Ghosh C, Jain I, Gaur S, Patel N, Upadhyay A, Chakraborty BS. Simultaneous estimation of atorvastatin and its two metabolites from human plasma by ESI-LC-MS/MS. *Drug Test Anal.* 2011;3(6):352-362.
 22. He BX, Shi L, Qiu J, Zeng XH, Tao L, Li R, Hong CJ, Gu XL, Dong FY, Yang L, Zhao SJ. Quantitative determination of atorvastatin and ortho-hydroxy atorvastatin in human plasma by liquid chromatography tandem mass spectrometry and pharmacokinetic evaluation. *Methods Find Exp Clin Pharmacol.* 2010;32(7):481-487.
 23. Nováková L, Vlcková H, Satínský D, Sadílek P, Solichová D, Bláha M, Bláha V, Solich P. Ultra high performance liquid chromatography tandem mass spectrometric detection in clinical analysis of simvastatin and atorvastatin. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009;877(22):2093-2103.
 24. Liu D, Jiang J, Zhou H, Hu P. Quantitative determination of atorvastatin and para-hydroxy atorvastatin in human plasma by LC-MS/MS. *J Chromatogr Sci.* 2008;46(10):862-866.
 25. Shah RP, Kumar V, Singh S. Liquid chromatography/mass spectrometric studies on atorvastatin and its stress degradation products. *Rapid Commun Mass Spectrom.* 2008;22(5):613-622.
 26. Erk N. Extractive spectrophotometric determination of atorvastatin in bulk and pharmaceutical formulation. *Analytical Letters* 2003;36(12):2699-2711.
 27. Darwish HW, Hassan SA, Salem MY, El-Zeiny BA. Three different spectrophotometric methods manipulating ratio spectra for determination of binary mixture of Amlodipine and Atorvastatin. *Spectrochim Acta A Mol Biomol Spectrosc.* 2011; 83(1):140-148.
 28. Sankar AS, Vetrichelvan T, Venkappaya D. Simultaneous estimation of ramipril, acetylsalicylic acid and atorvastatin calcium by chemometrics assisted UV-spectrophotometric method in capsules. *Acta Pharm.* 2011;61(3):283-296.
 29. Mazurek S, Szostak R, Mazurek S, Szostak R. Quantification of atorvastatin calcium in tablets by FT-Raman spectroscopy. *J Pharm Biomed Anal.* 2009;49:168-172.
 30. Skorda D, Kontoyannis CG. Identification and quantitative determination of atorvastatin calcium polymorph in tablets using FT-Raman spectroscopy. *Talanta* 2008;74:1066-1070.
 31. Kerzner B, Corbelli J, Sharp S, Lipka L, Melani L, LeBeaut A, Suresh R, Mukhopadhyay P, Veltri EP. Ezetimibe Study Group: Efficacy and safety of ezetimibe coadministered with lovastatin in primary hypercholesterolemia. *J. Am. Coll. Cardiol.* 2003;91:418-424.
 32. Darkes MJ, Poole RM, Goa KL. Ezetimibe, *Am J. Cardio Vasc. Drugs.* 2003;3(1):67-76.
 33. Sistla R, Tata V, Kashyap Y, Chandrasekar D. Development and validation of a reversed-phase HPLC method for the determination of ezetimibe in pharmaceutical dosage forms. *J Pharm Biomed Anal.* 2005;39:517-522.
 34. Singh S, Singh B, Bahuguna R, Wadhwa L, Saxena R, Stress degradation studies on ezetimibe and development of a validated stability indicating HPLC assay. *J Pharm Biomed Anal.* 2006;41:1037-1040.
 35. Doshi AS, Kachhadia PK, Joshi HS. Validation of a Stability-Indicating LC Method for Assay of Ezetimibe in Tablets and for Determination of Content Uniformity. *Chromatographia.* 2008;67:137-142.
 36. Kumar DA, Sujan DP, Vijayasree V, E Rao JVLNS. Simultaneous Determination of Simvastatin and Ezetimibe in Tablets by HPLC. *Journal of Chem.* 2009;6:541-544.
 37. Shuijun L, Gangyi L, Jingying J, Xiaochuan L, Chen Y. Liquid chromatography-negative ion electrospray tandem mass spectrometry method for the quantification of ezetimibe in human plasma. *Pharmaceut Biomed Anal.* 2006;40(4-3):987-992.
 38. Oswald S, Scheuch E, Cascorbid I, Siegmund W. A LC-MS/MS method to quantify the novel cholesterol lowering drug ezetimibe in human serum, urine and feces in healthy subjects genotyped for SLCO1B1. *J chromatogr B Analyt Technol Biomed Life Sci.* 2006;830(1):143-150.

39. Sharma M, Mhaske DV, Mahadik M, Kadam SS, Dhaneshwar SR. UV and three derivative spectrophotometric methods for determination of ezetimibe in tablet formulation. *Indian J Pharm Sci.* 2008;70(2):258-260.
40. Raj HA, Rajput SJ. Simultaneous estimation of Ezetimibe and Losavastatin by derivative spectroscopy. *PharmTech.* 2009;1(3):894-899.
41. <http://www.rxlist.com/antara-drug.htm>
42. United States Pharmacopoeia 32 (USP 32). United States Pharmacopeia Convention: Rockville. 2009;pp.2351.
43. The British Pharmacopoeia (2007) British Pharmacopoeial Commission, London. 2007;Vol II:pp.854.
44. Komsta L, Misztal G. Determination of fenofibrate and gemfibrozil in pharmaceuticals by densitometric and videodensitometric thin-layer chromatography. *J AOAC Int.* 2005;88(5):1517-1524.
45. Dixit RP, Barhate CR, Nagarsenker MS. Stability-Indicating HPTLC Method for Simultaneous Determination of Ezetimibe and Simvastatin. *Chromatographia* 2008;67(1-2):101-107.
46. Lacroix PM, Dawson BA, Sears RW, Black DB, Cyr TD, Ethier JC. Fenofibrate raw materials: HPLC methods for assay and purity and an NMR method for purity. *J Pharm Biomed Anal.* 1998;18(3):383-402.
47. Misztal G, Komsta L. Study of the liquid chromatography retention of some fibrate-type antihyperlipidemic drugs on C18 and CN columns: application for quantitation in pharmaceutical formulations. *J AOAC Int.* 2005;88(2):479-89.
48. El-Gindy A, Emara S, Mesbah MK, Hadad GM. Spectrophotometric and liquid chromatographic determination of fenofibrate and vinpocetine and their hydrolysis products. *Farmaco.* 2005;60(5):425-38.
49. Straka RJ, Burkhardt RT, Fisher JE. Determination of fenofibric acid concentrations by HPLC after anion exchange solid-phase extraction from human serum. *Ther. Drug Monit.* 2007;29(2):197-202.
50. Zzaman MT, Khan SA, Arora A, Ahmad O. Method development and validation of Fenofibrate by HPLC using human plasma. *Electron. J. Biomed.* 2009;3:41-54.
51. Kublin E, Kaczmarek-Graczyk B, Malanowicz E, Mazurek AP. Methods of chromatographic determination of medicines decreasing the level of cholesterol. *Acta Pol Pharm.* 2010;67(5):455-461.
52. Bhavesh D, Shah S, Shivprakash. Determination of fenofibric acid in human plasma by ultra-performance liquid chromatography-electrospray ionization mass spectrometry: application to a bioequivalence study, *Biomed Chromatogr.* 2009;23(9):922-928.
53. Trivedi RK, Kallem RR, Mullangi R, Srinivas NR. Simultaneous determination of rosuvastatin and fenofibric acid in human plasma by LC-MS/MS with electrospray ionization: assay development, validation and application to a clinical study. *J Pharm Biomed Anal.* 2005;39(3-4):661-669.
54. Komsta L, Misztal G. Application of UV-derivative spectra for determination of four antihyperlipidaemic drugs in pharmaceutical formulations. *Acta Pol Pharm.* 2004;61(1):9-13.
55. Le Y, Chen JF, Pu M. Electronic structure and UV spectrum of fenofibrate in solutions. *Int. J. Pharm. Sci.* 2008;358(1-2):214-218.
56. Komsta Ł, Misztal G, Majchrzak E, Hauzer A. Separation of fibrate-type antihyperlipidemic drugs by capillary electrophoresis and their quantitation in pharmaceuticals. *J Pharm Biomed Anal.* 2006;41(2):408-414.
57. Koh KK, Quon MJ, Han SH, Chung WJ, Ahn JY, et al. Additive Beneficial Effects of Fenofibrate Combined With Atorvastatin in the Treatment of Combined Hyperlipidemia. *J Am Coll Cardiol* 2005;45(10):1649-1653.
58. Farnier M, Freeman MW, Macdonell G, Perevozskaya I, Davies MJ, et al. Efficacy and safety of the coadministration of ezetimibe with fenofibrate in patients with mixed hyperlipidaemia. *Eur Heart J* 2005;26:897-905.
59. Nikalje AG, Choudhari VP. Validated TLC Method for Simultaneous Quantitation of Atorvastatin, Ezetimibe, and Fenofibrate in Bulk Drug and Formulations. *Acta Chromatographica.* 2011;23(2):267-280.
60. Choudhari VP, Nikalje AG. Simultaneous Estimation of Atorvastatin, Ezetimibe and Fenofibrate in Pharmaceutical Formulation by RP-LC-PDA. *Pharm Anal Acta* 2010;1:111.

61. Patel A, Macwana C, Parmar V, Patel S. Simultaneous determination of atorvastatin calcium, ezetimibe, and fenofibrate in a tablet formulation by HPLC. J AOAC Int. 2012;95(2):419-423.
 62. ICH (ICH Q2B). Validation of Analytical Procedures: Methodology International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland. 1996.
 63. Dinc, E, Onur, F. Application of a new spectrophotometric method for the analysis of a ternary mixture containing metamizol, paracetamol and caffeine in tablets. Anal. Chim. Acta. 1998;359:93-106,
 64. Dinc, E, Baleanu, D. Spectrophotometric quantitative determination of cilazapril and hydrochlorothiazide in tablets by chemometric methods. J. Pharm. Biomed. Anal. 2002;30:715-723.
 65. Pathak A, Rajput SJ. Simultaneous determination of a ternary mixture of doxylamine succinate, pyridoxine hydrochloride, and folic acid by the ratio spectra-zero-crossing, double divisor-ratio spectra derivative, and column high-performance liquid chromatographic methods. J AOAC Int. 2008;91(5):1059-1069.
- Ruikar DB, Rajput SJ, George RK. Chemometric Simultaneous Estimation of Clopidogrel Bisulphate and Aspirin from Combined Dosage Form. Indian J Pharm Sci. 2008;70(4):450-454.



54878478451001254



Submit your next manuscript to *IAJPR* and take advantage of:

- Access Online first
- Double blind peer review policy
- No space constraints
- Rapid publication
- International recognition

Submit your manuscript at: editorinchief@iajpr.com