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A NOVEL REVERSE PHASE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ATORVASTATIN, EZETIMIBE AND FENOFIBRATE IN BULK AND TABLET DOSAGE FORM

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Keywords:

Atorvastatin Calcium, Ezetimibe, Fenofibrate, RP-HPL and Method validation

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ABSTRACT: A novel reverse phase liquid chromatographic method has been developed for the simultaneous estimation of Atorvastatin Calcium, Ezetimibe and Fenofibrate in bulk and Pharmaceutical formulations by using reverse phase Agilent 100-5 C_{18} column [250mm x 4.6mm]. The mobile phase (Methanol: water) in the ratio of 70:30% v/v was pumped at a flow rate of 1ml/min and the column effluents were monitored at 250nm using Variable Wavelength UV detector. Linearity was obtained in the concentration range of 20-100 µg/ml for Atorvastatin and Ezetimibe and 50-250µg/ml for Fenofibrate. The established method was statistically validated according to the ICH Q2B guidelines and the percentage relative standard deviation for precision, robustness and ruggedness was found to be less than 2% indicating high degree of precision and robustness. The percentage recovery for the accuracy was found to be 100.52%, 100.45% and 101.31% for Atorvastatin, Ezetimibe and Fenofibrate respectively which were within the specified limits of recovery. Assay for the marketed formulation proved that 99.86% of Atorvastatin, 99.61% of Ezetimibe and 99.83% of Fenofibrate. Hence due to its simplicity, rapidity, precision and accuracy the developed HPLC method can be applied for the estimation of Atorvastatin, Ezetimibe and Fenofibrate in pure and marketed formulations by a modern analyst.

INTRODUCTION: Atorvastatin calcium is chemically (3R,5R) – 7 - [2 - (4-Fluorophenyl) - 3-phenyl 4 (phenylcarbamoyl)-5-propan-2-ylpyrrol-1 -yl]-3,5-dihydroxyheptanoic acid(Fig.1.a) and is a competitive inhibitor of HMG-CoA reductase. HMC-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, an early rate-limiting step in cholesterol biosynthesis.

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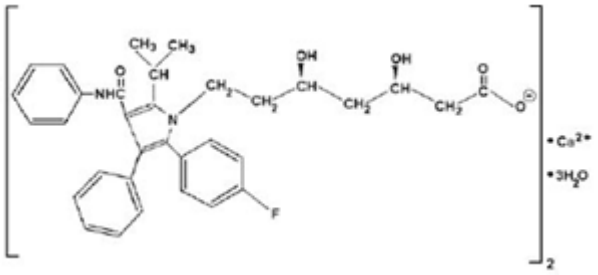
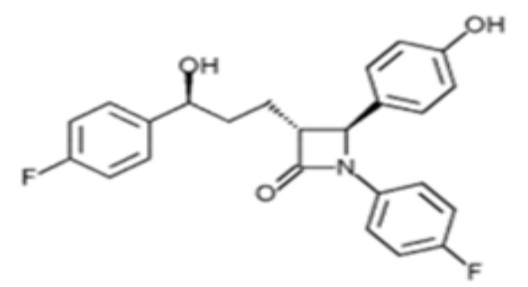
Ezetimibe is (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4 hydroxy phenyl)azetidin-2-one (**Fig.1.b**). Ezetimibe is a selective cholesterol absorption inhibito, which potently and selectively prevents absorption of cholesterol from dietary and biliary sources by preventin transport of cholesterol through the intestinal wall. Fenofibrate is propan-2-yl 2-{4-[(4-chlorophenyl) carbonyl] phenoxy}-2- methyl propanoate (**Fig.1.c**).

The active moiety of Fenofibrate is fenofibric acid. The effects of fenofibric acid seen in clinical practice have been explained *in vivo* in transgenic mice and *in vitro* in human hepatocyte cultures by the activation of peroxisome proliferator activated

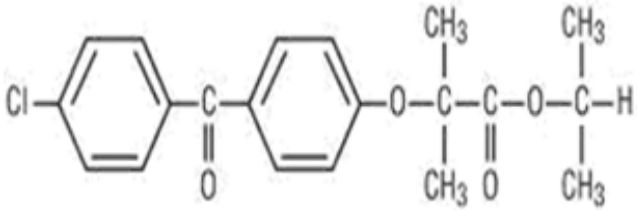
receptor α (PPARα). Through this mechanism, Fenofibrate increases lipolysis and elimination of triglyceride-rich particles from plasma by activating lipoprotein lipase and reducing production of apoprotein C-III (an inhibitor of lipoprotein lipase activity) [1-6].



a) ATORVASTATIN CALCIUM



b) EZETIMIBE



c) FENOFIBRATE

FIG 1: CHEMICAL STRUCTURES of a) ATORVASTATIN CALCIUM b) EZETIMIBE c) FENOFIBRATE

Extensive literature search revealed that very few methods were reported for the estimation of combination of Atorvastatin, Ezetimibe and Fenofibrate [7-21]. The objective of the present work was to design a validation procedure which can determine the three drugs in tablet dosage form with a economical and ecofriendly mobile phase, with good resolution and peak symmetry. The present established liquid chromatographic method was validated by following ICH Q2B guidelines.

MATERIALS AND METHODS:

Equipment used: The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20µl fixed loop. A reverse phase C18[Agilent 100-5 column, 250mm × 4.5 mm]was used. Lab India 3000^{+}double beam UV visible spectrophotometer and Axis AGN204-PO electronic balance was used for spectrophotometric determinations and weighing purposes respectively.

Reagents and chemicals: Pharmaceutical grade pure Atorvastatin from Mylan Laboratories Hyderabad, Ezetimibe and Fenofibrate gift samples were procured from Yarrow chem products, Mumbai. Marketed formulation Tablets with dose of 10mg of Atorvastatin, 10mg of Ezetimibe and 160mg of Fenofibrate were procured from local market. (Mfd. by Sun Pharma). HPLC grade Acetonitrile and Water were procured from Merck specialties private limited, Mumbai.

Chromatographic conditions: Agilent 100-5C_{18} column [250mm x 4.6mm] was used for the chromatographic separation at a detection wave length of 250 nm. Mobile phase of composition Methanol: water in a ratio of 70:30 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was adjusted to 1ml/min and the injection volume was 20µl.

Preparation of Mobile Phase: Mobile phase was prepared by mixing Methanol and Water in the ratio of 70:30 and was initially filtered through 0.45µm Millipore membrane filter and sonicated for 15 min before use.

Preparation of Standard solutions: 25mg each of Atorvastatin, Ezetimibe and Fenofibrate were accurately weighed and transferred into three 25ml volumetric flasks, dissolved using mobile phase and the volume was made up with the same solvent to obtain primary stock solutions A (Atorvastatin), B (Ezetimibe) and C(Fenofibrate) of concentration 1000µg/ml of each drug. From the primary stock solutions, 1ml of each were pipette out from A, B and C respectively, transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 100 µg/ml of each drug individually and this solution is (working stock solution A).

Preparation of Sample Solution: Twenty tablets of Atorvastatin, Ezetimibe and Fenofibrate were weighed and crushed. Tablet powder equivalent to 10mg of Atorvastatin, 10mg of Ezetimibe and 250mg of Fenofibrate was weighed accurately and transferred to a 10ml volumetric flask. The content was dissolved with 5ml of mobile phase and then sonicated for 15min. The volume was made up with the mobile phase and filtered with 0.45µ membrane filter and sonicated for 20min. 1ml of this solution was pipette out and transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain a concentration of 100 µg/ml of Atorvastatin, 100µg/ml of Ezetimibe and 250µg/ml of Fenofibrate (working stock solution B).

Optimization of RP-HPLC method: The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Atorvastatin, Ezetimibe and Fenofibrate. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with Methanol and Water (70:30 v/v) using Agilent 100-$5C_{18}$ column [250mm x 4.6mm].

Validation of the RP-HPLC method: Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability: System suitability was carried out with five injections of solution of 100% concentration having 100µg/ml of Atorvastatin and Ezetimibe and 250µg/ml of Fenofibrate in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factors (T) were reported in **Table 1**.

Linearity: For the determination of linearity, appropriate aliquots were pipetted out from working stock solution A to a series of 10ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 20-100µg/ml of Atorvastatin, 20-100µg/ml of Ezetimibe and 50-250µg/ml of Fenofibrate. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Atorvastatin, Ezetimibe and Fenofibrate were shown in **Fig. 3, 4** and **5**. Their corresponding linearity parameters were given in **Table 2.**

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in **Table 2.**

Precision: The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (100µg/ml of Atorvastatin and Ezetimibe and 250µg/ml of Fenofibrate) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were given in **Table 3.**

Accuracy: To ensure the reliability and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug was added to pre-analyzed sample and contents were reanalyzed by the proposed method and the percent recovery was reported. The results were given in **Table 4.**

Specificity: Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients. The optimized chromatogram of Atorvastatin, Ezetimibe and Fenofibrate without any interference was shown in **Fig. 2.**

Robustness: Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, wave length detection, flow rate, etc. and the % RSD should be reported. Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ±2nm in the detection wave length and ±0.2ml/min in the flow rate, were tried individually. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. %RSD was reported in the **Table 5.**

Assay of Marketed Formulations: 20µl of sample solution of concentration 100µg/ml of Atorvastatin, 100µg/ml of Ezetimibe, and 250µg/ml of Fenofibrate was injected into chromatographic system and the peak responses were measured. The solution was injected three times in to the column. The amount of drug present and percentage purity was calculated by comparing the peak areas of the standards with that of test samples. A typical chromatogram for assay of marketed formulation was shown in **Fig. 6** and the obtained values were reported in the **Table 6**.

RESULTS AND DISCUSSION: After a number of trials with mobile phases of different composition, Methanol and Water in the ratio 70:30v/v was selected as mobile phase because of better resolution and symmetric peaks. Atorvastatin, Ezetimibe and Fenofibrate were found to show appreciable absorbance at 250nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Atorvastatin, Ezetimibe and Fenofibrate at different R_Ts was shown in **Fig. 2.**

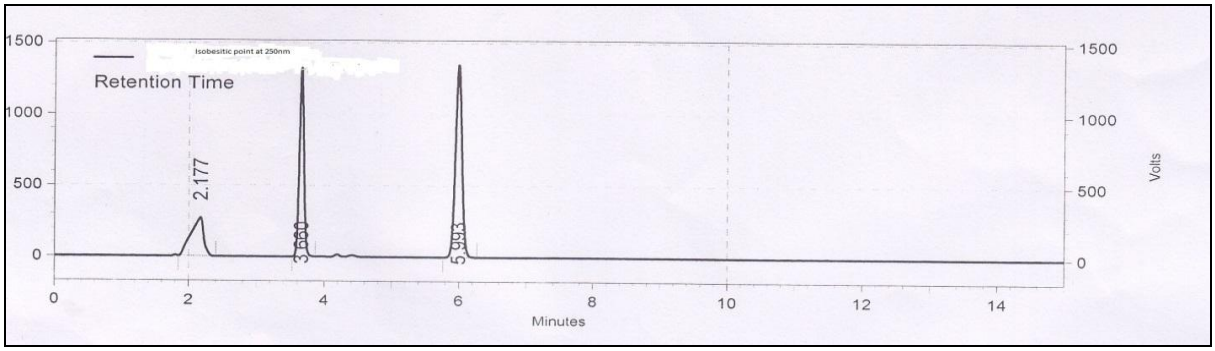


FIG. 2: OPTIMIZED CHROMATOGRAM OF ATORVASTATIN, EZETIMIBE AND FENOFIBRATE

System suitability was carried out by injecting 5 replicate injections of 100% test concentration, number of theoretical plates, HETP and resolution were satisfactory. The chromatograms confirm the presence of Atorvastatin, Ezetimibe and Fenofibrate at 2.1, 3.6min and 5.8min respectively without any interference. The parameters were given in **Table 1.**

TABLE 1: SYSTEM SUITABILITY PARAMETERS (n=5)

Parameters	Atorvastatin	Ezetimibe	Fenofibrate
Retention Time (min)	2.103	3.660	5.987
Tailing Factor (T)	1.3	1.1	1.2
Theoretical Plates (N)	7251	8652	9643
Resolution (R_s)		1.988	

∗n= No. of determinants

Concentration range of 20-100µg/ml for Atorvastatin and Ezetimibe and 50-250µg/ml for Hydrochlorothiazide were found to be linear with correlation coefficients 0.999, 0.998 and 0.999 for Atorvastatin, Ezetimibe and Fenofibrate respectively. Respective calibration curves were shown in **Fig. 3, 4** and **5**. The results were given in **Table 2.**

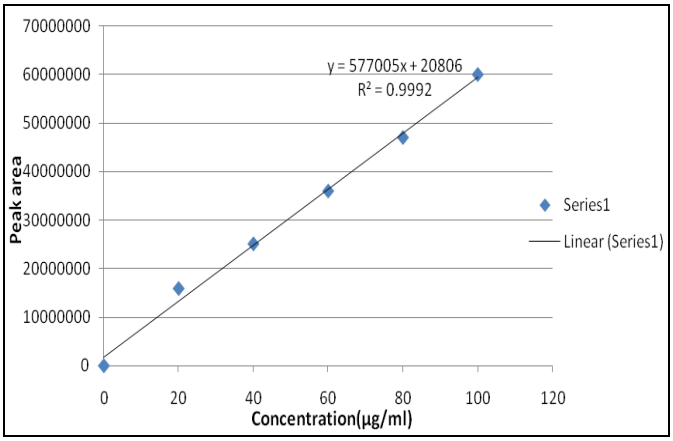


FIG. 3: CALIBRATION PLOT OF ATORVASTATIN

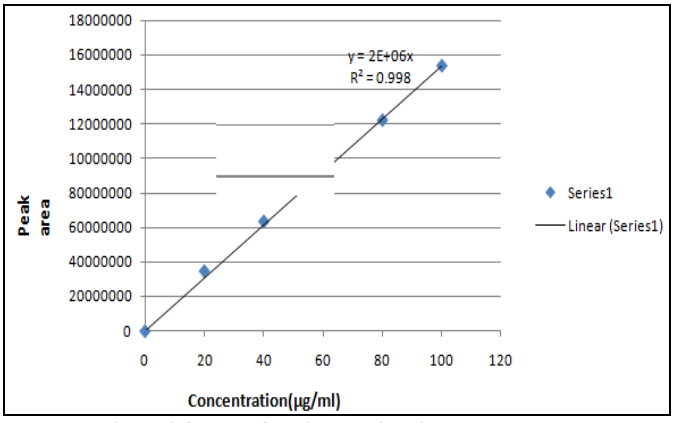


FIG. 4: CALIBRATION PLOT OF EZETIMIBE

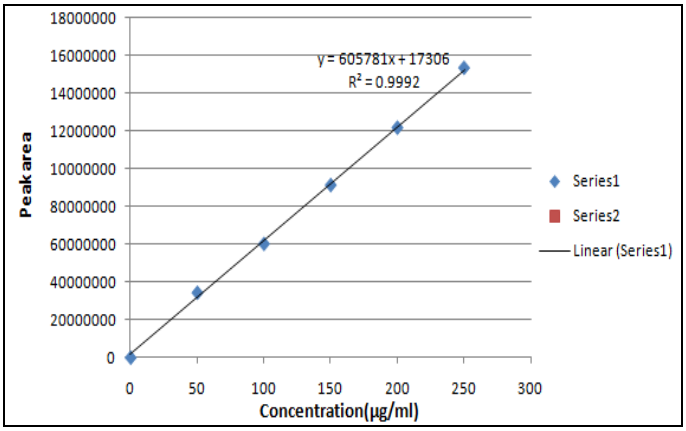


FIG. 5: CALIBRATION PLOT OF FENOFIBRATE

The limits of detection for Atorvastatin, Ezetimibe and Fenofibrate were found to be 0.658µg/ml, 0.544µg/ml and 0.548µg/ml respectively and the limits of Quantitation were 2.171µg/ml, 1.795µg/ml and 1.808µg/ml respectively. Values were represented in **Table 2.**

TABLE 2: RESULTS FOR LINEARITY (n=3)

Parameter	Atorvastatin	Ezetimibe	Fenofibrate
Linearity Range (µg/ml)	20-100	40-120	50-250
Regression Equation	y = 577005x + 20806	y = 863182x+30810	y = 605781x+17306
Slope (m)	577005	863182	605781
Intercept (c)	20806	308106	17306
Regression Coefficient (r^2)	0.9992	0.998	0.9992
Limit of Detection (µg/ml)	0.658	0.544	0.548
Limit of Quantitation (µg/ml)	2.171	1.795	1.808

*n= No. of determinants

The proposed method was found to be precise and reproducible with %RSD of 0.58, 0.77 and 0.86 for Atorvastatin, Ezetimibe and Fenofibrate respectively. %RSD was reported in **Table 3**.

TABLE 3: RESULTS OF PRECISION (n=6)

Drug	Intraday Precision (%RSD)	Interday Precision (%RSD)
Atorvastatin	0.58	0.61
Ezetimibe	0.77	0.79
Fenofibrate	0.86	0.88

*n= No. of determinants

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 99.78% to 100.52% for Atorvastatin, 99.5 to 99.9% for Ezetimibe and 99.3 to 100.5 % for Fenofibrate. This indicates that the method was accurate. Values obtained were given in **Table 4.**

TABLE 4: RESULTS FOR ACCURACY (n=3)

Recovery level	Amount of Standard drug added (µg/ml)			Amount of test added (µg/ml)			Total Amount Recovered (µg/ml)			% Recovery w/w		
	Ator	Ezet	Fen	Ator	Ezet	Fen	Ator	Ezet	Fen	Ator	Ezet	Fen
80%	20	20	50	60	60	150	79.8	79.6	198.7	99.78	99.5	99.3
100%	40	40	100	60	60	150	100.4	99.8	249.6	100.45	99.8	99.8
120%	60	60	150	60	60	150	120.6	120.1	301.5	100.52	99.9	100.5

*n= No. of determinant

The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in **Table 5.**

TABLE 5: RESULTS FOR ROBUSTNESS (n=3)

Parameters (n=3)	%RSD		
	Atorvastatin	Ezetimibe	Fenofibrate
Detection wavelength at 248nm	0.60	0.18	0.751
Detection wavelength at 252nm	0.32	0.75	0.815
Flow rate 0.8ml/min	0.588	0.70	0.744
Flow rate 1.2ml/min	0.604	1.416	0.353

∗n= No. of determinant

The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 98.69% for Atorvastatin, 99.61% for Ezetimibe and 99.56% for Fenofibrate. The typical chromatogram for assay of marketed formulations was shown in **Fig.6** and Values obtained were given in **Table 6.**

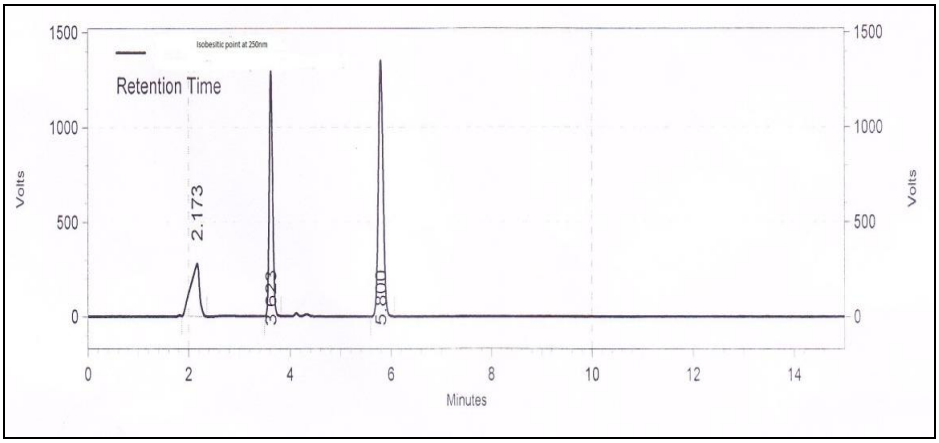


FIG. 6: A TYPICAL CHROMATOGRAM FOR ASSAY OF MARKETED FORMULATION CONTAINING 10µg/ml OF ATORVASTATIN, 10µg/ml OF EZETIMIBE AND 250µg/ml OF FENOFIBRATE

TABLE 6: RESULTS FOR ASSAY (n=3) OF MARKETED FORMULATION

S. no.	Label claim (mg/tab)			Amount Recovered (mg/tab)			% Amount found in drug		
	ATV	EZT	FEN	ATV	EZT	FEN	ATV	FEN	ATV
1	10	10	160	9.869	9.961	159.3	98.69	99.61	99.5

∗n= No. of determinants

CONCLUSION: The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Atorvastatin, Ezetimibe and Fenofibrate from their formulations. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the method was found to be simple, accurate, precise, rugged, robust, economical and ecofriendly. So the established method can be employed in the routine analysis of the marketed formulations.

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REFERENCES:

1. Mendham, Deny and Thomas. Vogels Textbook of Quantitative Analysis. Pearson Education Limited. 6th Edition 2004.
2. A.H.Beckett and J.B. Stenlake. Practical Pharmaceutical Chemistry. CBS Publishers and Distributors, 4th Edition 2002.
3. ICH: Q2 (R1), Validation of Analytical Procedures. Text and Methodology 2005.
4. Indian Pharmacopoeia. Indian Pharmacopoeia Commission, Ghaziabad, Vol. II 2014.

5. The Merck Index. An encyclopedia of Chemicals, Drugs and Biologicals. Merck research laboratories, 14th Edition 2013.
6. ICH Harmonized Tripartite Guideline. Validation of Analytical Procedure Methodology. Q2B 1996.
7. Swetha Chandrika Gurram. RP-HPLC Method Development and Validation for the Simultaneous Estimation of Atorvastatin, Fenofibrate and Ezetimibe in a Pharmaceutical Dosage Form. Biochem Anal Biochem 2015; 4 (2):1-4.
8. Patel A, Macwana C, Patel S. Simultaneous determination of Atorvastatin calcium, Ezetimibe, and Fenofibrate in a tablet formulation by HPLC. J AOAC Int. 2014; 95(2): 419-423.
9. S. Ashutosh Kumar, Manidipa Debnath, J.V.L.N. Seshagiri Rao and D. Gowri Sankar. New Validated Stability-Indicating RP-HPLC Method for Simultaneous Estimation of Atorvastatin and Ezetimibe in Human Plasma by Using PDA Detector. Adv Pharm Bull 2015; 5(3): 385–391.
10. S. Sahu, UL. Narayan, B. Garnaik and SK. Patro. Stability Indicating RP-HPLC Method for Determination of Ezetimibe in Pure and Pharmaceutical Formulation. International Journal of Research in Pharmaceutical and Biomedical Sciences 2013; 4(4): 1249-1255.
11. K Raja Rajeswari, GG Sankar, AL Rao and JVLN Seshagirirao. RP-HPLC method for the simultaneous determination of Atorvastatin and Amlodipine in tablet dosage form. Indian Journal of Pharmaceutical Sciences 2006; 68 (2); 275-277.
12. Amit Goel, Sanjula Baboota, Jasjeet K. Sahni, Kona S. Srinivas, Ravi S. Gupta, Vinod P. Semwal, and Javed. Ali. Development and Validation of Stability-Indicating Assay Method by UPLC for a Fixed Dose Combination of Atorvastatin and Ezetimibe. Journal of Chromatographic Sciences 2013; 51(3): 222-228.
13. Dillip Kumar Sahoo, Prafulla Kumar Sahu and Chandra Sekhar Patro. New validated isocratic RP-HPLC method for assay of Fenofibrate. International Journal of Pharmacy and Pharmaceutical Sciences 2014; 6(2):169-172.
14. G.krishnaveni and PVV Satyannarayana. Method Development and Validation for Simultaneous determination of Ezetimibe and Simvastatin in combined Pharmaceutical dosage form by RP-HPLC method. International Journal of Pharmaceutical and Life Sciences 2013; 2(2): 60-69.
15. Tarek S. Belal, Hoda G. Daabees, Magdi M. Abdel-kalek, Mohamed S. Mahrous and Mona M. Khamis. New simple spectrophotometric method for determination of the binary mixtures atorvastatin calcium and ezetimibe; candesartan cilexetil and hydrochlorothiazide in tablets. Journal of Pharmaceutical Analysis 2013; 3(2): 118–126.
16. Praveen Kumar, Yusra Ahmad and Amitav Ghosh. A stability indicating RP-HPLC method development for determination of Ezetimibe in tablet dosage form. Der Pharma Chemica 2012; 4(4): 1415-1423.
17. R N.Kane, PS. Bhokare and DS. Desai. Simultaneous Spectrophotometric Estimation of Atorvastatin and Fenofibrate in Bulk Drug and Dosage Form by Using Dual Wavelength Method. International Journal of Research in Pharmaceutical and Biomedical Sciences 2012; 3(4): 1448-1453.
18. R Savithri, N Sai Sree Bindu, P Shiva Bhargavi, D H Theja and P Ramalingam. Dual Wavelength UV - Spectrophotometric Method for Simultaneous Estimation of Atorvastatin and Ezetimibe in bulk and their combined Tablet Dosage Form. Der Pharmacia Sinica 2011; 2(5): 251-258.
19. Ankit Ajmera, Shrikalp Despande, Pranav Patel, Keyur Patel, Sagar Solanki and Kinjal Rathod. Reverse phase high performance liquid chromatographic (HPLC) Method for simultaneous determination of Atorvastatin, Ezetimibe and Fenofibrate in commercial tablets. International Journal of Pharmacy and Pharmaceutical Sciences 2012; 4(1): 206-209.
20. R R Sevda, A S Ravetkar and P J Shirote. UV Spectrophotometric estimation of Rosuvastatin Calcium and Fenofibrate in bulk Drug and Dosage Form using Simultaneous Equation Method. Int.J. ChemTech Res 2011; 3(2): 629-635.
21. Jayapal Reddy Sama, K. Rama Rao, V. Surya Narayana Rao and P. Reddanna. Simultaneous Estimation of Atorvastatin and Ezetimibe in Pharmaceutical Formulations by RP-HPLC Method. Der Pharmacia Lettre 2010; 2 (1): 427-436.

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