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Research Article

New Validated Stability-Indicating Rp-HPLC Method for Simultaneous Estimation of Atorvastatin and Ezetimibe in Human Plasma by Using PDA Detector

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Abstract

Purpose: This paper describes a simple, precise and accurate RP-HPLC method for simultaneous estimation of atorvastatin and ezetimibe in plasma.

Methods: The chromatographic separation of the drugs were performed on an X-Terra C_8 (4.6 x 150 mm, 3.5 µm), with phosphate buffer [pH 3.5 with Ortho Phosphoric Acid] – acetonitrile 40:60 (v/v) as mobile phase. The detection was performed at 235 nm. The flow rate was maintained at 1.2 mL/min. The run time was 8.0 min.

Results: The accuracy and reliability of the method was assessed by evaluation of linearity (5-25 µg/mL for both atorvastatin calcium and ezetimibe), precision (intra-day RSD 0.57 % and inter-day RSD 0.02 % for atorvastatin calcium and intra-day RSD 0.56 % and inter-day RSD 0.1 % for ezetimibe), accuracy (100.08- 100.84 % for atorvastatin calcium and 100.56- 101.00 % for ezetimibe), and specificity, in accordance with ICH guidelines. The LLOQ obtained by the proposed method were 1.294 and 1.384 µg/mL for atorvastatin and ezetimibe respectively.

Conclusion: Overall the proposed method was found to be suitable and accurate for the quantitative determination in plasma. The method was effectively separated the drug from plasma.

Introduction

Atorvastatin (ATV)[1] is chemically [R-(R*, R*)]-2-(4-flurophenyl)-β, δ-dihydroxy-5- (1-methylethyl)-3-phenyl-4-[(phenyl amino) carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt trihydrate. Atorvastatin calcium is an inhibitor of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase. This enzyme catalyses the conversion of HMG-CoA to mevalonate, an early and rate limiting step in cholesterol biosynthesis. Ezetimibe (EZE)[2,3] is [(3R, 4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone. It is a selective cholesterol absorption inhibitor used in the treatment of primary hypercholesterolemia. It inhibits the absorption of biliary and dietary cholesterol from small intestine without affecting absorption of fat soluble vitamins, triglycerides and bile acids. Ezetimibe does not have significant pharmacokinetic interactions with other lipid lowering drugs as it does not influence the activity of cytochrome P450. EZE is administered at the dose of 10 mg with and without atorvastatin. A literature survey regarding quantitative analysis of these drugs revealed that attempts were made to develop analytical methods for atorvastatin using extractive spectrophotometry, HPLC, HPTLC, UPLC.[4-12] A liquid chromatography/ mass spectrometry method for the simultaneous quantitation of rosuvastatin and ezetimibe in human plasma was reported.[13] LC and UPLC MS-MS simultaneous determination of atorvastatin and ezetimibe in human plasma were also reported in the literature.[14,15]

From the above literature, it was found that, there are lots of chromatographic methods available for the simultaneous estimation of atorvastatin and ezetimibe in their combined dosage form along with other drugs also; this work holds a challenge for developing a new method in high performance liquid chromatography. Moreover, among the existing liquid chromatographic methods, there exists no method in which the drugs were eluted below 4 min. Hence HPLC was selected in order to reduce the elution time of both the drugs which in turn reduce the consumption of mobile phase and time of analysis.

Hence, the objective of this work was to develop a simple, precise, reliable and rapid high performance liquid chromatographic analytical method for simultaneous estimation of atorvastatin and ezetimibe in plasma, to validate the method in accordance with ICH[16-22] guidelines. The validation procedure followed the

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guidelines of USP. The chemical structures were represented in Figure 1.

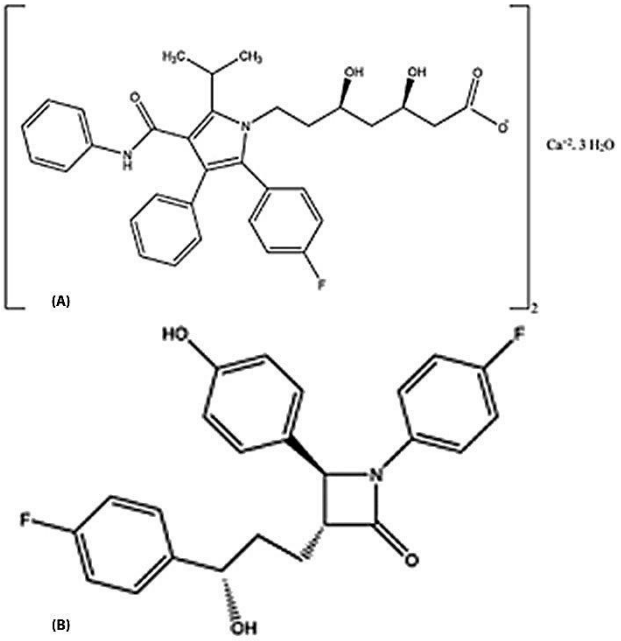

Figure 1. Chemical structures of (A) Atorvastatin and (B) Ezetimibe

Materials and Methods[23]

Chemicals and Reagents Used

Atorvastatin and Ezetimibe [working standards] were obtained from M/s. Pharma Train, Hyderabad, Telagana, India. The commercially available formulation brand name Liptruzet (Atorvastatin 10 mg and Ezetimibe 10 mg) manufactured by Merck Sharp & Dohme Corp., Mumbai., was purchased from the local market. The HPLC grade water was obtained from Millipore. Methanol of HPLC grade was obtained from E. Merck. (India) Ltd., Mumbai. Ortho phosphoric acid of analytical grade was purchased from Standard Solutions, Hyderabad, Telagana, India. The processed plasma was collected from M/s. Pharma Train, Hyderabad, Telagana, India.

0.45 µ membrane filters (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) were used for filtration of various solvents and solutions intended for injection into the column.

Apparatus and Chromatographic Conditions

The equipment used was High Performance Liquid Chromatography Equipped with Auto Sampler and DAD or UV Detector. The column X-Terra C_8 (4.6 x 150 mm, 3.5 µm) was selected. The flow rate was monitored at 1.2 mL per min. The detection was carried out at 235 nm. The injection volume selected 20 µL, the temperature of the column oven was maintained at 25 °C, the detector used was Photo diode array and the run time was 8.0 min.

The ultra violet spectra of the drugs used for the investigation were taken on a Lab India UV 3000 spectrophotometer for finding out their λ_{max} values.

Solubility of the compounds was enhanced by sonication on an ultra sonicator (Power Sonic 510, (Hwashin Technology).

All the weighings in the experiments were done with an Afcoset electronic balance. The Hermle microlitre centrifuge Z100 (model no 292 P01) was used for the centrifugation process and Remi equipments (model no-CM101DX) Cyclomixer was used.

Glassware

All the volumetric glassware used in the study was of Grade A quality Borosil.

Preparation of Phosphate buffer[24]

The buffer solution was prepared by weighing 7.0 grams of KH_2PO_4 into a 1000mL beaker, dissolved and diluted to 1000mL of water [HPLC grade]. Then the pH was adjusted to 3.5 with ortho phosphoric acid.

Preparation of mobile phase

The mobile phase was prepared by mixing a mixture of above buffer 400 mL (40 %) and 600 mL of acetonitrile HPLC (60 %) and degas in ultrasonic water bath for 5 minutes. Then, the solution was filtered through a 0.45 µ filter under vacuum filtration.

Preparation of standard solution of atorvastatin and ezetimibe

About 10 mg atorvastatin was weighed accurately and transferred into a 100 mL clean and dry volumetric flask. Initially, the drug was mixed with 70 mL of diluent. The solution was sonicated for 15 min for complete dissolution of the drug. The final volume was made up to the mark with the same solvent. Similarly, about 10 mg ezetimibe was weighed accurately and transferred into a 100 mL clean and dry volumetric flask. Initially, the drug was mixed with 70 mL of diluent. The solution was sonicated for 15 min for complete dissolution of the drug. The final volume was made up to the mark with the same solvent.

From the above prepared stock solutions 1.5 mL of atorvastatin and 1.5 mL ezetimibe were pipetted out into a 10 mL clean and dry volumetric flask and it was diluted up to the mark with diluent. This mixed stock solution contains 15.0 µg/mL of atorvastatin and 15.0 µg/mL of ezetimibe.

Spiking of atorvastatin and ezetimibe into plasma and their extraction from plasma

From the above prepared mixed stock solution (15.0 µg/mL of atorvastatin and 15.0 µg/mL of ezetimibe), 0.5 mL was pipetted out and spiked into 0.5 mL of plasma in a polypropylene tube (Torson's). Then the tube was cyclo mixed for 5 min. Then 1.0 mL of acetonitrile was added to the tube and centrifuged for 20 min at 3000 rpm. Further the supernatant liquids were collected in another Eppendorf tube and 20 µL supernatant was injected into the analytical column.

Protein precipitation

When a drug strongly binds to the plasma proteins (in case of plasma samples) it is often difficult extract the drug from plasma by any means. Then protein precipitation followed by extraction is only the process to extract the drug from plasma samples. This separation technique removes proteins from the samples by denaturating them directly. The protein precipitation is usually done by the addition of a water miscible organic solvent (e.g. methanol, ethanol, acetonitrile or acetone) or a strong acid such as trichloroacetic acid. The denatured proteins are then removed from the sample by centrifugation. Efficient centrifugation will give clear and safe samples for injection.

Validation Development

Selectivity

An aqueous mixture of atorvastatin and ezetimibe (15.0 µg/mL of atorvastatin and 15.0 µg/mL of ezetimibe)was prepared and injected into the column and the retention times were checked and any interference at the retention times were checked by comparing the response in the blank. No interference was observed at the retention times for atorvastatin and ezetimibe extracted from plasma. The method was found to be precise and specific. A typical overlay chromatogram of atorvastatin and ezetimibe in plasma is shown in Figure 2.

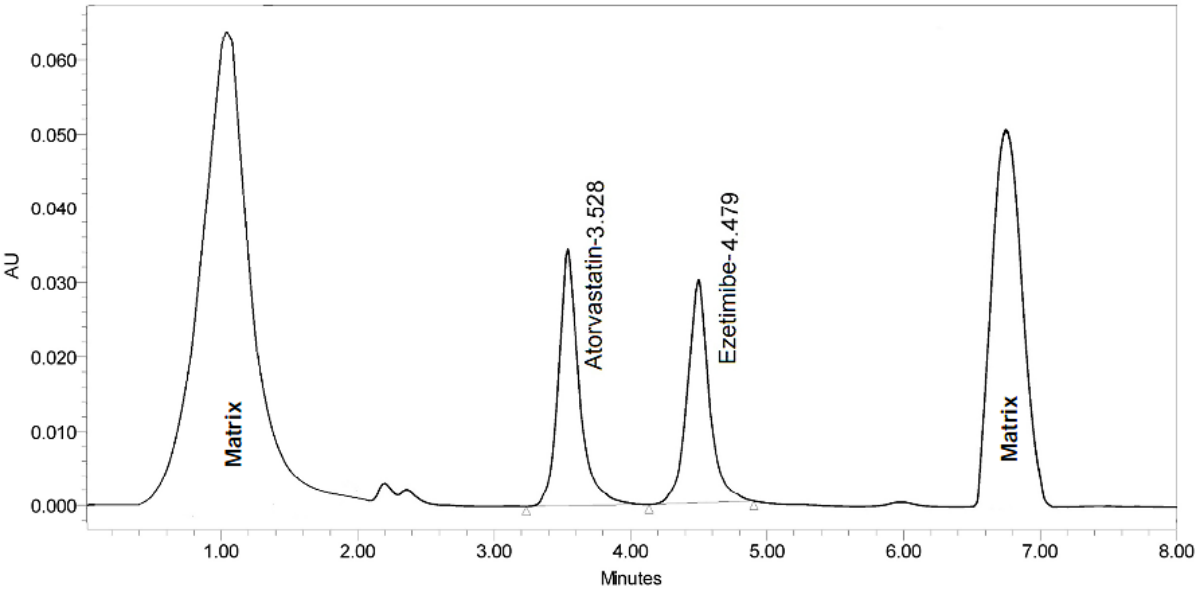

Figure 2. A typical overlay chromatogram of Atorvastatin and Ezetimibe in plasma

Sensitivity

To determine the sensitivity in terms of LLOQ, 'Lower Limit of Quantification' where the response of LLOQ must be at least five times greater than the response of interference in blank matrix at the retention time of the analyte(s). The LLOQ obtained by the proposed method were 1.294 and 1.384 µg/mL for atorvastatin and ezetimibe respectively.

Precision

To check the intra and inter-day variations of the method, solutions containing 15.0 µg/mL of atorvastatin and 15.0 µg/mL of ezetimibe were subjected to the proposed HPLC method of analysis and results obtained were noted. The precision of the proposed method i.e. the intra and inter-day variations in the peak areas of the drugs solutions in plasma were calculated in terms of percent RSD and the results are represented in Table 1 and 2. A statistical evaluation revealed that the relative standard deviation of the drugs at linearity level for 6 injections was less than 2.0.

Table 1. Intra-day precision of the proposed method for Atorvastatin and Ezetimibe in plasma

Injection	Retention Time for Atorvastatin	Area for Atorvastatin	Retention Time for Ezetimibe	Area for Ezetimibe
Injection-1	3.538	36949	4.470	37329
Injection-2	3.538	37126	4.469	37509
Injection-3	3.536	37001	4.468	37379
Injection-4	3.535	36821	4.466	37217
Injection-5	3.535	36665	4.467	36987
Injection-6	3.541	36566	4.474	37003
Average	3.537	36854.7	4.469	37237.3
S.D	0.002	211.8	0.003	209.9
% RSD	0.1	0.57	0.1	0.56

Table 2. Inter-day precision of the proposed method for Atorvastatin and Ezetimibe (on three consecutive days n = 6) in plasma

Days	Retention Time for Atorvastatin	Area for Atorvastatin	Retention Time for Ezetimibe	Area for Ezetimibe
Day-1*	3.542	36729	4.484	37044
Day -2*	3.539	36719	4.484	37013
Day -3*	3.541	36734	4.485	36997
Average	3.540	36727	4.484	37018
S. D	0.001	7.6	0.001	23.9
%RSD	0.04	0.02	0.013	0.1

*Average of Six injections

Accuracy

To determine the accuracy of the proposed method, recovery studies were carried out by analyzing (8.0, 10.0, 12.0 mg of atorvastatin and ezetimibe) of pure drugs. The drugs solutions were diluted at linearity level (15 µg/mL of atorvastatin and 15 µg/mL of ezetimibe).Then each dilution was injected thrice (n=3). The percent recoveries of the drugs were determined. The results are shown in Table 3 and 4.

Table 3. Accuracy data of the proposed method for Atorvastatin in plasma

Conc. Level	% Recovery	Avg. % Recovery	Amount Recovered	SD	% RSD
80%	101.39 99.32 100.34	100.35	8.11 7.95 8.03	0.080	1.0
100%	101.39 98.50 100.34	100.08	10.14 9.85 10.03	0.066	1.46
120%	101.39 100.78 100.34	100.84	12.17 12.09 12.04	0.066	0.54

Table 4. Accuracy data of the proposed method for Ezetimibe in plasma

Conc. Level	% Recovery	Avg. % Recovery	Amount Recovered	SD	% RSD
80%	101.50 100.86 100.55	100.97	8.12 8.07 8.04	0.04	0.04
100%	100.28 100.86 100.55	100.56	10.03 10.09 10.06	0.040	0.50
120%	101.59 100.86 100.55	101.00	12.19 12.1 12.07	0.062	0.52

Linearity

In order to find out the linearity range of the proposed HPLC method in plasma, curves were constructed by plotting peak areas obtained for the analyte against their concentrations. A good linear relationship (r^2=0.994) was observed between the concentrations of atorvastatin and ezetimibe and their corresponding peak areas. The relevant regression equations were y = 2442.x + 467(r^2=0.994) for atorvastatin and y = 2520.x - 343.8 (r^2=0.995) for ezetimibe (where y is the peak area and x is the concentration of atorvastatin and ezetimibe (µg/mL)). The slope, intercept and the correlation coefficient of the plots are shown in Table 5.

Stability[25]

All stability determinations used a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. The stock solutions of the analyte for stability evaluation were prepared in an appropriate solvent at known concentrations. To test the stability of the drug extract, it was subjected to

(a) Freeze and thaw stability at -20 °C ± 2 °C,
(b) Short term stability for period of 24 hours stored at room temperature,
(c) Long term stability for period of 15 days stored at 4°C.
Similar to the preparation of the standard preparation, the above samples were spiked into the plasma and extracted and collected in vial and injected into HPLC system. All the stability samples compared against the standard stock solution assessed for stability. The results are presented in Table 6 and 7 (the Figures in the Table are in peak area units).

Table 5. Linearity range of Atorvastatin and Ezetimibe in plasma

Concentration (µg/mL)	Area for Atorvastatin	Statistical Analysis for Atorvastatin	Area for Ezetimibe	Statistical Analysis for Ezetimibe
5.0	13361		12829	
10.0	26258	Slope= 2442	25249	Slope= 2520
15.0	39186	Intercept= 467	36598	Intercept=343.8
20.0	52356	C. C= 0.994	48355	C.C= 0.995
25.0	65381		64289	

Table 6. The Stability data for Atorvastatin in plasma

Sr. No.	Standard Sample	Freeze and Thaw Stability Sample	Short Term Stability Sample	Long Term Stability Sample
1.	36180	35673.48	35439.44	35298.42
2.	36108	35687	35418	35284
3.	36189	35684	35458	35274
Mean	36159	35681	35438	35285
SD	44	7	20	12
% RSD	0.12	0.02	0.06	0.03
Assay	-	98.68	98.01	97.58

Table 7. The Stability data for Ezetimibe in plasma

Sr. No.	Standard Sample	Freeze and Thaw Stability Sample	Short Term Stability Sample	Long Term Stability Sample
1.	36592	35898	35857.25	35614.62
2.	36584	35882.12	35025	35658
3.	36554	35785	35865	34915
Mean	36577	35855	35582	35396
SD	20	61	483	417
% RSD	0.05	0.17	1.36	1.18
Assay	-	98.03	97.34	96.77

Results and Discussion

To optimize the mobile phase, various proportions of phosphate buffer (pH 3.5) with acetonitrile (HPLC Grade) were tested. The use of phosphate buffer (pH 3.5) and acetonitrile (HPLC Grade) in the ratio of 40:60 (v/v) resulted in peak with good shapes and resolution. A flow rate of 1.2mL /min was found to be optimum in the 0.4-1.5 mL/min range resulting in short retention time, baseline stability and minimum noise.
The LLOQ obtained for atorvastatin and ezetimibe by the proposed method in plasma were 1.294 and 1.384 µg/mL respectively. The retention times obtained for atorvastatin and ezetimibe in plasma were observed at 3.528 and 4.479 min respectively. Quantitative linearity of drugs in plasma was obeyed in the concentration ranges of 5.0-25.0 µg/mL for atorvastatin and 5.0-25.0 µg/mL for ezetimibe respectively. The relevant regression equations were y = 2442.x + 467for atorvastatin (r^2=0.994) and y = 2520.x - 343.8 for ezetimibe (r^2=0.995) (where y is the peak area and x is the concentration of atorvastatin and ezetimibe (µg/mL))**.**The intra-day and inter-day drugs variations in plasma by the proposed method in plasma showed an RSD less than 2 %, indicating that the method is precise. The corresponding mean recoveries of the drugs in plasma were 100.08-101.00 %. This reveals that the method is quite accurate. The RSD obtained for the drugs spiked in plasma for stability studies were less than 2 %.

Conclusion

The proposed HPLC method was found to be simple, precise, accurate and sensitive for the simultaneous determination of atorvastatin and ezetimibe. The method

was validated as per ICH guidelines and all the parameters met within the acceptance criteria. Applicability of this method for simultaneous estimation of atorvastatin and ezetimibe in plasma was confirmed. Hence, this method is specific and can be easily and conveniently adopted for routine quality control analysis of the above drugs.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

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