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Development and validation of a reversed-phase HPLC method for the determination of ezetimibe in pharmaceutical dosage forms

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Abstract

Ezetimibe belongs to a group of selective and very effective 2-azetidione cholesterol absorption inhibitors that act on the level of cholesterol entry into enterocytes. A rapid, specific reversed-phase HPLC method has been developed for assaying ezetimibe in pharmaceutical dosage forms. The assay involved an isocratic elution of ezetimibe in a Kromasil 100 C_{18} column using a mobile phase composition of water (pH 6.8, 0.05%, w/v 1-heptane sulfonic acid) and acetonitrile (30:70, v/v). The flow rate was 0.5 ml/min and the analyte monitored at 232 nm. The assay method was found to be linear from 0.5 to 50 μg/ml. All the validation parameters were within the acceptance range. The developed method was successfully applied to estimate the amount of ezetimibe in tablets.

Keywords: Ezetimibe; Reversed-phase HPLC; Hypercholesterolemia; Tablets

1. Introduction

Ezetimibe (SCH 58235) [(3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone] (Fig. 1) is a selective cholesterol absorption inhibitor, which potently inhibits the absorption of biliary and dietary cholesterol [1] from the small intestine without affecting the absorption of fat-soluble vitamins [2], triglyceride or bile acids. Ezetimibe reduces the small intestinal enterocyte uptake and absorption of cholesterol that keeps the cholesterol in the intestinal lumen for excretion [3]. Ezetimibe is rapidly absorbed and primarily metabolized in the small intestine and liver to its glucuronide, both of which undergo enterohepatic recycling in humans [4,5]. Since ezetimibe does not influence the activities of CYP 450 enzymes, significant pharmacokinetic interactions with other medications including statins, fibrates, digoxin and warfarin have not been found [6]. Ezetimibe complements the lipid lowering effects of other therapies, such as statins. Clinical studies have shown that co-administration of ezetimibe with statins could provide significant reductions in both the low-density lipoproteins (LDL) and the total cholesterol with slight increase in the high-density lipoproteins (HDL) [7–10]. Also co-administration of ezetimibe with statins could significantly reduce the risk of coronary heart disease (CHD) events in patients with hypercholesterolemia [11]. Hence, ezetimibe is administered at a dose of 10 mg once daily either alone or in combination with an HMG-CoA reductase inhibitor as adjunctive therapy to diet for reduction of elevated total cholesterol, LDL cholesterol and APO B in patients with primary hypercholesterolemia. It is available in the market as 10 mg tablets (Zetia, Merck/Schering-Plough Pharmaceuticals, USA; Ezzicad, Glen mark Pharmaceuticals, Mumbai, India; Ezta, Hetero Drugs Pvt. Ltd., Hyderabad, India).

No chromatographic methods have been published till date for the quantitation of ezetimibe except for a pharmacokinetic study in which ezetimibe and its glucuronide conjugate (ezetimibe-glucuronide) were determined in human plasma using a liquid chromatography-mass spectrometric method [12]. Quality control has become a stringent tool in

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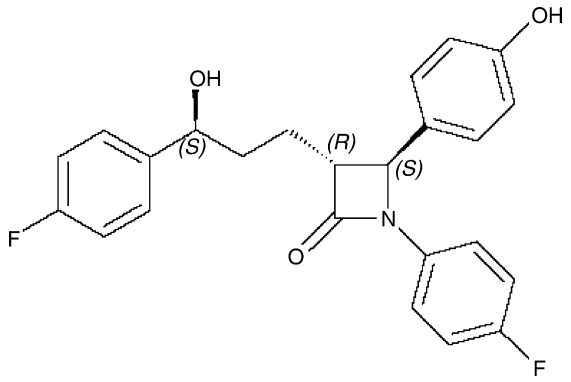

Fig. 1. Structure of ezetimibe (SCH 58235), 1-(4-fluorophenyl)-3-[3-(4-fluorophenyl)-3-hydroxy-propyl]-4-(4-hydroxy-phenyl)-azetidin-2-one.

pharmaceuticals in order to minimize batch-to-batch variation and assure quality. Since there are no official methods available for the estimation of ezetimibe, there is an immense need to develop a sensitive, specific and validated analytical method for the routine analysis of the drug in pharmaceutical dosage forms.

In the present study, a rapid, specific, precise and validated HPLC method for the quantitative estimation of ezetimibe in pharmaceutical dosage forms is reported.

2. Experimental

2.1. Chemicals and reagents

Ezetimibe (99.5% pure) was a gift sample from MSN Laboratories (Hyderabad, India). Amoxycillin trihydrate (99.72% pure, Internal standard) was purchased from Ranbaxy Laboratories (New Delhi, India). Acetonitrile (HPLC grade) was obtained from Qualigens Fine Chemicals (Mumbai, India). 1-Heptane sulfonic acid was purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade deionized water (NanoPure Diamond, Barnstead Thermolyne, USA) was used throughout the analysis. All the other chemicals used were of analytical grade.

2.2. Instrumentation

The HPLC system consisted of a Waters Alliance (Waters Corporation, MA, USA) equipped with a Waters 2695 solvent delivery module in a quaternary gradient mode and a Waters 2669 PDA detector. Data acquisition was performed by the Millennium 32® bit software operated on a Pentium® IV microprocessor. Analysis was carried out at 232 nm with a Kromasil 100 C_{18} reversed-phase column of 250 mm × 4 mm i.d., 5 μm dimensions (VDS Optilab, Chromatographie technik GmbH, Germany) at ambient temperature. The mobile phase consisted of water (with 0.05%, w/v of 1-heptane sulfonic acid, pH 6.8) and acetonitrile (30:70, v/v) that was set at a flow rate of 0.5 ml/min.

2.3. Preparation of solutions

2.3.1. Drug stock solution and internal standard

Stock solution of ezetimibe was prepared by dissolving accurately weighed 10 mg of the drug in 10 ml of acetonitrile (final concentration, 1 mg/ml). The prepared stock solution was stored at 4 °C protected from light. Amoxycillin trihydrate (IS) stock solution was made at a final concentration of 1 mg/ml using acetonitrile: water (50:50, v/v) and stored at 4 °C protected from light. From this stock solution standard 100 μg/ml was freshly prepared during the analysis day.

2.3.2. Calibration standards and quality control samples

Calibration standards were prepared at concentrations of 0.5, 1, 2, 3, 5, 8, 10, 20 and 50 μg/ml from a standard solution of 100 μg/ml by appropriate dilution with mobile phase. Four quality control (QC) samples at the concentrations of 0.5, 1.5, 25 and 40 μg/ml representing the LLOQ, low, medium and high concentrations, respectively, of the linearity range were prepared from the standard solution.

2.4. Sample preparation

For the preparation of linearity curve, calibration standards were added with 100 μl of IS (final concentration, 10 μg/ml) and the volume made up to 1 ml with the mobile phase to get the linearity range of 0.5, 1, 2, 3, 5, 8, 10, 20 and 50 μg/ml. Concentrations of 1.5, 25.0 and 40.0 μg/ml were taken as quality control samples. An aliquot of 20 μl of this solution was injected for the HPLC analysis. For the estimation in dosage form, 20 tablets from each batch were randomly selected and powdered. Amount equivalent to 10 mg of ezetimibe from powdered formulation was accurately weighed and taken in an extraction flask; suitable quantity of IS was added followed by 10 ml of acetonitrile. This mixture was subjected to vigorous shaking for 30 min for complete extraction of the drug, and then centrifuged at 10,000 rpm for 10 min (Biofuge Stratos Heraeus, Kendro Laboratory Products, Germany). The clear supernatant was taken and diluted with mobile phase (1/100) and 20 μl of this solution was injected for HPLC analysis.

2.5. Method validation

2.5.1. System suitability

The system suitability was assessed by six replicate analyses of the drug at a concentration of 5.0 μg/ml. The acceptance criterion was ±2% for the percent coefficient of variation (%CV) for the peak area and retention times for both drug and IS.

2.5.2. Detection and quantitation limits (sensitivity)

Limits of detection (LOD) and quantitation (LOQ) were estimated from the signal-to-noise ratio. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation

limit was defined as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than 10, with precision (%CV) and accuracy (%bias) within ±10%.

2.5.3. Linearity (calibration curve)

The calibration curves were constructed with eight concentrations including the LLOQ ranging from 0.5 to 50 μg/ml. The peak area ratio of the drug to the IS was considered for plotting the linearity graph. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

2.5.4. Accuracy and precision

Accuracy of the assay method was determined for both intra-day and inter-day variations using the triplicate analysis of the QC samples. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time that was evaluated by assaying the QC samples during the same day. Intermediate precision was assessed by comparing the assays on different days (3 days).

2.5.5. Specificity

Specificity of the method was determined by subjecting the sample solution (0.1 mg/ml) to accelerated degradation by heat (60 °C) after addition of 1N NaOH in order to verify that none of the degradation products interfered with the quantitation of drug.

2.5.6. Stability

The stability of the drug solution was determined using the QC 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.

2.5.7. Stress testing

Stress testing of the drug substance can help identify the likely degradation products, the stability of the molecule and also validate the stability and specificity of the analytical procedures [13]. For degradation studies, 5 mg of ezetimibe was accurately weighed and transferred to a 50 ml volumetric flask (step 1). To this, 1 ml of 1N NaOH (for alkaline degradation) or 1N HCl (for acid degradation) or Zn + 1N HCl (for reductive degradation) was added and placed in a water bath maintained at 60 °C for 1 h. The mixture was cooled, dissolved and made to volume with mobile phase. An aliquot of 1 ml of this solution was transferred to a 10-ml volumetric flask containing 1 ml of 0.1 mg/ml solution of IS and the volume made up with mobile phase. For oxidative degradation, 1 ml of hydrogen peroxide (1/20) solution was added in step 1. For photolytic degradation, solution prepared as in step 1 was exposed to UV light at 254 nm for 8 h. One milliliter of each of these solutions was transferred to a 10 ml volumetric flask containing 1 ml of 0.1 mg/ml solution of IS and the volume made up with mobile phase. The final solution was injected for analysis.

3. Results and discussion

3.1. Method development and optimization

Ezetimibe is hydrophobic and is almost insoluble in aqueous solutions, whereas it is freely soluble in organic solvents like methanol and acetonitrile. During the development phase, the use of acetonitrile and water as the mobile phase resulted in asymmetric peak with a greater tailing factor (>2). Ion-pair chromatography is a good approach to minimize this effect. The successful use of 1-heptane sulfonic acid as an ion-pair reagent for the separation of biochemicals and pharmaceuticals is described elsewhere [14–17]. The addition of 1-heptane sulfonic acid to the mobile phase resulted in drastic reduction of peak tailing. At the reported concentration (0.05%, w/v), the tailing factor was within the acceptable limit (1.2) resulting in good peak symmetry and resolution. Increasing the flow rate to 0.8 ml/min and 1 ml/min resulted in poor resolution between the drug and IS. A flow rate of 0.3 ml/min resulted in drug retention time beyond 10 min that was more time consuming. Hence, the mobile phase was optimized at 0.5 ml/min with the retention time of the drug around 6.0 min and that of the IS was around 2.6 min. Also, the low flow rate and less run time consumes comparatively less mobile phase solvents that will prove cost-effective during routine analysis of drug samples. There were no interferences at the drug and IS peaks. The peak shape and symmetry were found to be good when a mobile phase composition of 30:70 (v/v, water: acetonitrile) was used with better resolution of the drug and IS.

3.2. Method validation

3.2.1. System suitability

The %CV of peak area and retention time for both drug and IS are within 2% indicating the suitability of the system (Table 1). The efficiency of the column as expressed by number of theoretical plates for the six replicate injections was 9124.5 ± 1% (mean ± %CV) and the USP tailing factor was 1.2 ± 0.2% (mean ± %CV).

Table 1
System suitability study

	Ezetimibe (5 μg/ml)		Internal standard (10 μg/ml)	
	Retention time (min)	Peak area	Retention time (min)	Peak area
Mean (n = 6)	5.97	491082.83	2.66	539447.83
S.D.	0.0009	1455.76	0.0004	1238.14
%CV	0.02	0.30	0.02	0.23

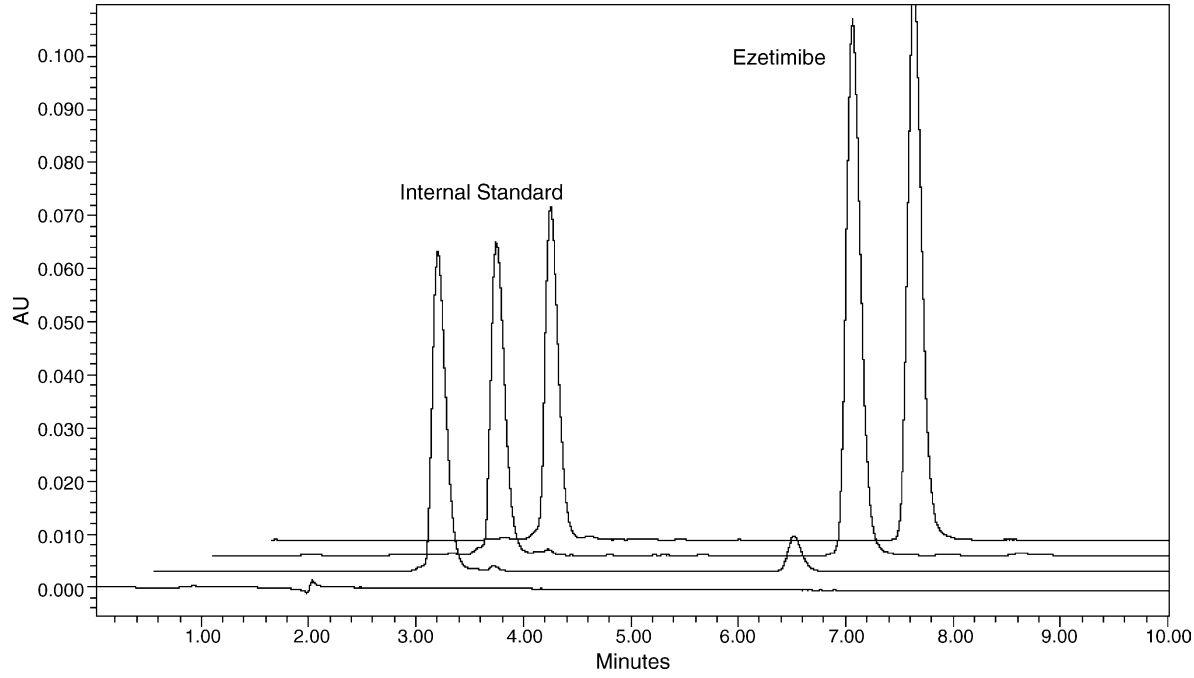

Fig. 2. Chromatograms of ezetimibe (from top to bottom) extracted from formulation (10 μg/ml), standard solution (10 μg/ml), LLOQ and mobile phase devoid of ezetimibe. Ezetimibe elutes approximately at 5.9 min and the internal standard at 2.6 min.

3.2.2. Detection and quantitation limits (sensitivity)

Fig. 2 illustrates the chromatograms of the mobile phase (diluent) LLOQ of the drug, standard solution and the drug extracted from formulation. The method was found to be sensitive as determined from the six replicate injections of the LLOQ where the %CV was 2.66% (Table 3, day1).

3.2.3. Linearity

The calibration curve constructed was evaluated by its correlation coefficient. The peak area ratio of the drug to IS was linear in the range of 0.5–50 μg/ml. Standard deviations of the slope and intercept for the calibration curves generated on six different days were 0.002 and 0.012, respectively. The correlation coefficient (r^2) of all the calibration curves were consistently greater than 0.999 (Table 2).

3.2.4. Accuracy and precision

Accuracy and precision calculated for the QC samples during the intra- and inter-day run are given in Table 3. The intra-day (day 1) accuracy ranged from −8.67% to −3.33% and precision from 0.05% to 1.26%. The results obtained from intermediate precision (inter-day) also indicated a good method precision. All the data were within the acceptance criteria of 5% (except 10% for LLOQ).

Table 2
Results of regression analysis of the linearity data of ezetimibe

	Mean ± S.D. (n = 6)
Slope	0.1757 ± 0.002
Intercept	0.0097 ± 0.012
Correlation coefficient (r^2)	0.9999 ± 0.0001

3.2.5. Specificity

From peak purity angle and peak purity threshold (Fig. 3 and Table 6), the co-elution of the degradation product can be excluded in alkaline media. The specificity of the analytical method was indicated in Fig. 3, where the alkaline degradation product of ezetimibe (indicated by an arrow) did not interfere with the retention time of the drug and IS.

3.2.6. Stability

Stability studies indicated that the samples were stable when kept at bench top for 12 h (short-term), in auto-sampler

Table 3
Intra- and inter-day accuracy and precision of HPLC assay for ezetimibe

	Nominal concentration			
	0.5 μg/ml	1.5 μg/ml	25 μg/ml	40 μg/ml
Day 1				
Mean	0.46	1.45	24.13	38.08
S.D.	0.006	0.010	0.167	0.017
%CV	1.26	0.69	0.69	0.05
%Bias	−8.67	−3.33	−3.48	−4.80
Day 2				
Mean	0.46	1.48	26.15	39.91
S.D.	0.010	0.046	0.624	1.493
%CV	2.17	3.10	2.39	3.74
%Bias	−8.00	−1.33	4.60	−0.22
Day 3				
Mean	0.49	1.43	24.77	40.35
S.D.	0.021	0.021	1.039	1.196
%CV	4.38	1.45	4.20	2.96
%Bias	−2.00	−4.44	−0.92	0.88

Each mean value is the result of triplicate analysis.

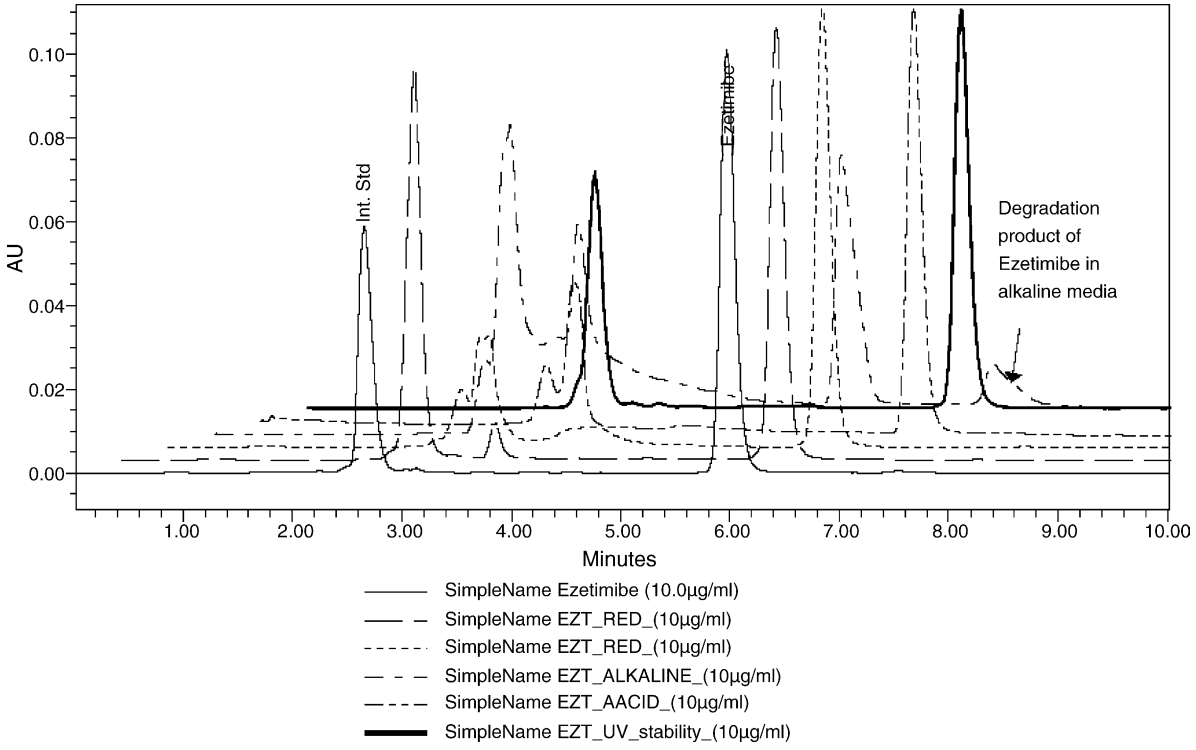

Fig. 3. Chromatograms depicting the specificity of ezetimibe.

for 24 h and refrigerated at 4 °C for 30 days (long-term). The results of these stability studies are given in Table 4, where the percent ratios are within the acceptance range of 90–110%.

3.2.7. Stress testing

The stress studies involving heat and acid, light (UV), oxidation and reduction revealed that ezetimibe was not fully degraded (Table 5). However, in alkaline conditions (1N NaOH), the drug was unstable and the degradation peak eluted at approximately 7.25 min (Fig. 3) accompanied with a drastic reduction in the number of theoretical plates (3354.67) and increased tailing (1.75) (Table 6). Except for alkaline conditions, the drug content was within 90–110% for all stress conditions indicating the stability and specificity of the analytical method to differentiate the degradation peaks.

Table 4
Short-term, long-term and auto-sampler stability of ezetimibe

	Nominal concentration			
	0.5 μg/ml	1.5 μg/ml	25 μg/ml	40 μg/ml
Short-term stability				
Mean	104.22	101.04	96.89	96.48
S.D.	7.148	6.610	5.106	2.699
%CV	6.86	6.54	5.27	2.80
Long-term stability				
Mean	94.44	92.23	95.10	96.56
S.D.	6.646	7.572	6.017	2.440
%CV	7.04	8.21	6.33	2.53
Auto-sampler stability				
Mean	106.69	93.80	94.35	97.31
S.D.	5.996	8.513	6.029	1.766
%CV	5.62	9.08	6.39	1.81

Each value is the result of triplicate analysis.

3.3. Application of the method to dosage forms

The HPLC method developed is sensitive and specific for the quantitative determination of ezetimibe. Also, the method is validated for different parameters, hence has been applied for the estimation of drug in pharmaceutical dosage forms. Ezetimibe tablets of 10 mg strength from two different manufacturers (Ezzicad, Glen mark Pharmaceuticals Ltd., Mumbai, India and Ezta, Hetero Drugs Pvt. Ltd., Hyderabad, India) were evaluated for the amount of ezetimibe present in the formulation. Each sample was analyzed in triplicate after extracting the drug as mentioned in the sample preparation of the experimental section. The amount of ezetimibe in 'Ezzicad' was 95.65% and in 'Ezta' was 96.85%. None of the tablet ingredients interfered with the analyte peak as seen in Fig. 2. The spectrum of ezetimibe extracted from the

Table 5
Results of ezetimibe exposed to different degradative pathways

	Mean ± S.D.	%CV
Oxidation	101.34 ± 0.286	0.28
Reduction	108.99 ± 4.920	4.51
Alkaline	80.02 ± 1.426	1.78
Acid	102.14 ± 1.280	1.25
UV	96.54 ± 0.605	0.63

Each value is the result of triplicate analysis.

Table 6
Specificity data

Sample name	Retention time	Area	Purity1 angle	Purity1 threshold	Purity1 flag	USP plate count	USP tailing
Ezetimibe standard (10 μg/ml)	5.97	956585	0.08	0.25	No	9192.69	1.24
Ezetimibe oxidation (10 μg/ml)	6.00	1001818	0.11	0.28	No	9235.68	1.22
Ezetimibe reduction (10 μg/ml)	6.00	1033044	0.10	0.28	No	8857.40	1.23
Ezetimibe alkaline (10 μg/ml)	5.75	768871	0.37	0.26	Yes	3354.67	1.75
Ezetimibe acid (10 μg/ml)	5.99	967910	0.13	0.33	No	8829.89	1.25
Ezetimibe UV (10 μg/ml)	6.00	926920	0.12	0.24	No	8781.21	1.24

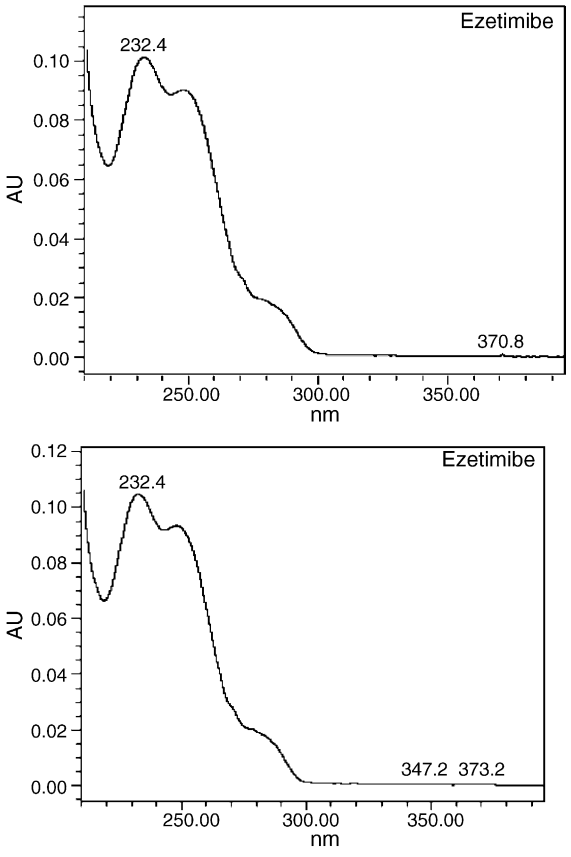

Fig. 4. UV spectra of ezetimibe extracted form formulation (above) and that of standard ezetimibe (below) derived from diode array detector.

tablets was matching with that of standard ezetimibe (Fig. 4) showing the purity of peak of ezetimibe in tablets.

4. Conclusion

A rapid, specific isocratic HPLC method has been developed for the determination of ezetimibe using a UV detector. The method was validated for accuracy, precision, linearity and stability. The method uses a simple mobile phase composition, easy to prepare with little or no variation. The rapid run time of 10 min and the relatively low flow rate (0.5 ml/min) allows the analysis of large number of samples with less mobile phase that proves to be cost-effective. Hence, this HPLC-UV method can be used for the routine drug analysis.

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