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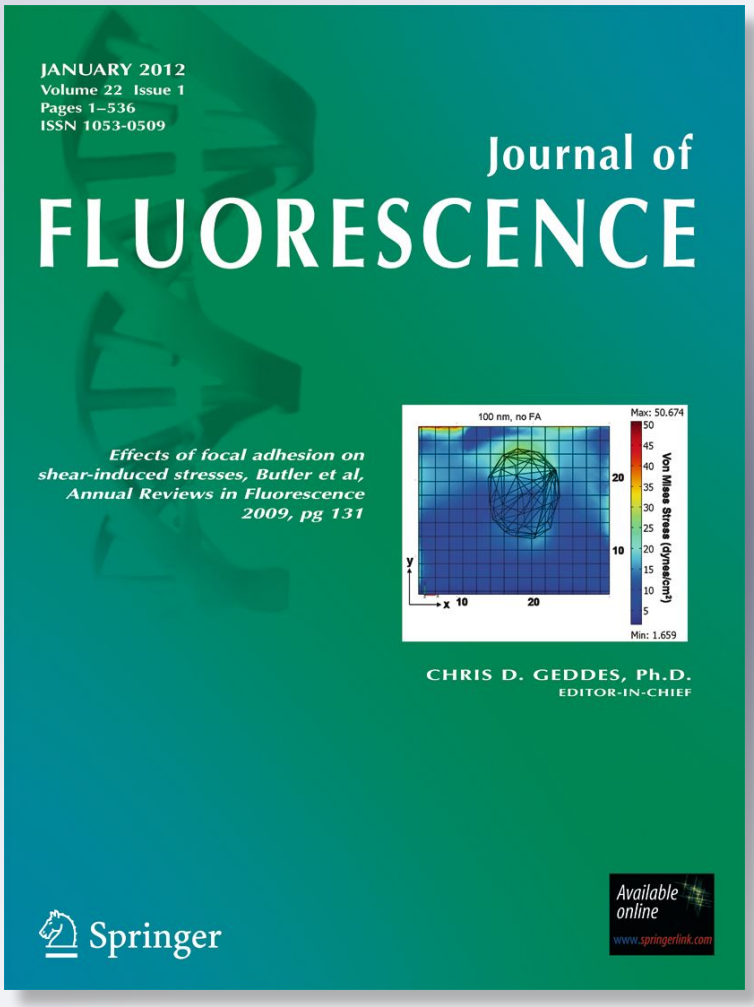

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FLUORESCENCE NEWS ARTICLE

Micelle-Enhanced Spectrofluorimetric Method for Determination of Cholesterol-Reducing Drug Ezetimibe in Dosage Forms

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Abstract A simple and sensitive spectrofluorimetric method was developed for the determination of ezetimibe in its pharmaceutical formulations. The proposed method is based on investigation of the fluorescence spectral behavior of ezetimibe in sodium dodecyl sulfate (SDS) micellar system. In aqueous solution of acetate buffer pH 5.0, the fluorescence intensity of ezetimibe was greatly enhanced, 200% enhancement, in the presence of SDS. The fluorescence intensity of ezetimibe was measured at 380 nm after excitation at 268 nm. The fluorescence-concentration plot was rectilinear over the range of 0.03–3.0 μg/mL with lower detection limit of 3.08×10^{-3} μg/mL. The method was successfully applied to the analysis of ezetimibe in its commercial tablets; the results were in good agreement with those obtained with the reported method. The application of the proposed method was extended to the stability studies of ezetimibe after exposure to different forced degradation conditions, such as acidic, alkaline, photo and oxidative conditions, according to ICH guidelines.

Keywords Ezetimibe · Spectrofluorimetry · Pharmaceutical preparations · Stability-indicating

Introduction

Ezetimibe (Fig. 1(A)) is a selective absorption inhibitor that effectively blocks intestinal absorption of dietary and biliary cholesterol. This reduces the overall delivery of cholesterol to the liver, thereby promoting the synthesis of low density lipoprotein receptors and the subsequent reduction in serum low density lipoprotein cholesterol [1, 2]. Chemically known as 1-(4-fluoro phenyl)-3(R)-[3-(4-fluoro phenyl)-3(S)-hydroxy propyl]-4(S)-(4-hydroxy phenyl)-2-azetidinone [3]. Several methods have been reported for the determination of ezetimibe viz., high performance liquid chromatography [4–8], gas-chromatography-mass spectrometry [9], liquid chromatography-mass spectrometry [10–12], spectrophotometry [13], and potentiometry using glassy carbon nanotubes [14] and ion selective electrodes [15]. Up to date, no fluorimetric or official methods have been reported for the determination of ezetimibe.

Spectrofluorimetry has been widely used in the determination of pharmaceutical compounds because it is highly sensitive, selective, easily operated and economic technique. The main goal of the present study is to investigate the native fluorescence characteristics of ezetimibe in aqueous medium and in SDS micellar medium. Based on the micellar-enhancement effect of SDS on the native fluorescence of ezetimibe, a simple and sensitive spectrofluorimetric method has been developed for the determination of ezetimibe in tablets. The present study is more simple and time saving with no need for derivatization reaction of ezetimibe. The stability-indicating capability of the proposed spectrofluorimetric method is evaluated through forced degradation studies of ezetimibe drug under various degradation conditions according to ICH guidelines [16].

Experimental

Apparatus

The fluorescence intensity was measured on a Perkin-Elmer model LS-55 luminescence Spectrometer (UK), equipped

N. A. Alarfaj (✉) · F. A. Aly
Department of Chemistry, College of Science,
King Saud University,
P.O. Box 22452, Riyadh 11495, Saudi Arabia
e-mail: nalarfaj@hotmail.com

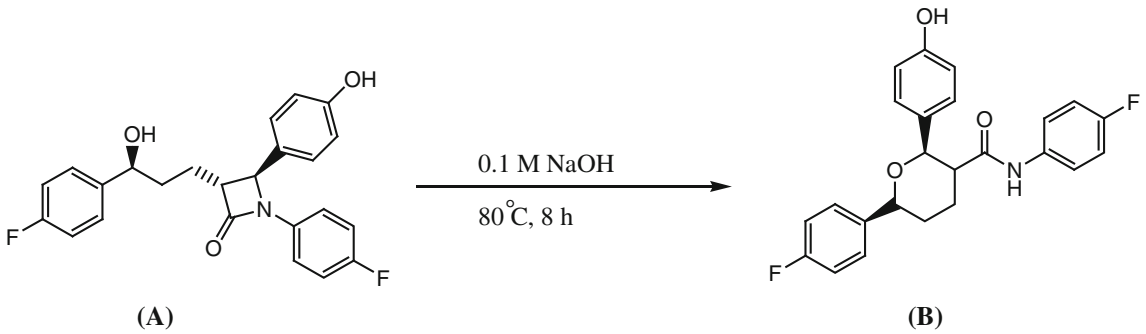

Fig. 1 Ezetimibe alkaline degradation pathway

with a 150 W xenon arc lamp, grating excitation and emission monochromators and a Perkin-Elmer recorder. Slit widths for excitation and emission monochromators were set at 5.0 and 7.0 nm, respectively. A 1-cm quartz cell was used. HANNA pH meter (Romania) was used for pH adjustments.

Materials and Reagents

All reagents were of analytical grade and distilled water was used throughout the work. Pure grade ezetimibe was kindly supplied from Saudi Pharmaceutical Industries and Medical Appliances Corporation, Al-Qassim Pharmaceutical Plant (SPIMACO) Saudi Arabia. The pharmaceutical preparation (Ezetrol® 10 mg/tablet) was provided by Schering-Plough Company, USA. Sodium dodecyl sulfate (SDS, 95.0%), cetyltrimethyl ammonium bromide (CTAB, 99.0%), Tween 80, methyl cellulose, *B*-cyclodextrin (*B*-CD), sodium acetate trihydrate, boric acid and sodium hydroxide were purchased from Winlan (UK). Methanol (99.5%), Ethanol (96.0%) and n-propanol (99.5%) were obtained from Sigma-Aldrich (Germany). Acetonitrile was obtained from Merck (Germany). Dimethyl sulfoxide, dimethyl formamide, glacial acetic acid and hydrochloric acid were purchased from BDH laboratory supplies (England). Ezetrol® 10 mg/tablet was purchased from local drug stores.

Standard Solution

Stock solution of ezetimibe was prepared by dissolving 10.0 mg of the drug in 100 mL of methanol. Further dilutions were done with the same solvent as appropriate. The standard solution was stable for 15 days when kept in the refrigerator.

General Procedures

Procedure for Calibration Graph

Aliquots of methanolic ezetimibe standard solution were transferred into a series of 10-mL volumetric flasks to give final concentrations of 0.03–3.0 μg/mL, and the total volume of methanol was adjusted to 2.0 mL in all flasks. 1 mL of 0.2 M acetate buffer solution of pH 5.0 was added to each flask followed by 2 mL of 2.0% w/v SDS. The solution was mixed well and the fluorescence intensity was measured at 380 nm after excitation at 268 nm. The fluorescence intensity was plotted vs. the final drug concentration (μg/mL) to get the calibration graph. Alternatively, the corresponding regression equation was derived.

Procedures for Tablets

The total content of ten tablets was ground to a fine powder. A weighed quantity of the powdered tablets equivalent to 10.0 mg of ezetimibe was transferred into 100-mL volumetric flask, about 80.0 mL of methanol was added and the flask was sonicated for 30 min. The solution was diluted to volume with methanol, mixed and filtered. Serial dilutions covering the working concentration range of 0.03–3.0 μg/mL were transferred into 10-mL volumetric flasks. Procedure described under "Procedures for calibration graph" was performed. The nominal content of the tablets was calculated using the calibration graph or the corresponding regression equation.

Procedures for Stability Study

Acid and Base Induced Degradation Studies

Aliquots of methanolic ezetimibe stock solutions (equivalent to 100 μg of the drug) were transferred into a series of small conical flasks; 5 mL aliquots of 0.1 or 1.0 mol L^{-1} hydrochloric acid was added and refluxed at 80 °C for 8 h. The studies under alkaline conditions were carried out in 0.1 mol L^{-1} sodium hydroxide and the solution was refluxed for 8 h at 80 °C. 1.0 mL of resulting solutions were then transferred into 10-mL volumetric flasks and proceeded as described above under "Procedure for Calibration Graph".

Oxidative Degradation

Aliquots of methanolic ezetimibe stock solution (equivalent to 100.0 μg of the drug) were transferred into a series of 10-mL volumetric flasks; different volumes of 6% H_2O_2 were added and the volume was completed with water. 1.0 mL of each of these solutions was transferred into 10-mL volumetric flasks and the procedure for the calibration graph was then followed.

Photo Degradation

Aliquots of methanolic ezetimibe stock solution (containing 100.0 μg of the drug) were transferred into 10-mL volumetric flasks; completed to volume with methanol, water or methanol/water mixture (50:50). The volumetric flasks were exposed to UV-lamp with a wavelength of 254 nm at a distance of 15 cm placed in wooden cabient for 24 h. 1.0 mL of each solution was transferred into a 10-mL volumetric flask and proceeded as described under "Procedure for Calibration Graph".

Results and Discussion

Ezetimibe was found to exhibit an emission band at 380 nm in aqueous acetate buffer pH 5.0, after excitation at 268 nm. It was aimed to enhance this emission band, attempting to explore a new methodology for the analysis of ezetimibe in its pharmaceutical preparations. It is well known that the addition of a surfactant at a concentration above its critical micellar concentration to a given fluorophore solution increases the molar absorptivity and/or the fluorescence quantum yield of the fluorophore in many cases [17]. This fact has been used to improve the performance of spectrofluorimetric methods of various analytes. The fluorescence properties of ezetimibe in various micellar media were studied, there was an enhancement of the fluorescence intensity in presence of SDS compared with aqueous solution (about 2.0 fold enhancement), and so that SDS was used as a fluorescence enhancer in order to develop a new spectrofluorimetric method for the determination of ezetimibe.

Optimization of Experimental Conditions

Fluorescence Spectra of Ezetimibe in Aqueous and SDS System

The fluorescence spectra of ezetimibe in both aqueous and SDS systems were studied. Figure 2, illustrates the obtained fluorescence spectra of ezetimibe in the two systems. One of them was aqueous acetate buffer of pH 5.0, while the second one was the same acetate buffer in presence of SDS as a fluorescence enhancer. The percentage of fluorescence enhancement in presence of SDS was 200% compared with the native fluorescence intensity (FI) of the drug in aqueous medium.

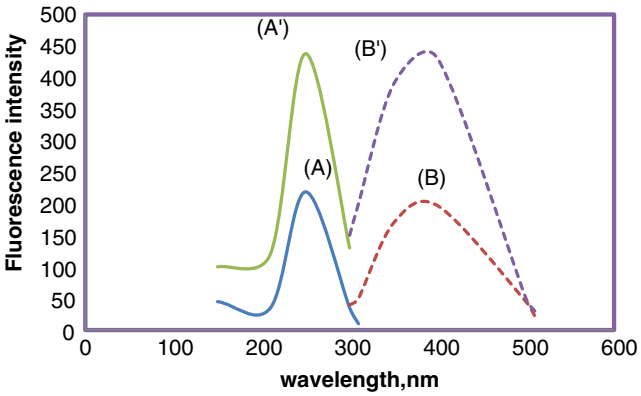

Fig. 2 Fluorescence spectra of: (A, B) ezetimibe (1 μg/mL) in acetate buffer of pH 5.0, (A′, B′) ezetimibe (1 μg/mL) in acetate buffer of pH 5.0/SDS system. Where: (A, A′) are the excitation spectra and (B, B′) are the emission spectra

Effect of Surfactants

The fluorescence properties of ezetimibe in various micellar media were studied using anionic surfactant (SDS), cationic surfactant (CTAB) and non ionic surfactant (tween-80, methyl cellulose and *B*-Cyclodextrin (*B*-CD). All the studied organized media caused decrease in native fluorescence or even have no significant effect in the FI of ezetimibe, while SDS system gave a substantial enhancement effect on the FI of the ezetimibe drug (Fig. 3).

Effect of SDS Volume

The influence of SDS on the FI was studied using increasing volumes of 2.0% w/v SDS. It was found that increasing

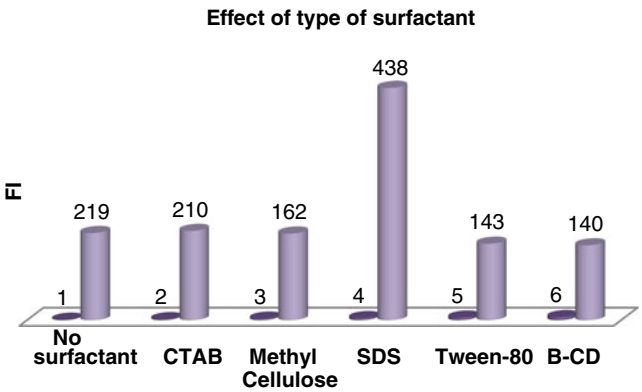

Fig. 3 Effect of type of surfactant (1 mL of 2.0% solution of each surfactant) on the FI of ezetimibe (1 μg/mL)

volumes of SDS solution resulted in corresponding increase in FI up to 1.0 mL, after which no further increase in FI was attained. Therefore, 2 mL of SDS was chosen as the optimum volume for ezetimibe (Fig. 4).

Effect of pH

The influence of pH on the micelle-enhanced fluorescence of ezetimibe can be observed in Fig. 5. Different types of buffers covering the whole pH range, such as 0.2 mol L^{-1} acetate buffer over the pH range of 3.5–5.6 and 0.2 mol L^{-1} borate buffer ranging from pH 6.5–9.0. It was found that maximum FI was achieved over the pH range of 4.4–5.0 for ezetimibe drug, so that, pH 5.0 was chosen as the optimum pH value. Increasing the pH value above pH 5.0 caused gradual decrease in the FI. As the fluorescence intensity for protonated species is always higher than that for neutral species, it can be inferred that the protonated species form interacts more strongly with the anionic micelles of SDS than the neutral form of the drug [18].

Effect of Diluting Solvent

The effect of different diluting solvents on FI of ezetimibe in presence of SDS was investigated using water, methanol, acetonitrile, n-propanol, dimethyl sulfoxide and dimethyl formamide. It was found that water was the best solvent for dilution as it gave the highest FI. Distinct and sharp increase in the fluorescence intensities was observed in SDS system upon using methanol, acetonitrile or n-propanol. This effect was attributed to their denaturating effect on the micelles, where, short-chain alcohols (methanol and n-propanol) were solubilizied mainly in the aqueous phase and affect the micellization process by modifying the solvent properties. Addition of these alcohols also results in reduction of size of the micelles, but with a progressive breakdown of the surfactant aggregate at very high concentration [19]. Each of dimethyl sulfoxide and dimethyl formamide decreased the fluorescence intensity of ezetimibe, since they initiated intersystem crossing process (similar to heavy atom effect) [20].

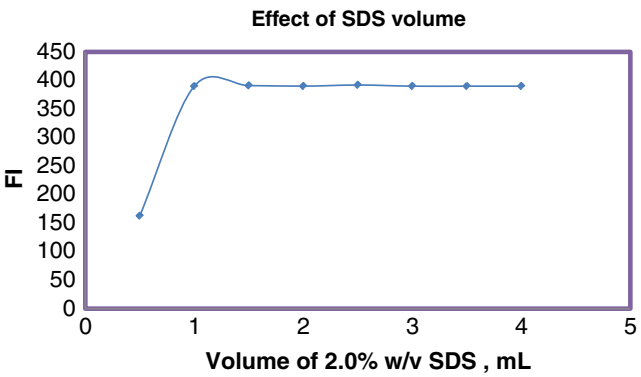

Fig. 4 Effect of the volume of 2.0% SDS on the FI of ezetimibe (1 μg/mL)

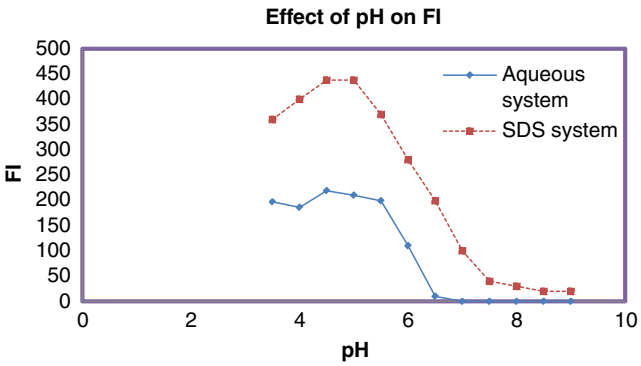

Fig. 5 Effect of pH on the FI of ezetimibe (1 μg/mL) in presence of 0.4% SDS and in aqueous medium

Effect of Methanol Volume

The effect of the total methanol volume was also investigated using increasing volumes of methanol and completing to the mark with distilled water. It was found that, increasing the volume of methanol above 2.0 mL hinders the micelle formation causing complete loss of the micelle-enhancement effect of SDS on the native fluorescence of ezetimibe, so that, the total volume of methanol was kept at 2.0 mL in all measurements.

Effect of Time

The effect of time on the FI of the drug was also studied. It was found that the fluorescence intensity was immediately developed and remained stable for more than three hours.

Method Validation

Linearity

For the micelle enhanced fluorescence system, a series of six standard solutions (three replicates for each one) of ezetimibe were measured by following the procedure described in the "Experimental section". Table 1, resumed the results obtained from statistical analysis of data obtained. The calibration graph of FI (*Y*) vs. ezetimibe concentration expressed in μg/mL (X) was found to be linear in the range of 0.03–3.0 μg/mL. The proposed method was evaluated by calculating the accuracy as relative error and precision as relative standard deviation [16].

Table 1 Performance data for the proposed spectrofluorimetric method

Parameter	Ezetimibe
Concentration range (μg/mL)	0.03–3.0
Limit of detection (LOD) (μg/mL)	3.08×10^{-3}
Limit of quantification (LOQ) (μg/mL)	0.01
Correlation coefficient (r)	0.9995
Slope	116.5
Intercept	203.6
%RSD	0.52
% Error[a]	0.13

[a] % Error = SD/√n

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The limit of quantification (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH Q2 (R1) recommendation [14] below which the calibration graph is nonlinear. The limit of detection (LOD) was determined by evaluating the lowest concentration of the analyte that can be readily detected. The results were summarized in Table 1.

Accuracy and Precision

Statistical analysis [21] of the results obtained by the proposed and the reference methods (Spectrophotometric determination of ezetimibe using Fe (III) chloride in the presence of 0.1 mol L^{-1} hydrochloric acid at 740 nm) [22] using student's *t*-test and variance ratio F-test showed no significant differences between the two methods regarding accuracy and precision, respectively, (Table 2). The intraday precision was evaluated by determination of three concentration of ezetimibe drug in pure form on three successive occasions. The interday precision was also evaluated through replicate analysis of three concentrations for a period of 3 successive days. The results of intraday and interday precision are summarized in Table 3.

Robustness of the Method

The robustness of the adopted method was demonstrated by the constancy of the FI with minor changes in the experimental conditions, such as the pH, 5.0±0.2, the change in the volume of SDS, 2±0.5. These minor changes that may take place during the experimental operations did not affect the FI.

Table 2 Application of the proposed and comparison methods to the determination of ezetimibe in pure form and its pharmaceutical dosage form

Ezetimibe						
	Proposed method			Reported method [22]		
	Taken (μg/mL)	Found (μg/mL)	% Found	Taken (μg/mL)	Found (μg/mL)	% Found
Pure form	0.03	0.0299	99.67	0.03	0.0297	99.00
	0.10	0.1000	100.00	0.10	0.0998	99.80
	0.50	0.4982	99.64	0.50	0.4994	99.88
	1.00	0.9995	99.95	1.00	0.9987	99.87
	1.50	1.4989	99.93	1.50	1.4993	99.95
	2.00	1.9896	99.48	2.00	1.9796	98.98
Mean ± SD	99.78±0.21			99.58±0.46		
t-test	0.99(2.23)*					
F-test	4.77(5.05)*					
Ezetrol® 10 mg/tablet	0.03	0.0296	98.67	0.03	0.0299	99.67
	0.10	0.0998	99.80	0.10	0.0989	98.90
	0.50	0.4894	97.88	0.50	0.4997	99.94
	1.00	0.9998	99.98	1.00	0.9982	99.82
	1.50	1.4976	99.84	1.50	1.4987	99.91
	2.00	1.9985	99.93	2.00	1.9799	98.99
Mean ± SD	99.35±0.87			99.54±0.47		
t-test	0.47 (2.23)*					
F-test	3.17 (5.05)*					

*Figures in parentheses are the tabulated t and F values at confidence limits 95% [21]

Table 3 Validation of the proposed method for the determination of ezetimibe in pure form

Ezetimibe				
Conc. (μg/mL)	Found (μg/mL)	% Recovery	%RSD	%Error
Intraday precision				
1.0	0.99	99.00±0.92	0.92	0.37
1.5	1.49	99.33±0.87	0.87	0.35
2.0	1.99	99.50±0.65	0.65	0.27
Interday precision				
1.0	0.98	98.00±0.74	0.74	0.30
1.5	1.48	98.67±0.68	0.68	0.28
2.0	1.97	98.50±0.48	0.48	0.20

Results of Stability Studies

Fluorimetric studies of ezetimibe samples obtained during the stress testing under different conditions suggested the following degradation behaviors.

Acid Induced Degradation

The rate of degradation in acid was slower as compared with that in alkali. Initially 0.1 mol L^{-1} hydrochloric acid was used at 80 °C for acid degradation of ezetimibe drug but since no degradation was observed, the strength of acid was increased, then 10%–20% degradation was observed by heating the drug solution with 1 mol L^{-1} hydrochloric acid at 80 °C.

Alkaline Induced Degradation

The drug was found to undergo alkaline degradation very readily. The reaction in 0.1 mol L^{-1} sodium hydroxide at 80 °C was so fast that around 80% of the drug was degraded in 5 min. subsequently; studies were performed in 0.01 mol L^{-1} sodium hydroxide at 40 °C. The drug showed a degradation of around 20% within 30 min at 40 °C. Figure 1 shows a proposal of alkaline degradation of ezetimibe . It is supposed that the drug undergoes base—catalyzed rearrangement of β-lactam ring to tetrahydropyrane ring.

Hydrogen Peroxide Induced Degradation

Oxidative degradation of ezetimibe with hydrogen peroxide was studied. It was found that treating the drug with 6% H_2O_2 at room temperature, according to the procedure described above, it was found that the drug was stable.

Photo Degradation

The effect of UV-light on the stability of ezetimibe was studied, it was found that no considerable degradation was observed even after exposure of the drug to the UV-light for 24 h.

Conclusion

A simple and sensitive spectrofluorimetric method was developed for the determination of ezetimibe. The proposed method was accurate, fast and precise. Its main advantage over HPLC methods is its rapidity (no complex pre-treatments are needed, and measurements of fluorescence are nearly instantaneous). On the basis of accuracy and range sample concentrations, the proposed method was successfully applied for the determination of ezetimibe in bulk drug and in its pharmaceutical dosage form.

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