Short Communication

Stability Indicating Liquid Chromatographic Method for the Simultaneous Determination of Rosuvastatin and Ezetimibe in Pharmaceutical Formulations

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Abstract

Purpose: A simple stability indicating reverse phase liquid chromatographic method was developed for the simultaneous determination of rosuvastatin and ezetimibe in pharmaceutical formulations.

Methods: Best chromatographic response was achieved with C18 column (250 X 4.6 mm, 5μm) with photo diode array (PDA) detector. The mobile phase was composed of a mixture of sodium acetate buffer (pH 4.0) and acetonitrile (30:70, %v/v) with a flow rate of 1.2 mL/min. (UV detection at 254 nm). Rosuvastatin and ezetimibe were subjected to stress conditions of degradation and the method was validated as per ICH guidelines.

Results: The method shows linearity over a concentration range of 0.5–250 μg/ml for both rosuvastatin (r^2 = 0.9993) and ezetimibe (r^2 = 0.9996). Both the drugs are highly sensitive towards alkaline conditions in comparison to other stress conditions.

Conclusion: The proposed method can be successfully applied to perform long-term and accelerated stability studies for the simultaneous determination of rosuvastatin and ezetimibe in pharmaceutical formulations.

Introduction

Rosuvastatin (RST) is chemically designated as (3R, 5S, 6E) - 7 - [4 - (4 - fluorophenyl) - 2 - (N - methylmethanesulfonamido) - 6 - (propan - 2 - yl) pyrimidin - 5 - yl] - 3, 5 - dihydroxyhept - 6 - enoic acid (Figure 1A).[1] It is a member of the drug class of statins. It is used in the treatment of Hyperlipidemia. Rosuvastatin Calcium is a selective and competitive inhibitor of hydroxyl methyl glutaryl coenzyme A (HMG CoA) reductase (a precursor of cholesterol), the rate-limiting enzyme that converts 3-hydroxyl-3-methylglutaryl coenzyme A to mevalonate. It reduces levels of low-density lipoprotein, apolipoprotein B and triglycerides in the blood, while increasing levels of high-density lipoprotein in the management of hyper lipidaemias.[2] Ezetimibe (EZT) chemically designated as (3R, 4S) - 1 - (4 - fluorophenyl) - 3 - [(3S) - 3 - (4 - fluorophenyl) - 3 - hydroxypropyl] - 4 - (4 - hydroxyphenyl) azetidin - 2 - one (Figure 1B).[1] It is a selective cholesterol absorption inhibitor, used for the treatment of hyperlipidemia, which potentially inhibits the absorption of biliary and dietary cholesterol. Ezetimibe prevents intestinal absorption of cholesterol without affecting absorption of triglycerides, fatty acids and fat-soluble vitamins.[3-5]
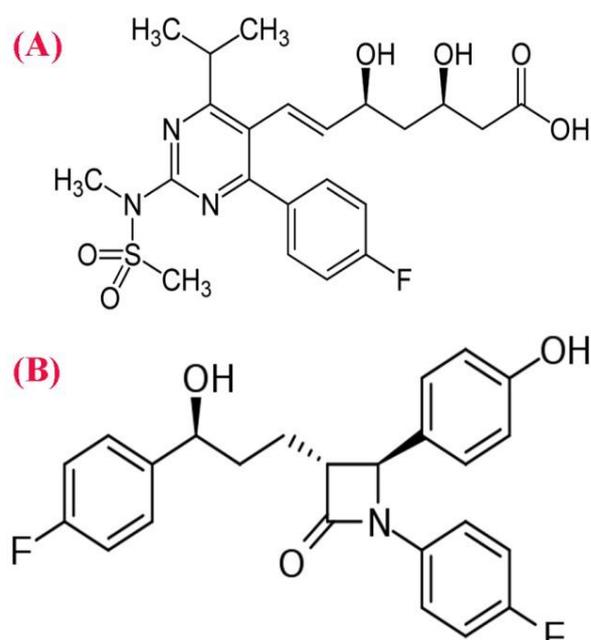
Figure 1. Chemical structures of rosuvastatin **[A]** and ezetimibe **[B]**.

Various analytical techniques such as micellar liquid chromatography,[6] HPLC,[7-13] HPTLC,[14-15] densitometric

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TLC,[16] spectrophotometry[17-19] and spectrofluorimetry[20] have been developed for the simultaneous determination of rosuvastatin and ezetimibe in pharmaceutical formulations. In the present study an attempt has been made to develop a validated stability indicating RP-HPLC method for the simultaneous determination of rosuvastatin and ezetimibe in pharmaceutical formulations as per ICH guidelines.[21]

Materials and Methods
Chemicals
Reference standards of rosuvastatin (purity 99%) and ezetimibe (purity 99.5 %) were obtained from Glenmark Pharmaceuticals Ltd., India. The combination of rosuvastatin and ezetimibe is available as film-coated tablets (10 mg of rosuvastatin and 10 mg of ezetimibe) with brand names RAZEL-EZ® (Glenmark Pharmaceuticals Ltd., India) and ROSUVAS-EZ ® (Ranbaxy Laboratories Ltd., India) and were procured from the local pharmacy store. HPLC grade (Merck) solvents and chemicals were used for the entire study.

Instrumentation and chromatographic conditions
Chromatographic separation was attained by means of a Shimadzu Model CBM-20A/20 Alite HPLC system, equipped with SPD M20A prominence photodiode array detector and a Rheodyne injection valve with a 20 µL loop. The experimental conditions were optimized on a C18 (250 mm × 4.6 mm i.d., 5 µm particle size) column maintained at 25 °C. Isocratic elution was performed using sodium acetate buffer (pH 4.0) and acetonitrile (30:70, %v/v). The overall run time was 10 min. The flow rate was 1.2 ml/min. 20 µl of sample was injected into the HPLC system and all chromatographic conditions were performed at room temperature (25°C ± 2°C).

Preparation of sodium acetate buffer solution (pH 4.0)
The buffer solution was prepared by mixing 28.6 ml of glacial acetic acid with 10ml of 50% w/v NaOH in to a 1000 ml volumetric flask, dissolving and diluting to volume with HPLC grade water.

Preparation of rosuvastatin and ezetimibe stock solutions
Stock solutions of rosuvastatin (1000 µg/ml) and ezetimibe (1000 µg/ml) were prepared by accurately transferring 25 mg of rosuvastatin and ezetimibe separately in two 25 ml volumetric flasks and the volume was made up to volume with mobile phase. Working solutions for HPLC injections were prepared on a daily basis from the stock solution with mobile phase containing sodium acetate buffer and acetonitrile (30:70, % v/v). Solutions were filtered through a 0.45 µm membrane filter prior to injection.

Assay of marketed formulations
Twenty tablets from each marketed brands (RAZEL-EZ® and ROSUVAS-EZ®) were procured from pharmacy store and tablet powder equivalent to 25 mg of each of rosuvastatin and ezetimibe was accurately transferred to 25ml volumetric flask and extracted with acetonitrile. The solution was sonicated and filtered and the filtrate was further diluted with mobile phase as per the requirement. All the solutions were filtered through 0.45 mm nylon filter prior injecting in to HPLC system.

Method Validation
Linearity
A series of drug solutions were prepared containing both rosuvastatin and ezetimibe together from their stock solutions and 20 µL of each mixture was injected in to the HPLC system. The peak area of each drug in the chromatogram was recorded. A calibration curve was plotted by taking the concentration on the x-axis and the corresponding peak area on the y-axis for each drug separately.

Accuracy
The accuracy of the assay method was evaluated in triplicate at three concentration levels (50, 100 and 150%), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of rosuvastatin and ezetimibe in the marketed product. The study was carried out in triplicate at 150, 200 and 250 µg/ml and the percentage recovery in each case was calculated.

Precision
The intra-day and inter-day precision of the method were evaluated at three different concentration levels (10, 50 and 100 µg/ml) (n=3) against a qualified reference standard. The intra-day precision study was conducted on the same day where as the inter-day precision study was conducted on three different days i.e. day 1, day 2 and day 3.

Robustness
The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis.[21] The robustness of the method was evaluated by assaying the drug solution by deliberately changing the different analytical parameters such as the detection wavelength (252 and 256 nm i.e. ± 2 nm), the mobile phase composition (sodium acetate buffer: acetonitrile, 32:68 and 28:72 i.e. ± 2 %, v/v), the flow rate (1.1 and 1.3 ml/min i.e. ± 0.1 ml/min) etc. and the % RSD was calculated.

Sensitivity/ Limit of detection and limit of quantitation
The limit of quantification (LOQ) and limit of detection (LOD) were based on the standard deviation of the response and the slope of the constructed calibration curve (n=3), as described in International Conference on Harmonization guidelines Q2 (R1).[21] Sensitivity of the method was established with respect to limit of detection

(LOD) and LOQ for analytes. LOD and LOQ were established by slope method as mentioned below.

$$LOD = \frac{3.3 \times \text{standard deviation of calibration curve}}{\text{Slope of the calibration curve}}$$

$$LOQ = \frac{10 \times \text{standard deviation of calibration curve}}{\text{Slope of the calibration curve}}$$

Forced Degradation Studies
Stress studies were performed to evaluate the specificity of the method.[22] All samples were diluted with mobile phase to give a final concentration 100 µg/ml and filtered through 0.45 µm nylon filter before injection.

Acidic degradation
Acidic degradation was performed by treating the drug solution mixture (containing each of 1 mg/ml rosuvastatin and ezetimibe) with 0.1 N hydrochloric acid for 30 min in a thermostat maintained at 80 °C. The drug solution mixture was cooled, neutralized with 0.1 N sodium hydroxide and then diluted with mobile phase as per the requirement and 20 µL of the solution was injected in to the HPLC system.

Alkaline degradation
Alkaline degradation was performed by treating the drug solution mixture (containing each of 1 mg/ml rosuvastatin and ezetimibe) with 0.1 N sodium hydroxide for 30 min in a thermostat maintained at 80 °C. The drug solution mixture was cooled, neutralized with 0.1 N hydrochloric acid and then diluted with mobile phase as per the requirement and 20 µL of the solution was injected in to the HPLC system.

Oxidation degradation
Oxidation degradation was performed by treating the drug solution mixture (containing each of 1 mg/ml rosuvastatin and ezetimibe) with 30% H_2O_2 for 30 min in a thermostat maintained at 80 °C. The drug solution mixture was cooled and then diluted with mobile phase as per the requirement and 20 µL of the solution was injected in to the HPLC system.

Photolytic degradation
The drug solution mixture (containing each of 1 mg/ml rosuvastatin and ezetimibe) was exposed to UV light (365 nm) for 3 hours, diluted with mobile phase as per the requirement and 20 µL of the solution was injected in to the HPLC system.

Thermal degradation
The drug solution mixture (containing each of 1 mg/ml rosuvastatin and ezetimibe) was in a thermostat maintained at 80 °C for 10 hours, cooled and 20 µL of the solution was injected in to the HPLC system after necessary dilution with mobile phase.

Results and Discussion
HPLC method development and optimization
Initially the stressed samples were analyzed using a mobile phase consisting of sodium acetate: acetonitrile (60:40, % v/v) at a flow rate of 1.2 mL/min. Under these conditions, a broad peak was eluted for rosuvastatin at 9.775 min and therefore the mobile phase composition was changed to 55: 45, v/v) with the same flow rate under which a sharp peak was observed with slight tailing. Finally the mobile phase composition was changed as 30:70, % v/v where a sharp peak was eluted at 2.563 min for rosuvastatin. In similar conditions ezetimibe was also eluted at 3.629 min and therefore mobile phase consisting of sodium acetate: acetonitrile (30:70, % v/v) with a flow rate of 1.2 mL/min was chosen as the best chromatographic response for the simultaneous determination of rosuvastatin and ezetimibe. UV detection was carried out at 254 nm (PDA detector).

Method Validation
Linearity
The combination of rosuvastatin and ezetimibe shows linearity over a concentration range of 0.5 to 250 µg/ml and the linear regression equations were found to be y = 30291x + 53405 (r^2 = 0.9993) and y = 39595x + 53321 (r^2 = 0.9996) for rosuvastatin and ezetimibe respectively. The chromatogram of the mobile phase (blank) and that of the combination of rosuvastatin and ezetimibe was shown in Figure 2.
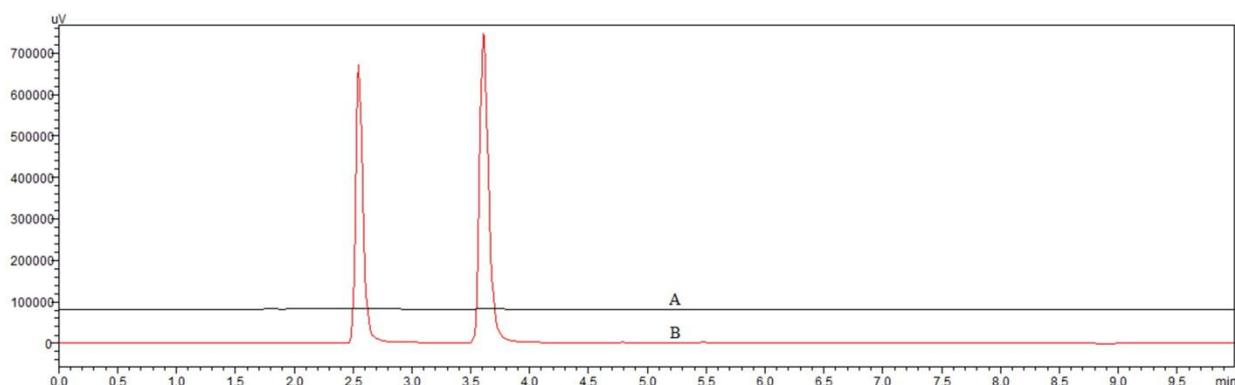
Figure 2. Typical chromatograms of blank [A], rosuvastatin and ezetimibe [B].

Accuracy

The method accuracy was proved by the recovery test. A known amount of rosuvastatin and ezetimibe standards (100 μg/ml) were added to aliquots of sample solutions and then diluted to yield the total concentrations of 150, 200 and 250 μg/ml as described in Table 1. The assay was repeated over three consecutive days and the resultant % RSD was found to be 0.21-0.41 and 0.50-0.73 with a recovery of 98.29-101.48 % and 99.41-99.81 % for rosuvastatin and ezetimibe respectively.

Table 1. Accuracy studies of rosuvastatin and ezetimibe

Drugs	Conc. (μg/mL)			*Mean peak area ± SD (%RSD)	Drug found (μg/mL)	% Recovery
	Formulation	Pure drug	Total			
Rosuvastatin	100	50	150	4602347.33±15847.32 (0.35)	150.21	100.14
	100	100	200	6202425.00±25385.87 (0.41)	202.95	101.48
	100	150	250	7500280.33±15623.15 (0.21)	245.73	98.29
Ezetimibe	100	50	150	5956160.67±35196.04 (0.60)	149.11	99.41
	100	100	200	7932586.67±57655.07 (0.73)	198.97	99.48
	100	150	250	9936336.33±49615.788 (0.50)	249.51	99.81

* Mean of three replicates

Precision

The intra-day precision of the method was determined by assaying three samples of each at three different concentration levels (10, 50 and 100 μg/ml) on the same day. The inter-day precision was calculated by assaying three samples of each at three different concentration levels (10, 50 and 100 μg/mL) on three different days. The % RSD for intra-day precision was found to be 0.41-0.94 and 0.31-0.59 for rosuvastatin and ezetimibe respectively whereas the inter-day precision was found to be 0.68-0.95 and 0.68-1.02 for rosuvastatin and ezetimibe respectively (Table 2).

Table 2. Precision studies of rosuvastatin and Ezetimibe

Drugs	Conc. (μg/mL)	Intra-day precision		Inter-day precision	
		Conc. (μg/mL) found	% RSD	Conc. (μg/mL) found	% RSD
Rosuvastatin	10	9.93	0.94	9.97	0.89
	50	50.05	0.74	49.91	0.76
	100	100.28	0.41	100.03	0.68
Ezetimibe	10	9.94	0.44	9.91	0.68
	50	49.98	0.59	50.06	0.89
	100	100.11	0.31	100.04	1.02

Sensitivity/ Limit of quantification (LOQ) and limit of detection (LOD)

The LOD and LOQ were found to be 0.1149 μg/ml and 0.3483 μg/ml for rosuvastatin and the LOD and LOQ for Ezetimibe were 0.1365 μg/ml and 0.4135 μg/ml respectively.

Robustness

The results of the robustness study were given in Table 3. The slight changes in flow rate, mobile phase composition etc. affects the chromatographic response such as retention time, tailing factor and theoretical plates etc. The % RSD obtained was 0.52-0.95 % and 0.71-1.38 % for rosuvastatin and ezetimibe respectively (< 2.0%) indicating that the proposed method is robust.

Analysis of commercial formulations

The proposed method was applied for the determination of rosuvastatin and ezetimibe in marketed formulations available (RAZEL-EZ® and ROSUVAS-EZ®). The % recovery was found to be 96.48 and 96.97 respectively.

Forced Degradation Studies

The specificity of the developed method can be determined from the stress studies and the percentage drug recovery was calculated from the peak area of the resultant chromatograms.

30.27 % of rosuvastatin has undergone alkaline degradation. The carboxylic acid group present in the rosuvastatin chemical structure is highly responsible for the alkaline degradation. Lactonization of the β-hydroxy acid and lactone ring formation can takes place at the same time and reports on such interconversion process have been seen in the literature for statins (Ex: Atorvastatin).[23] Ezetimibe also has undergone alkaline degradation (43.94 %) and the phenolic hydroxyl group present in the chemical structure may be responsible for it. During the oxidation an extra peak was observed at 1.915 min. During the acidic, oxidative, photolytic and thermal degradations the percentage of decomposition was found to be less than 20.0 %. The chromatograms obtained during the stress degradation conditions were shown in Figure 3A-3E.

Table 3. Robustness studies of rosuvastatin (100 µg/mL) and ezetimibe (100 µg/mL)

	Parameter	Condition	Conc. (µg/mL) found	% RSD	% Assay
Rosuvastatin	Flow rate (mL/min)	1.1 1.2 1.3	99.84	0.52	99.84
	Detection wavelength (nm)	252 254 256	99.67	0.42	99.67
	Mobile phase composition (v/v)	32:68 30:70 28:72	100.17	0.76	100.17
	pH ± 0.1	3.9 4.0 4.1	99.28	0.95	99.28
Ezetimibe	Flow rate (mL/min)	1.1 1.2 1.3	99.40	0.71	99.40
	Detection wavelength (nm)	252 254 256	98.88	0.99	98.88
	Mobile phase composition (v/v)	32:68 30:70 28:72	100.21	1.38	100.21
	pH ± 0.1	3.9 4.0 4.1	99.60	0.93	99.60

Figure 3. Typical Chromatograms of rosuvastatin and ezetimibe on acidic [A], alkali [B], oxidation [C], thermal [D] and photolytic [E] degradations.

The system suitability parameters for all the degradation studies were shown in Table 4. The number of theoretical plates (N) is used to determine the performance and effectiveness of the column. The efficiency of a column can be measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates / meter are ideal for a good system. Efficiency can be calculated by using the formula: $N = 5.54 \, [R/W_{h/2}]^2$

Where 'W' is the peak width, 'h' is the height of the peak and 'R_t' is the retention time of the drug peak. The theoretical plates were found to be more than 2000 and the tailing factor was less than <1.5 –2 or <2 indicating that the method is more selective and specific.

Table 4. Forced degradation studies of rosuvastatin and ezetimibe

	Stress conditions	% Drug recovered	% Drug decomposed	Theoretical plates	Tailing factor	% RSD
Rosuvastatin	Standard drug	100	0	6091.624	1.471	0.21
	Acidic degradation	98.92	1.08	6021.477	1.485	0.35
	Alkaline degradation	69.73	30.27	5923.741	1.495	0.23
	Oxidative degradation	97.61	2.39	6137.202	1.480	0.54
	Photolytic degradation	87.92	12.08	5546.035	1.450	0.48
	Thermal degradation	99.63	0.37	6017.274	1.488	0.57
Ezetimibe	Standard drug	100	0	8432.386	1.415	0.42
	Acidic degradation	80.95	19.05	8600.699	1.479	0.85
	Alkaline degradation	56.06	43.94	9001.826	1.432	0.74
	Oxidative degradation	99.43	0.57	8807.580	1.423	0.56
	Photolytic degradation	98.67	1.33	8528.807	1.414	0.23
	Thermal degradation	96.22	3.78	8385.751	1.436	0.62

* Mean of three replicates

Conclusion

The proposed method for the simultaneous determination of rosuvastatin and ezetimibe is simple, specific, precise, accurate, and robust and validated as per the ICH guidelines and can be applied for the long term stability studies as well as for the kinetic studies of the pharmaceutical formulations. The method was validated as per the ICH guidelines.

Acknowledgements

Conflict of interest

The authors report no conflicts of interest.

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