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Method development and validation of LC and Densitometric-TLC for simultaneous estimation of rosuvastatin calcium and ezetimibe in combined dosage forms

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ABSTRACT

Two simple and accurate methods to determine rosuvastatin calcium (ROS) and ezetimibe (EZE), in combined dosage form, were developed and validated using liquid chromatography (LC) and densitometric-thin layer chromatography (TLC). The LC separation was achieved on a Phenomenex Luna C_{18} column (250 mm, 4.6mm i.d., 5µm), in the isocratic mode using 0.65 M ammonium acetate buffer: methanol: acetonitrile: (30: 40: 30, v/v/v), pH 7.2 ± 0.05 at a flow rate of 1 mL/min. The retention times were about 3.60 and 6.99 min for ROS and EZE, respectively. Quantification was achieved with Photodiode array (PDA) detector at 239 nm over the concentration range of 0.5-5 µg/mL for each, with recoveries in the range of 98.91-99.82 % and 99.27-100.12 % for ROS and EZE, respectively. The TLC separation was achieved on silica gel 60 F254 HPTLC plates using toluene: acetone: glacial acetic acid (64.6: 35.0: 0.4, v/v/v), as the mobile phase. The Rf values were about 0.39 and 0.72 for ROS and EZE, respectively. Quantification was achieved with ultraviolet (UV) detection at 239 nm over the concentration range of 50-500 ng/spot for each, with recoveries in the range of 98.92-100.09 % and 98.80-100.01 % for ROS and EZE, respectively. Both methods were validated, and the results were compared statistically. They were found to be simple, specific, accurate, precise and robust. The methods were successfully applied for the determination of ROS and EZE in Combined dosage form without any interference from common excipients.

Key words: Rosuvastatin calcium, Ezetimibe, Combined dosage form, Liquid Chromatography and Densitometric-Thin Layer Chromatography

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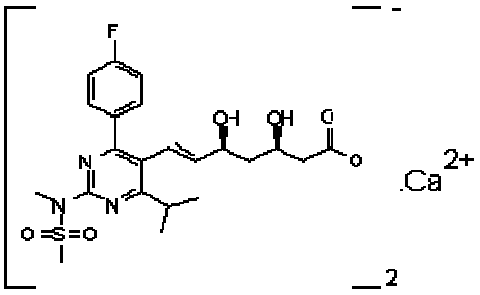
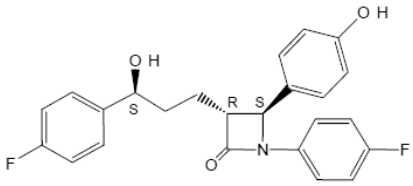
INTRODUCTION

Rosuvastatin (ROS), Bis[(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl(methyl sulfonyl)amino] pyrimidin-5-yl](3R,5S)-3,5-dihydroxyhept-6-enoic acid] calcium salt (Fig. 1 (a)), is a fully synthetic statin which has a potent cholesterol-lowering action than other drugs in its class. ROS is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, used as the calcium salt in the treatment of hyperlipidemia [1, 2]. Ezetimibe (EZE, 1-(4-fluorophenyl)-3-[3-(4-fluorophenyl)-3-hydroxy-propyl]-4-(4-hydroxyphenyl)-azetidin-2-one (Fig. 1 (b)), is a lipid lowering agent which selectively inhibits the intestinal absorption of cholesterol and related phytosterols [3].



(a)



(b)

Figure 1. Chemical structure of (a) ROS and (b) EZE

Literature survey revealed several analytical methods such as spectrophotometry [4-5], simple high performance thin layer chromatography (HPTLC) [6-7], simple and column-switching high performance liquid chromatography (HPLC) with UV and PDA detection [8-10], capillary zone electrophoresis [11], stability indicating HPLC [12], LC-MS/MS [13-14] and LC-ESI-MS [15] have been reported for the determination of ROS in pharmaceutical dosage forms and or in biological samples.

Literature survey revealed several analytical methods such as simple and stability indicating Spectrophotometry [16-17], simple and stability indicating HPTLC [18-20], simple and column-switching HPLC with UV detection [21-23], stability indicating HPLC [24-25], LC-MS/MS [26-27] and LC-ESI-MS [28-29] have been reported for the determination of EZE in pharmaceutical dosage forms and biological samples.

The present investigation describes a simple, accurate, sensitive and precise Liquid chromatography and Densitometric-thin layer chromatographic methods for the simultaneous determination of ROS and EZE in tablet dosage form.

MATERIALS AND METHODS

Reagents and materials

ROS and EZE powder with 99.94 and 99.96 % purity, respectively. LC grade methanol, acetonitrile and ammonium acetate were from SD Fine Chemicals Pvt. Ltd. (Ahmedabad, India). The water for LC was prepared by triple glass distillation and filtered through nylon 0.45µm-47mm membrane filter (Millipore, Bedford, MA). For TLC, aluminium backed silica gel 60 F_{254} 20 x 20 cm HPTLC plates (E. Merck KGaA, Darmstadt, Germany) with 0.2 mm layer thickness. AR grade methanol, toluene, acetone and glacial acetic acid were from SD Fine Chemicals Pvt. Ltd. (Ahmedabad, India).

Apparatus and chromatographic conditions

LC

A Shimadzu (Kyoto, Japan) LC system (LC-2010CHT) equipped with auto sampler, UV detector and Phenomenex (Torrence, CA) Luna C_{18} column (250mm, 4.6mm i.d., 5µm) was used. A Sartorius CP224S (Gottingen, Germany) analytical balance, and an ultra sonic cleaner (Frontline FS 4, Mumbai, India) were used. The LC system was operated isocratically at 25 ± 2 ^{0}C using mobile phase comprised of 0.65 M ammonium acetate buffer: methanol: acetonitrile: (30: 40: 30, v/v/v), pH 7.2 ± 0.05, at a flow rate of 1 mL/min. The mobile phase was filtered through nylon 0.45µm-47mm membrane filter and was degassed before use. The determination was performed at 239 nm using LC solution (v 1.2; Shimadzu) software. The injection volume was 20 µL and the total run time was 10 min.

TLC

A Camag (Muttenz, Switzerland) TLC system equipped with Linomat V auto sprayer, Scanner-III, flat bottom twin trough developing chamber (20 x 10 cm) and UV cabinet with dual wavelength (254 nm and 366 nm) UV lamp. Before use the plates were washed with methanol and activated at 110°C for 5 min. The mobile phase was

comprised of toluene: acetone: glacial acetic acid (64.6: 35.0: 0.4, v/v/v). Samples were applied to the plates as 5 mm bands, with a Hamilton (Reno, Nevada, USA) HPTLC syringe (100µL), keeping distance (5 mm) between bands, distance (158 mm) from the plate side edge and distance (15 mm) from the bottom of the plate. A sample application rate of 10µL/s was used. The chamber saturation time was 20 min at temperature 25 ± 2^0C. The development distance was 7 cm. Plates were removed from chamber, dried by means of hot air. The densitometric scanning was performed at 239 nm in absorbance-reflectance mode with winCATS software (v 1.3.3; Camag). The slit dimensions were 5.00 × 0.45 mm and the scanning speed was 100nm/s. The radiation source was a deuterium lamp emitting continuous UV radiation between 190 and 360 nm.

Preparation of ROS and EZE Standard solutions

LC

Accurately weighed ROS (10 mg) and EZE (10 mg) standards were transferred to a 50 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard stock solution (200 µg/mL) for ROS and EZE, each. An aliquot (2.5 mL) of the solution was transferred to a 50 mL volumetric flask, and diluted to the mark with mobile phase to obtain a working standard solution (10 µg/mL) for ROS and EZE, each.

TLC

Accurately weighed ROS (10 mg) and EZE (10 mg) standards were transferred to 50 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain standard stock solution (200 µg/mL) for ROS and EZE, each. An aliquot (5 mL) of the solution were transferred to 50 mL volumetric flask, and diluted to the mark with methanol to obtain working standard solution (20 µg/mL) for ROS and EZE, each.

Preparation of Sample solution

Twenty tablets were powdered and average weight was calculated. Accurately weighed amount of tablet powder equivalent to 10 mg ROS and 10 mg EZE was transferred to a 50 mL volumetric flask and methanol (30 mL) was added. The flask was sonicated for 15 min. The flask was allowed to stand at room temperature for 5 min, and the volume was diluted to the mark with methanol to obtain the sample stock solution (200 µg/mL) of ROS and EZE, each. For LC, the solution was filtered through 0.45µm-47mm membrane filter. An aliquot (2.5 mL) from sample stock solution was transferred to a 50 mL volumetric flask, and diluted to the mark with mobile phase to obtain working sample solution (10 µg/mL) for ROS and EZE, each. An aliquot from working sample solution (2 mL) was transferred to a 10 mL volumetric flask, and diluted to the mark with mobile phase to obtain the sample solution (2 µg/mL) for ROS and EZE, each. For TLC, an aliquot (5 mL) of the sample stock solution was transferred to 50 mL volumetric flask, and diluted to the mark with methanol to obtain working sample solution (20 µg/mL) for ROS and EZE, each. An aliquot of working sample solution (10 µL equivalent to 200 ng/spot for ROS and EZE, each) was applied to the plate.

Method validation

The methods were validated for the following parameters following the International Conference on Harmonization (ICH) guidelines [30].

Specificity

The specificity of the methods was established by comparing the chromatograms and measuring the peak purities of ROS and EZE from standard and sample solutions of the combined dosage form. For LC, the specificity was established by comparison of chromatogram of standard and sample ROS and EZE. For TLC, the peak purity of ROS and EZE were assessed by comparing spectra acquired at the peak start (S), peak apex (M), and peak end (E) of a spot. Correlation between ROS and EZE spectra from standard and sample was also established.

Linearity (Calibration curve)

LC

Aliquots (0.5, 1, 2, 3, 4 and 5 mL) of mixed working standard solution (equivalent to 0.5, 1, 2, 3, 4 and 5 µg/mL for ROS and EZE, each) were transferred in a series of 10 mL volumetric flasks, and the volume was made up to the mark with mobile phase. An aliquot (20 µL) of each solution was injected under the operating chromatographic conditions as described above. Responses were recorded. Calibration curves were constructed by plotting the peak areas versus the concentrations, and the regression equations were calculated. Each response was average of three determinations.

TLC

The Calibration curves were plotted over the concentration range of 50-500 ng/spot for ROS and EZE, each. Aliquots (2.5, 5, 10, 15, 20 and 25 µL) from the working standard solutions (equivalent to 50, 100, 200, 300, 400 and 500 ng/spot for ROS and EZE, each), were applied on plates. The plates were developed and scanned as

described above. Responses were recorded. Calibration curves were constructed by plotting peak areas vs. concentrations of ROS and EZE, and the regression equations were calculated. Each response was average of three determinations.

Accuracy (% Recovery)
The accuracy of the methods was determined by calculating recoveries of ROS and EZE by the standard addition method. Known amount of standard solutions of ROS (0, 0.5, 1.5 and 2.5 µg/mL) and EZE (0, 0.5, 1.5 and 2.5 µg/mL) for LC, and ROS (0, 100, 200 and 300 ng/spot) and EZE (0, 100, 200 and 300 ng/spot) for TLC were added to a prequantified sample solutions of ROS and EZE (2 µg/mL, each) for LC and (200 ng/spot, each) for TLC. Each solution was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equations of the calibration curves.

Precision
The precision of the instruments was checked by repeatedly injecting (n = 6) solution of ROS and EZE (2 µg/mL, each) for LC; while ROS and EZE (200 ng/spot, each) for TLC. For TLC, the repeatability of sample application was checked by measuring area of six bands having same concentration of ROS (200 ng/spot) and EZE (200 ng/spot) applied on the same plate, while the repeatability of measurement of peak area was checked by repeatedly measuring (n = 6) area of one band of ROS (200 ng/spot) and EZE (200 ng/spot) applied on the same plate without changing the position of plate. The results are reported in terms of relative standard deviation.

The intraday and interday precisions of the proposed methods were determined by estimating the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of ROS (1, 3 and 5 µg/mL) and EZE (1, 3 and 5 µg/mL) for LC, and ROS (100, 300 and 500 ng/spot) and EZE (100, 300 and 500 ng/spot) for TLC. The results are reported in terms of relative standard deviation.

Limit of Detection and Limit of Quantification
The limit of detection (LOD) and the limit of quantification (LOQ) of the ROS and EZE, for both LC and TLC, were calculated using the standard deviation of responses and slopes using signal-to-noise ratio.

Robustness
The robustness was studied by analysing the same samples of ROS and EZE by deliberate variation in the method parameters. The change in the responses of ROS and EZE were noted.

LC
Robustness of the method was studied by changing the extraction time of ROS and EZE from Combined dosage form by ± 2 min, composition of mobile phase by ± 2 % of organic solvent, flow rate by ± 0.2 mL/min.

TLC
Robustness of the method was studied by changing the extraction time of ROS and EZE from combined dosage form by ± 2 min, composition of mobile phase by ± 0.2 mL of organic solvent, development distance by ± 1 cm.

System-Suitability Test
System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were asymmetry of the chromatographic peak, peak resolution and tailing factor.

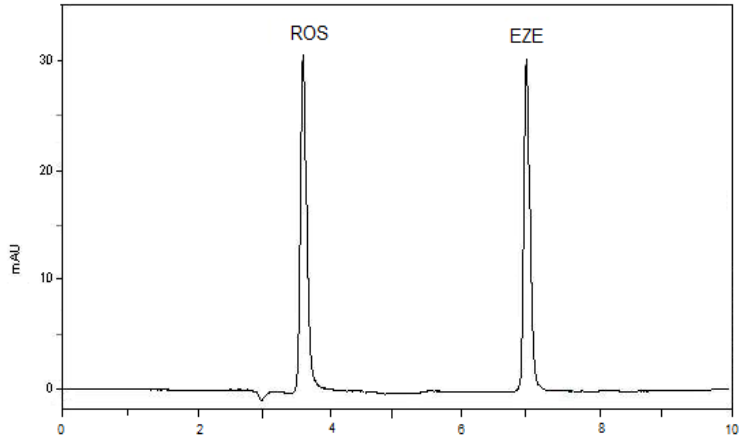
Determination of ROS and EZE in Combined dosage form
The responses of sample solutions were measured at 239 nm for quantitation of ROS and EZE by the proposed methods. The amount of ROS and EZE present in the sample solutions were determined by fitting the responses into the regression equations of the calibration curve for ROS and EZE, respectively.

RESULTS AND DISCUSSION

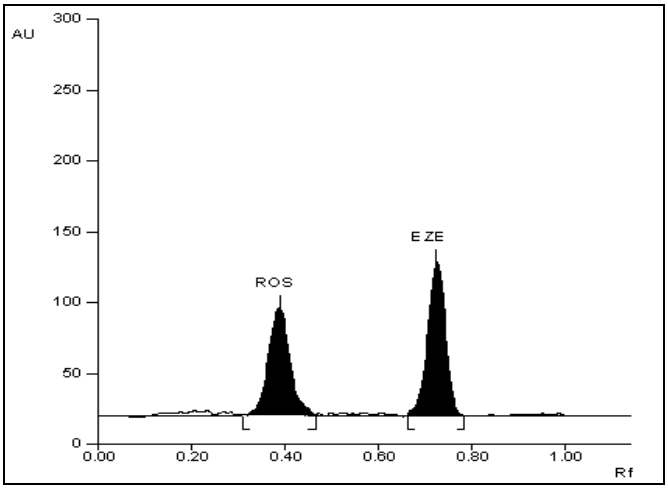
LC
The mobile phase consisting of 0.65 M ammonium acetate buffer: methanol: acetonitrile: (30: 40: 30, v/v/v), pH 7.2 ± 0.05 at a flow rate of 1 mL/min, was found to be satisfactory to obtain good peak symmetry, better reproducibility and repeatability for ROS and EZE. Quantification was achieved with UV detector at 239 nm based on peak area. The retention times were about 3.60 and 6.99 min for ROS and EZE, respectively (Figure 2).

TLC

The mobile phase consisting of toluene: acetone: glacial acetic acid (64.6: 35.0: 0.4, v/v/v) was found to be satisfactory to obtain good peak symmetry, better reproducibility and repeatability for ROS and EZE. Quantification was achieved with ultraviolet detection at 239 nm based on peak area. The Rf values were about 0.39 and 0.72 for ROS and EZE, respectively (Figure 2).



(a)



(b)

Figure 2. (a) Liquid chromatogram of ROS (2 µg/mL) and EZE (2 µg/mL) from Combined dosage form at 239 nm with retention time of 3.60 and 6.99 min, respectively; (b) Thin Layer chromatogram of ROS (200 ng/spot) and EZE (200 ng/spot) from Combined dosage form at 239 nm with R_f of 0.39 and 0.72, respectively

Method validation

Specificity

Both methods were found to be specific as no significant change in the responses of ROS and EZE was observed after 24 h for LC and TLC. For TLC, the peak purities of ROS and EZE from sample solution were >0.99. The peak purity r(S, M) = 0.9996 and r(M, E) = 0.9996 for ROS and r(S, M) = 0.9997 and r(M, E) = 0.9996 for EZE were found. Also good correlation (r = 0.9997) for ROS and (r = 0.9998) for EZE were obtained between standard and sample spectra. Peak purity > 0.99 indicates the methods specificity.

Linearity (Calibration curve)

Linear correlation was obtained between peak area and concentration for ROS and EZE each, in the range of 0.5-5 µg/mL for LC, and 50-500 ng/spot for TLC. The linearity of the calibration curves were validated by the value of correlation coefficient of the regression (*r*). The regression analysis of the calibration curves is shown in Table 1.

Table 1. Regression analysis of the calibration curves for ROS and EZE by the proposed LC and TLC-Densitometric methods (*n*=3)

Parameter	LC		TLC	
	ROS	EZE	ROS	EZE
Linearity range	0.5-5 µg/mL	0.5-5 µg/mL	50-500 ng/spot	50-500 ng/spot
Slope	49913.60	442146.36	5.22	7.49
Standard deviation of slope	48.1396	29.6039	1.0418	1.0319
Intercept	1087.98	15809.75	351.74	155.15
Standard deviation of intercept	210.8213	127.1639	8.1440	7.8947
Correlation coefficient, *r*	0.9995	0.9993	0.9989	0.9990

Accuracy (% Recovery)

The recovery study was carried out by the standard addition method. The percent recoveries obtained for ROS and EZE were in the range of 98.91-99.82 % and 99.27-100.12 % for LC, while 98.92-100.09 % and 98.80-100.01 % for TLC, which were satisfactory (Table 2).

Table 2. Results of recovery study for ROS and EZE by the proposed LC and TLC-Densitometric methods (*n*=3)

Method	Drug	Amount taken	Amount added	Recovery, %	RSD, %
LC	ROS, µg	2	0	99.36	0.31
		2	0.5	99.82	0.14
		2	1.5	98.91	0.43
		2	2.5	99.73	0.29
	EZE, µg	2	0	100.12	0.20
		2	0.5	100.09	0.48
		2	1.5	99.27	0.35
		2	2.5	99.84	0.16
TLC	ROS, ng	200	0	100.09	0.58
		200	100	99.75	0.32
		200	200	99.63	0.40
		200	300	98.92	0.61
	EZE, ng	200	0	99.48	0.62
		200	100	100.01	0.55
		200	200	98.80	0.29
		200	300	99.73	0.37

Precision

For LC, % RSD for repeatability was found to be 0.56 and 0.32 for ROS and EZE, respectively. For TLC, % RSD for the repeatability of sample application were found to be 0.35 and 0.40; while for the repeatability of measurement of peak area were found to be 0.22 and 0.24 for ROS and EZE, respectively.

The value of % RSD for intraday and interday variations were found to be in range of 0.29-1.04 and 0.34-1.10 for ROS, and 0.37-1.25 and 0.45-1.33 for EZE, respectively for LC; while 0.55-1.27 and 0.65-1.32 for ROS, and 0.48-1.35 and 0.56-1.42 for EZE, respectively for TLC. The % RSD values indicate the proposed methods are precise.

LOD and LOQ

The LOD and LOQ were found to be 0.0362 and 0.1101 µg for ROS, 0.0211 and 0.0638 µg for EZE, respectively for LC; while 2.4581 ng and 7.4489 ng for ROS, and 1.7268 ng and 5.2368 ng for EZE, respectively for TLC.

Robustness

The methods are found to be robust as the results were not significantly affected by slight variation in the chromatographic conditions for both LC and TLC.

System-Suitability Test

The results are reported in Table 3.

Table 3. System suitability test parameters for ROS and EZE by the proposed LC method

Parameter	ROS ± % RSD	EZE ± % RSD
Retention time, min	3.60 ± 1.17	6.98 ± 0.12
Tailing factor	1.14 ± 0.45	1.12 ± 0.67
Asymmetry	1.15 ± 0.73	1.13 ± 0.46
Theoretical plates	9155.50 ± 0.87	8410.50 ± 0.58

Determination of ROS and EZE in Combined dosage form

The proposed Liquid chromatography and Densitometric-Thin Layer chromatography were successfully applied for determination of ROS and EZE in Combined dosage form. The results obtained for ROS and EZE were comparable with the corresponding labeled claim percentage (Table 4).

Table 4. Analysis results for ROS and EZE Combined dosage form by the proposed LC and TLC-Densitometric methods (*n*=5)

Method	ROS			EZE		
	Labelled amount (mg)	Amount found (mg)	ROS ± SD, %	Labelled amount (mg)	Amount found (mg)	EZE ± SD, %
LC	10	10.01	100.10 ± 0.12	10	9.98	99.76 ± 0.22
TLC	10	10.02	100.21 ± 0.10	10	9.96	99.58 ± 0.15

$t_{calculated} < t_{table}$ (P = 0.05)

COMPARISON OF THE PROPOSED METHODS

The assay results for ROS and EZE, in combined dosage form, obtained using Liquid chromatography and Densitometric-thin Layer chromatography were compared statistically by applying the paired *t*-test. The calculated *t*-values for ROS (0.51) and EZE (0.55) are less than the tabulated *t*-value for ROS (2.73) and EZE (2.73) at the 95% confidence interval. Therefore, no significant difference is found in the content of ROS and EZE determined by the proposed methods.

CONCLUSION

Two methods were developed for determination of ROS and EZE based on different analytical techniques. Both methods were validated and found to be simple, sensitive, specific, accurate, precise and robust. Statistical comparison of the assay results for ROS and EZE in Combined dosage form by both methods indicated no significant difference. Hence, both methods can be used successfully for the routine analysis of pharmaceutical dosage forms of ROS and EZE.

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