Stability-Indicating Liquid Chromatographic Method for the Simultaneous Determination of Rosuvastatin and Ezetimibe from Pharmaceuticals and Biological Samples

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Abstract: Fixed dose combinations of ezetimibe and rosuvastatin are well known to provide significantly superior efficacy to rosuvastatin alone in lowering total cholesterol, and triglyceride levels. Therefore, in this recent work, we prepared fixed dose (10 mg/10 mg) ezetimibe and rosuvastatin tablet formulations, and a simple, selective and we established and validated rapid chromatographic method for the sensitive and simultaneous determination of rosuvastatin and ezetimibe. We performed the separation on Zorbax SB-C18 (100 mm x 4.6 mm, 3.5 µm particle size) column at 30 °C with a mobile phase that has a composition as water containing 0.1% H_3PO_4:methanol:acetonitrile (50:25:25 v/v/v) at 1.2 mL/min flow rate. We further performed validation studies according to ICH guidelines. Furthermore, we exposed rosuvastatin and ezetimibe to degradation conditions. We plotted peak area vs. concentration graphs for rosuvastatin and ezetimibe and used for defining the linearity of the detector response. In this suggested method, we observed linear relationships varying from 0.05 to 50 µg/mL for ezetimibe concentrations and from 0.05 to 25 µg/mL for rosuvastatin concentrations. We found the limit of detection values for rosuvastatin and ezetimibe as 0.006 and 0.008 µg/mL, respectively. Moreover, we further used the established method for to the analysis of ezetimibe and rosuvastatin from real spiked samples of rabbit serum.
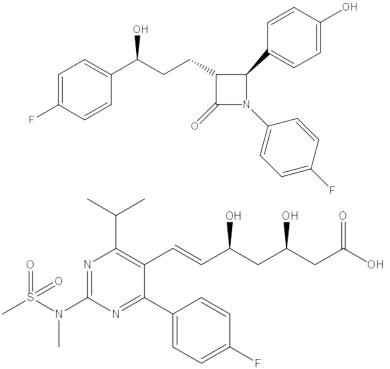
INTRODUCTION

Atherosclerosis is the main aspect of coronary heart disease (CHD), the most important reason for death and the main reason of illness all over the world (1,2). The main factors of risk for CHD (3,4) are hypercholesterolemia and higher low density lipoprotein cholesterol (LDLC) levels. Statins are generally used for lowering the cholesterol level. They inhibit the biosynthesis of cholesterol source; reduce the intracellular pools, and results in a reduction of plasma LDLC. However, an effective response cannot be obtained from a considerable fraction of hypercholesterolemic subjects, in the treatment of statin. Dose escalation and combination therapy of hypolipidemic drugs may be necessary to arrange LDLC blood levels (5,6). In LDLC reduction, drug combinations are successful hence; they may have some other effects. Nevertheless, the negligible efficacy of dose increase and present combination treatments using statins are limited by tolerance and compliance (7).

Ezetimibe, (EZE) ((3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3- (4-fluorophenyl)-3-hydroxyl propyl)] -4-(4-hydroxy phenyl)-2-azetidinone) blocks dietary and biliary cholesterol absorption by the small intestine. Hence, US Food and Drug Administration approved EZE in 2002 for the treatment of primary hypercholesterolemia. EZE is a lipid-lowering drug active compound, can selectively prevent the intestinal absorption of cholesterol and related phytosterols (7–9). Rosuvastatin, (ROS) bis((E)-7-(4-(4-flurophenyl)-6-isopropyl-2-(methyl(methylsulfonyl)amino)pyrimidin-5-yl)(3R,5S) -3,5-dihydroxyhept-6-enoic acid) calcium salt is used for the treatment of hyperlipidemia inhibiting 3-hydroxyl-3-methylglutaryl coenzyme A reductase (10,11). ROS is a cholesterol lowering agent, which used as precursor for cholesterol. In order to avoid cardiovascular disease, high cholesterol and associated situations ROS can be used for treatment (12, 13). In the market, ROS generally has dual combinations with other drug active compounds in pharmaceutical preparations such as amlodipine, fenofibric acid, salicyclic acid, olmesartan, etc. In the literature, high performance liquid chromatographic (HPLC) methods with UV detection exist for these combinations (14–17). As stated in the literature, the patients taking 5, 10, 20, or 40 mg rosuvastatin daily, mean plasma concentration of rosuvastatin was 1.6 ng/mL, 3.5 ng/mL, 6.3 ng/mL and 9.8 ng/mL, respectively (18). Moreover, after a single dose of 10 mg ezetimibe, mean ezetimibe peak plasma concentrations (C_{max}) of 3.4 to 5.5 ng/mL were obtained within 4 to 12

hours (19). Patients at risk of coronary CHD might not achieve the low-density lipoprotein cholesterol levels recommended in statin monotherapy. The usage of ROS/EZE combination therapy reduced LDL and enhanced other constituents of the lipid/lipoprotein profile more than ROS alone (−69.8% vs −57.1%, p <0.001) (6,20–22). in contrary, EZE does not affect the CYP enzyme system; the hydrophilic properties of ROS may reduce the need for extensive metabolism by CYP enzymes. Therefore, this overlaid pharmacokinetic effects, EZE and ROS combination is used in the therapy of LDLC (6). To identify, quantify, and separe the components, HPLC is the most widely used technique, which depends on high pressure to push solvents through the column. This method has some advantages towards other chromatographic methods such as reproducible response, high resolution, good peak shape, and high speed (23). HPLC methods are widely examined for the simultaneous determination of combined drug formulations in the biological matrix. Since routine analyses for clinical and pharmacological studies need fast and precise detection methods, in this research, determination of ROS and EZE using a stability indicated, fully validated method was provided to assay drugs in pharmaceutical dosage forms. Moreover, to demonstrate the applicability of the suggested chromatographic method, we detected EZE and ROS from prepared fixed dose combinations of EZE and ROS and spiked rabbit serum samples.

Scheme 1. Structures of Ezetimibe (top) and Rosuvastatin (bottom).

EXPERIMENTAL

Chemicals

We kindly obtained ROS and EZE from NUVOMED İLAÇ SAN. TİC. A.Ş. We received hydrogen peroxide, *o*-phosphoric acid, and microcrystalline cellulose from Sigma–Aldrich (St Louis, MO, USA). We purchased methanol, acetonitrile, sodium hydroxide, and hydrochloric acid, from Merck. To have ultra-pure water, we used Pure Flex water system (ELGA, Wycombe, UK). If not specified, all chemicals are of analytical reagent grade.

Instrumentation

We utilized the Agilent Hewlett-Packard 1100 HPLC system (Avondale, USA) consisting of DAD variable wavelength detector and data processing, which was supported with B.04.03 version of Chemstation®. We used Thermo Scientific Benchtop pH meter (Orion 3 Star™ Plus, USA) for the pH measurements. For the stationary phase, Zorbax SB-C18 (100 mm x 4.6 mm and 3.5 µm particle size), we purchased from Agilent Technologies (Agilent, USA)). For the preparation of pharmaceuticals, we used a tableting machine (Erweka EP-1, Germany).

MATERIAL AND METHODS

Chromatographic conditions

As the stationary phase, we used Zorbax SB-C18 (100 mm x 4.6 mm and 3.5 µm particle size (Agilent, USA)) column in isocratic mode at 30 °C and 1.2 mL/min flow rate with a mobile phase consisting of water containing 0.1% H_3PO_4:methanol:acetonitrile (50:25:25 v/v/v), with an injection volume of 10 µL. We used 5.0 M NaOH to adjust the pH of the mobile phase; we degassed and afterwards filtered the mobile phase. Prior to injections, we pre-conditioned the column for 30 min with the mobile phase. We washed the autosampler needle with mobile phase between each injection. We detected the samples at 242 nm.

Preparation of solutions

We prepared the stock solutions by dissolving ROS and EZE in methanol and diluted to desired concentrations with the mobile phase. We separated stock solutions of rabbit plasma using the solvent precipitation method. After addition of test solutions to the plasma, we mixed aliquots of 250 µL of plasma with 250 µL of cold acetonitrile and vortexed to precipitate the proteins. We then removed the precipitated proteins by centrifugation at 10000 rpm for 15 min. We withdrew 50 µL aliquots mixed with 950 µL mobile phase. We then placed aliquots of the resulting clear supernatants into the HPLC vials. We utilized KBr solution 0.01% (v/w) in water in chromatograms, to have the dead time (t_o).

Formulation and analyzing EZE–ROS tablets

We prepared tablets using direct compression method. Each tablet contained 10 mg of ROS and EZE as active substances and 230 mg of microcrystalline cellulose as the binder. Prior to compression, we screened granules using 300 µm sieve and then thoroughly blended in a mortar with pestle. For the preparation of tablets with an average weight of 250 mg, we utilized a single punch-tableting machine armed with flat-faced punches with a die, whose diameter was 11 mm.

System suitability test studies and validation of the method

We tested HPLC system using with USP criteria related to system suitability like capacity factor (from integrator), symmetry (at 10% height), tailing factor, and theoretical plate number. Subsequently, we achieved the validation of the proposed method and reported related to ICH guidelines and USP criteria (24–27). We injected different concentrations of ROS and EZE to the system to assess the linear range from triplicate injections. Thereafter, we accomplished the area of the detector response versus corresponding concentration to plot calibration graphs. We used least squares linear regression statistical analysis for the regression data analysis. Using the equations of $3.3 \times s/m$ and $10 \times s/m$, we calculated the limit of detection (LOD) and limit of quantification (LOQ), respectively. In these equations, (s) refers to the standard deviation of response and (m) to the slope of the calibration curve (25–27). We performed precision studies from averages of five measurements that were conducted in the same day and between days from 10 µg/mL for both standard and spiked rabbit serum samples. We confirmed the accuracy of the proposed method by recovery studies, which were performed using a standard addition procedure. We conducted recovery analysis by adding 5 µg/mL standard solutions of ROS and EZE on pharmaceutical dosage form to see the effect of ingredients. We expressed recovery studies in terms of the percent recovery, percent relative standard deviation and bias values. We performed recovery studies separately in mobile phase and serum samples.

According to ICH recommendations degradation studies consisted in terms of mild and drastic conditions (24,28). For thermal degradation of ROS and EZE, we located solid state of them in an oven at 75 ºC for 3 and 24 hours. We achieved acidic and oxidative degradation of 50 µg/mL ROS and EZE by exposing with hydrochloric acid (0.5 M HCl and 1 M HCl) and H_2O_2 (33% and 3%). We exposed these solutions for 3 hours under stress conditions. For alkaline hydrolysis, we exposed 50 µg/mL ROS and EZE to 0.5 M and 1 M NaOH for 3 hours.

RESULTS AND DISCUSSIONS

Method development
Prior to the validation studies, we improved the optimization of the HPLC detection parameters such as mobile phase composition in terms of % amount of H_3PO_4, methanol, and acetonitrile and pH; the flow rate and the temperature of the columns, (Data not shown) to have the optimum separation between ROS and EZE. Using Zorbax SB-C18 (100 mm x 4.6 mm, 3.5 µm particle size) column at 30 °C with the mobile phase composition as water containing 0.1% H_3PO_4:methanol:acetonitrile (50:25:25 v/v/v) at 1.2 mL/min flow rate we obtained an acceptable separation.

Parameters of System Suitability Tests
Prior to the chromatographic analysis we performed system suitability tests related to USP criteria (25–27). We examined capacity factor (>2), symmetry at 10% height (<2), USP tailing (<2), theoretical plate number (>2000), resolution (>1.5) parameters which were all intolerable limits (24, 25). Moreover, the retention time of ROS was 2.80 min and EZE was 4.50 min. We found resolution between ROS and EZE as 10.58 and we calculated selective factor as 1.90 between ROS and EZE. We summarized all the parameters related to system suitability tests in Table 1.

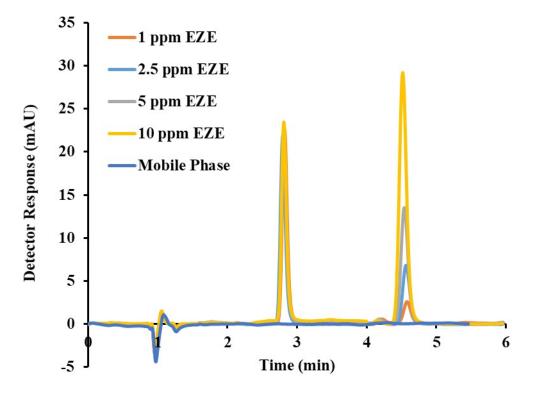**Table 1:** Parameters of System Suitability Tests.

Parameters	ROS	EZE	Recommended Value
Capacity Factor	2.05	3.90	>2
Selectivity to previous peak	-	1.90	>1
Resolution to previous peak	-	10.58	>2
Tailing Factor	1.21	1.17	<2
Symmetry at 10%height	1.09	1.16	<2
Theoretical number of plates	6904	9358	>2000

Method Validation

Linearity
The analytical performance of the method revealed by a linear relationship between ROS and EZE concentrations varying from 0.05 to 25 µg/mL for ROS, and 0.05 to 50 µg/mL for EZE. By means of the linearity equations, we obtained the LOD values as 0.006 µg/mL and 0.008 µg/mL for ROS and EZE, respectively. Readers can also follow Table 2 that, we obtained the calibration curves between 0.25-10 µg/mL for ROS and 0.5-10 µg/mL for EZE in a biological sample, spiked rabbit serum. We reported all statistical assessment of the calibration data in Table 2 and we presented chromatograms of increasing concentrations of EZE and ROS in Figure 1 in mobile phase and in Figure 2 in a biological sample, spiked rabbit serum.
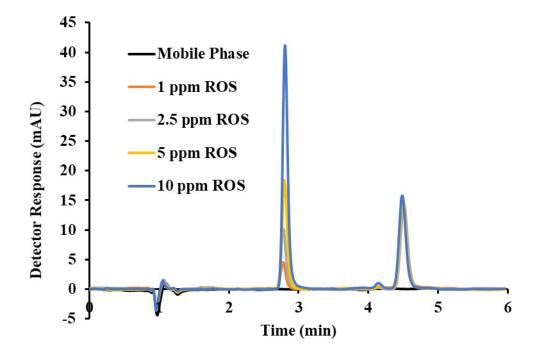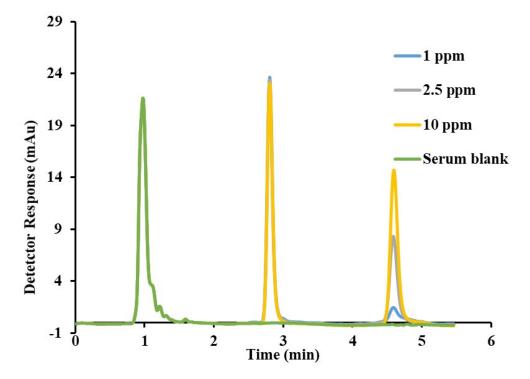
Figure 1: Chromatograms of increasing concentrations of EZE in the presence of 5 μg/mL ROS in mobile phase (top) and ROS in the presence of 5 μg/mL EZE in mobile phase (bottom).
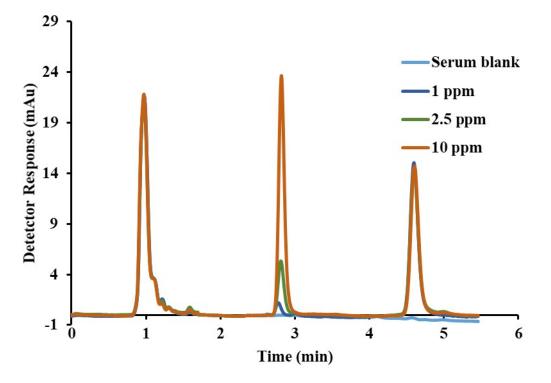
Figure 2: Chromatograms of increasing concentrations of EZE in the presence of 5 µg/mL ROS (top) and ROS in the presence of 10 µg/mL EZE (bottom) in spiked rabbit serum samples.

Precision: Repeatability studies

To have a precise method, we followed in day and between days repeatability studies and gave in terms of RSD %. It can be resulted from Table 2 that, low values of RSD % values indicate that the developed method is precise for the assay of both standard solutions and spiked rabbit serum samples of ROS and EZE.

Table 2: Statistical evaluation of the calibration data.

	Mobile Phase		Rabbit Serum	
Compounds	**ROS**	**EZE**	**ROS**	**EZE**
Linearity range (µg/mL)	0.05-25	0.05-50	0.25-10	0.5-10
Slope (mAU/µg.mL)	23.386	23.270	24.412	20.916
Intercept (mAU)	-3.362	-6.521	7.240	15.727
Correlation coefficient	0.999	0.999	0.999	0.999
LOD (µg/mL)	0.006	0.008	0.054	0.010
LOQ (µg/mL)	0.018	0.025	0.164	0.028
Within day Repeatability[a] (RSD %)	0.746	0.209	0.425	0.451
Between day Repeatability[a] (RSD %)	0.724	0.482	0.513	0.831

[a] Each value is the mean of five experiments.

Specificity: Degradation studies

We accomplished that degradation studies for 50 µg/mL ROS and EZE for specificity of the method. We designed degradation experiments at mild and drastic conditions with exposure to acidic and alkaline hydrolysis, hydrogen peroxide, UV, and heat. We presented related results in Table 3. It can be resulted that, since we found the degradation percentage more than 90 in heating and UV exposure, the method and the drug active compounds especially ROS are very sensitive to heating and UV exposure.
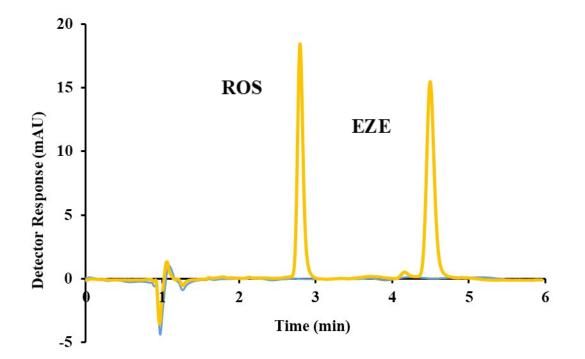
Table 3: The results of hydrolytic, oxidizing, thermal, and photolytic stressed under drastic and mild conditions of ROS and EZE using developed HPLC method.

	Response Stress Conditions	Degradation of ROS%	Degradation of EZE%
Drastic Conditions	HCl (1 M)	85.63	25.90
	NaOH (1 M)	75.25	35.98
	H_2O_2 (30 %)	77.79	36.02
	Thermal (24 hours at 75 °C)	96.72	57.39
	UV light exposure (24 hours at 254 nm)	97.92	56.56
Mild Conditions	HCl (0.5 M)	70.82	15.90
	NaOH (0.5 M)	52.56	23.19
	H_2O_2 (3 %)	44.52	22.40
	Thermal (3 hours at 75 °C)	96.94	51.83
	UV light exposure (3 hours at 254 nm)	97.63	52.81

Accuracy: Pharmaceutical analysis and recovery studies

We realized the determination of ROS and EZE as pharmaceutical dosage form (see Figure 3) and recoveries from the pharmaceutical dosage form and rabbit serum samples to conduct accuracy studies. In account of that, we used the related obtained equations for the calibration curves for the simultaneous determination of ROS and EZE. We added known amounts of standard solutions (5 μg/mL) over pharmaceutical dosage form samples and serum samples for the recovery studies. The results of recovery %, RSD % and Bias % were shown in the Table 4. We found mean of the spike recoveries

between 99-101% for tablet and serum samples that specifies a tolerable accuracy of the method. Accordingly, it can be noticed that the developed method was not affected from the possible interferences in the tablet and rabbit serum.

As a result, we herein suggested a powerful, suitable, and valuable method for analysis of ROS and EZE with fully validated conditions. Since in our method, the organic solvent consumption is only 3 mL per injection and analysis time is 5 min, greener approach (29–32) shorter analysis time (29, 30) was investigated when we compare the results within the literature.

Figure 3: The chromatogram of 5 μg/mL Rosuvastatin and Ezetimibe from prepared tablet formulation.

Table 4: Results of tablet and recovery analysis.

	Mobile Phase		Rabbit Serum	
	ROS	**EZE**	**ROS**	**EZE**
Label Prepared (mg)	10.00	10.00	-	-
Found	10.06	10.07	-	-
RSD (%)	0.95	0.49	-	-
Bias (%)	-0.63	-0.71	-	-
Added (mg)	5.00	5.00	5.00	5.00
Found[a] (mg)	5.04	5.05	4.98	5.01
Recovery (%)	100.80	101.00	99.60	100.20
RSD of recovery (%)	0.82	0.25	0.93	0.85
Bias (%)	-0.80	-1.00	0.40	-0.20

[a]Each value is the mean of five experiments.

CONCLUSIONS

In this study, we achieved a simultaneous determination of ROS and EZE with optimization and validation studies via a selective, sensitive, precise, and accurate HPLC method. The system suitability test parameters were also reports as capacity factor (ROS:2.05; EZE:3.90), symmetry at 10% height (ROS:1.09; EZE:1.16), USP tailing (ROS:1.21; EZE:1.17), theoretical plate number (ROS:6904; EZE:9358), resolution (EZE:10.58), and selectivity (EZE:1.90) parameters. After having the optimum conditions for separation such as Zorbax SB-C18 (100 mm x 4.6 mm, 3.5 μm particle size) column at 30 °C with a mobile phase that has a composition as water containing 0.1% H_3PO_4:methanol:acetonitrile (50:25:25 v/v/v) at 1.2 mL/min flow rate; we followed the full validation parameters. In order to show that the method is suitable for the detection of ROS and EZE, we further achieved the detection of ROS- and EZE-spiked rabbit serum samples. Furthermore, we also accomplished degradation studies and we utilized the stability indicating method to analyze ROS and EZE in spiked rabbit serum samples.

ACKNOWLEDGEMENT

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