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CHEMOMETRIC-ASSISTED SPECTROPHOTOMETRIC AND HPLC METHODS FOR THE SIMULTANEOUS ANALYSIS OF ATORVASTATIN CALCIUM AND EZETIMIBE

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ABSTRACT. This study aimed to develop new analytical strategies for the simultaneous determination of atorvastatin calcium (ATVC) and ezetimibe (EZE) in pharmaceutical formulations. The proposed methods included a chemometric approach using the partial least squares (PLS) technique with spectrophotometric data, first derivative zero-crossing, ratio spectra first derivative, and HPLC-DAD. PLS analysis was conducted using specialized software, with calibration performed in the 223–280 nm range at $\Delta\lambda$ = 4 nm intervals. In the first derivative zero-crossing method, absorbance values at 245.2 nm for ATVC and 224.8 nm for EZE were used. The ratio spectra first derivative method quantified ATVC at 232.6 nm and EZE at 223.1 nm. HPLC-DAD analysis was carried out using an XBridge C18 column with a mobile phase of 20 mM NaH_2PO_4:ACN (50:50 v/v), (240 nm, 1.0 mL/min). The linearity ranges were 1–20 µg/mL, 2–10 µg/mL, and 0.05–1.0 µg/mL, respectively. The methods were validated according to ICH guidelines, with no significant statistical differences observed (ANOVA, $p > 0.05$). Recovery from tablet formulations ranged from 95.43% to 102.28%. The PLS technique was highlighted as an environmentally friendly analytical approach. These validated methods demonstrated high sensitivity, accuracy, and reproducibility for routine pharmaceutical analysis.

Keywords: *Atorvastatin calcium, Ezetimibe, Chemometric, Spectrophotometry, HPLC, Validation, Green Chemistry*

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INTRODUCTION

Atorvastatin (ATV) is a reductase enzyme inhibitor. It is used for the prevention and treatment of cardiovascular disease in people at high risk. In pharmaceutical preparations ATV is usually used as the calcium salt atorvastatin calcium (ATVC). It is an enzyme inhibitor of 3-hydroxy-3 methyl glutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyses the conversion of HMG-CoA. ATV has the chemical formula C33H35FN2O5 (Figure 1) and is called (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid [1-3]**.**

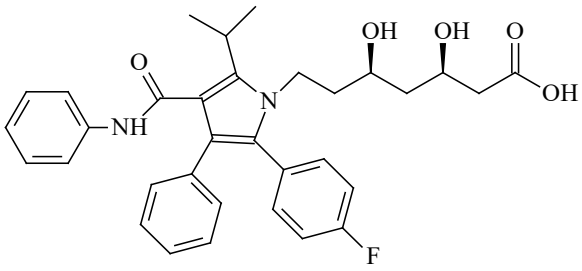


Figure 1. Chemical structure of ATV

Ezetimibe (EZE) is an active substance used to regulate high cholesterol levels in the blood. There are various generic preparations of different companies on the market. EZE has the chemical formula C24H12F2NO3 and is called 1-(4-fluorophenyl)-3R-[3(S)-(4-fluorophenyl)-3-hydroxy propyl]-4(S)(4 hydroxyphenyl) azetidin-2-one). (Figure 2) [4,5].

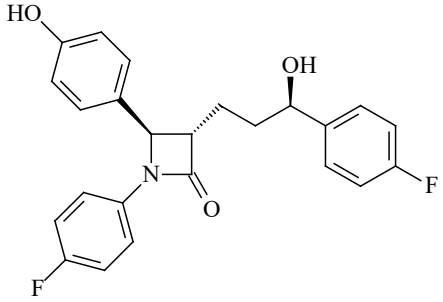


Figure 2. Chemical structure of EZE

Many studies have been conducted on the efficacy and safety of the combined use of ATV and EZE in patients with hyperlipidemia. Clinical studies have shown that the pharmacologic effect of the combined use of

EZE and ATV is more effective than the use of these two drugs alone [4,6]. Studies have also shown that the effect of ATV varies depending on the dose. For this reason, pharmaceutical preparations of ATV at various doses are available in the market [7].

One of the main reasons drugs have different responses in individuals is the genetic factors that affect drug pharmacokinetics. Therefore, drug analysis methods and applications are crucial in terms of drug dosage and the effectiveness of treatment response. ATVC and EZE mixtures have been extensively analyzed using various HPLC and other separation methods. Numerous studies in the literature focus on the determination of ATVC and EZE using UV and first derivative spectrophotometric methods for analyzing ATV and EZE either individually or in combination with other drugs [8-12]. Additionally, several chromatographic methods using HPLC-DAD/UV and LC-MS have been reported, which enable the simultaneous analysis of ATV/ATVC or EZE under different chromatographic conditions [13-18]. These methods not only allow the separation of the two drugs, ATVC and EZE, but in some cases, they achieve the separation of complex multi-component mixtures that include these two drugs. Only a limited number of studies are available for the determination of these two drugs using chemometrics [19]. However, no studies have been reported in the literature on using a chemometric approach with spectrophotometry for the simultaneous separation and quantification of both drugs.

The simplest method for determining the active ingredients in mixtures in pharmaceutical preparation is the chemometric approach. Because the concentration of each component can be calculated in just a few seconds without any separation techniques or other additional operational steps, using only a small number of solutions with varying active substance concentrations. Chemometric methods include mathematical operations that allow the analysis of substances without the use of any chemical reagents other than solvent that used for dissolving the active substance. For this reason, it can allow analysis without the need for solvents or chemicals discharged into the environment. While there are other techniques available for analytical purposes, partial least-squares (PLS) and principal component regression (PCR) are the most widely used methods [20] in chemometric analysis. Comparing two methods to other multicomponent analytes reveals certain advantages. The precise concentration of the preparation's active components can be ascertained by building matrices using variables like absorbance values and concentrations.

An extremely helpful analytical technique for qualitative and quantitative analysis from spectra made up of unresolved bands is derivative spectrophotometry. The zero-crossing approach involves measuring the

peak-to-peak distance from the x-axis, parallel to the ordinate. In this method, the absorbance of one drug is measured at the wavelength where the absorbance of the other drug crosses zero, allowing for the identification of a wavelength that provides a linear response with respect to concentration.

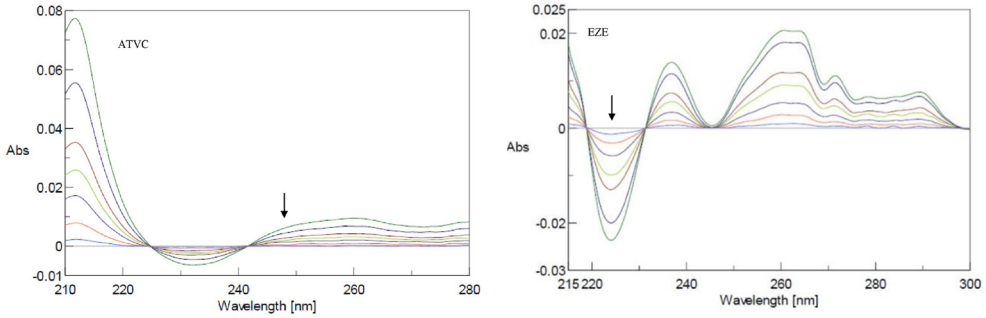
In the ratio spectra first derivative method, each spectrum is divided by the stored standard spectrum of either ATVC or EZE, and then the first derivative of the resulting ratio spectra is calculated. The ratio spectra of the solutions at various concentrations were generated using different mixtures of solutions that contain ATVC and EZE. The first derivative of these spectra is then identified. Chosen wavelength gives the linear relationship against the analyte concentration. Analyte concentrations that are unknown can be determined by analyzing the analytical responses at different intervals within the chosen wavelength range.

In this study, it was aimed to develop new four method for the analysis of ATVC, a cholesterol and triglyceride lowering agent, and EZE, another cholesterol and triglyceride lowering active substance, by a chemometric technique (PLS), first derivative zero-crossing, ratio spectra first derivative spectrophotometry, and HPLC methods and to apply the developed methods to the analysis of ATVC and EZE in Pharmaceutical formulations as tablets. Furthermore, to the best of our knowledge, no study in the literature has developed and compared four different analytical methods for the simultaneous determination of both drugs. Additionally, a comparative evaluation of these four techniques based on green analytical chemistry principles has not yet been conducted.

RESULTS AND DISCUSSION

For validation studies, specificity, limits of detection (LOD) and quantification (LOQ), selectivity, linearity, accuracy, sensitivity, intra-day and inter-day precision were investigated for developed methods. The developed methods were put to the test using the validation parameters for the purpose to demonstrate their validity. The accuracy of the procedure was evaluated by comparing the measured values with the actual values, while precision was assessed by analyzing three different concentration levels in six replicates each.

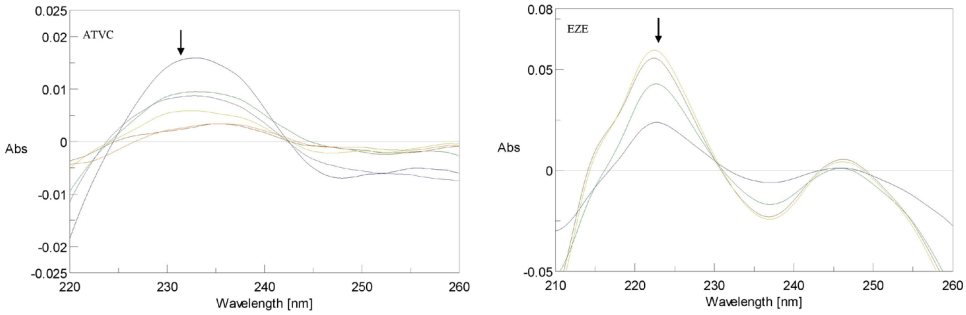
The first derivative zero-crossing method was found to be linear in the range 1 - 20 µg/mL for ATVC and EZE. With this method, quantification was performed at wavelength values of 245.2 nm for ATVC and 224.8 nm for EZE (Figure 3).



(a) **(b)**

Figure 3. First derivative zero-crossing spectrums for (a) ATVC and (b) EZE in ethanol:water (1:1 v/v).

The ratio spectra first derivative method was found to be linear in the range 2 - 15 µg/mL for ATVC and EZE. In this method, for the quantification of ATVC in mixed solutions, the concentration of ATVC was gradually increased while the concentration of EZE was kept constant at 2 µg/mL. Each spectrum was then divided by a fixed ATVC concentration (5 µg/mL), and the first-order derivative of the resulting absorbance spectra was calculated and used for quantification through spectral analysis. ATVC quantification was performed at a wavelength of 232.6 nm. Similarly, for EZE quantification, ATVC (2 µg/mL) concentration was kept constant and EZE concentration was increased and divided by a constant EZE (5 µg/mL) concentration value and the first order derivative of the absorbance spectra were obtained and quantified by using these spectra. EZE quantification was performed at a wavelength of 223.1 nm (Figure 4).



(a) **(b)**

Figure 4. Ratio spectra first derivative absorption spectrums obtained increasing concentrations (a) ATVC and (b) EZE in ethanol:water (1:1 v/v).

In the PLS method, the absorbance values of the calibration data set and the unknown samples were taken in the wavelength range 220-270 nm at the spectral conditions determined and the concentration of the unknown solution was calculated using the vectorial measurements of the absorbance values in the wavelength range 223-280 nm in the chemometric calibrations (Table 6).

In the developed HPLC-DAD method, method was found linear in the range 0.1-1.2 µg/mL for ATVC and EZE. The system suitability test for the standard solution containing 1µg/mL ATVC, EZE and Q; internal standard (IS) was evaluated, and parameters were accepted in terms of retention times, capacity factor (k'), tailing factor and theoretical number of plates (N) (Table 1). The tailing factor was found to be <2, capacity factor values in the range 1<k'<10 and N>2000.

Table 1. System suitability parameters

	Retention Time* (Min)	**Capacity Factor (k')**	**Tailing Factor**	**Theoretical Number of Plates**
IS	3.64 ± 0.01	2.65	1.93 ± 0.02	10438.08
ATVC	6.13 ± 0.01	5.11	1.17 ± 0.01	8620.80
EZE	8.80 ± 0.01	7.82	1.17 ± 0.01	11938.96

*n=6, mean ± standard deviation

The LOD and LOQ values for ATVC and EZE were found as 0.05 µg/mL and 0.15 µg/mL, respectively for first derivative spectra zero-crossing, ratio spectra first derivative spectrophotometric methods and PLS technique. For HPLC-DAD method LOD and LOQ values for ATVC and EZE were found as 0.01 µg/mL and 0.03 µg/mL, respectively. The calibration curve parameters generated from the data obtained from the methods are given in Table 2. The r^2; regression coefficient values from the curve equations were very close to 1.

Table 2. Calibration curve parameters (n=6)

	First derivative zero-crossing		**Ratio spectra first derivative**		**HPLC**	
Parameters	**ATVC**	**EZE**	**ATVC**	**EZE**	**ATVC**	**EZE**
λ (nm)	245.2	224.8	232.6	213.8	240.0	240.0
Linear range (µg/mL)	1.0-20.0	1.0-20.0	2.0-10.0	2.0-10.0	0.05-1.0	0.05-1.0
(y=mx+n) Slope (m)±SE[a]	0.0002± 0.00	0.0012± 0.00	-0.0008± 0.00	-0.0034± 0.00	0.831± 0.00	0.925± 0.00
(y=mx+n) intercept (n)±SE[a]	0.0001± 0.00	0.0005± 0.00	0.0104± 0.00	0.0027± 0.00	-0.0137± 0.00	0.0014± 0.00
r^2	0.9991	0.9961	0.9987	0.9969	0.9985	0.9996

SE[a] : Standard error

Precision studies were performed for each method according to the linear range. Intra-day and inter-day (n=6) evaluation results are given in Table 3. Figure 5 shows the representative chromatogram obtained for the standard solution containing 1µg/mL ATVC, EZE and Q (IS) under the selected analysis conditions. The retention times (mean ± standard deviation, n=6) for Q, ATVC and EZE were found to be 3.644 ± 0.005, 6.128 ± 0.008 and 8.796± 0.013 min. respectively.

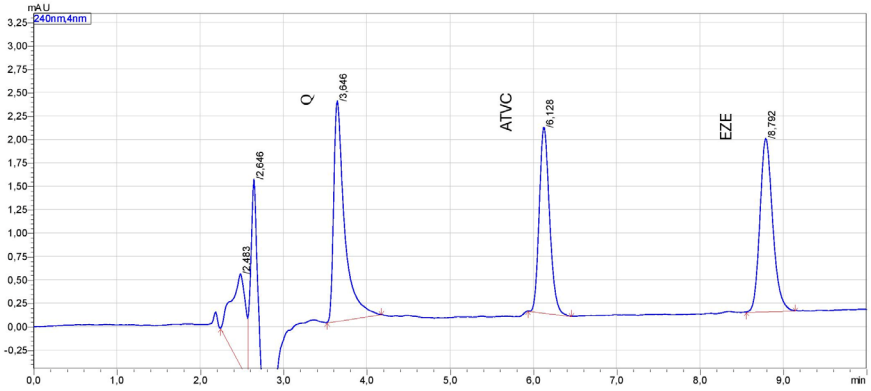


Figure 5. Chromatogram of standard containing 1.0 µg/mL ATVC, EZE, and Q (IS)

Table 3. Precision and accuracy results for inter-day and intra-day (n=6)

	Inter-day			Intra-day		
Added (µg/mL)	**Found[a] $\bar{x}$ (µg/mL)**	**Precision[b] RSD (%)**	**Accuracy[c] Bias (%)**	**Found[a] $\bar{x}$ (µg/mL)**	**Precision[b] RSD (%)**	**Accuracy[c] Bias (%)**
First derivative zero-crossing (EZE)						
2.5	2.48± 0.03	1.19	-0.58	2.49± 0.04	1.43	-0.41
7.5	7.52± 0.06	0.76	0.29	7.60± 0.04	0.55	1.38
10.0	9.99± 0.82	1.12	-0.09	9.95± 0.01	0.13	-0.44
First derivative zero-crossing (ATVC)						
2.5	2.54± 0.01	0.28	1.97	2.54± 0.03	1.10	1.59
7.5	7.44± 0.01	0.18	-0.74	7.45±0.07	0.88	-0.67
10.0	10.00±0.01	0.07	0.07	10.16±0.09	0.90	1.61
Ratio spectra first derivative (EZE)						
2.0	1.99± 0.02	1.07	-0.11	1.99± 0.01	0.36	-0.49
5.0	5.08± 0.01	0.07	1.68	5.06± 0.02	0.38	1.23
10.0	9.99± 0.13	1.34	-0.02	10.02±0.04	0.43	0.29
Ratio spectra first derivative (ATVC)						
2.0	1.99 ± 0.05	2.31	-0.08	1.99 ± 0.05	2.31	-0.08
5.0	5.07 ± 0.12	2.32	1.43	5.03 ± 0.04	0.82	0.63
10.0	9.97 ± 0.09	0.90	-0.29	10.01±0.08	0.76	0.17

	Inter-day			Intra-day		
Added (µg/mL)	**Found[a] $\bar{x}$ (µg/mL)**	**Precision[b] RSD (%)**	**Accuracy[c] Bias (%)**	**Found[a] $\bar{x}$ (µg/mL)**	**Precision[b] RSD (%)**	**Accuracy[c] Bias (%)**
HPLC (EZE)						
0.1	0.101±0.02	1.22	-0.12	0.097±0.06	0.75	-1.50
0.4	0.405±0.06	1.10	1.14	4.201±0.02	0.88	1.08
0.8	0.799±0.07	0.84	-0.06	0.816±0.03	1.03	2.00
HPLC (ATVC)						
0.1	0.097±0.01	0.71	-1.27	0.10 ± 0.01	0.76	0.92
0.4	0.383±0.04	0.46	-2.17	0.388±0.03	0.94	-1.09
0.8	0.807±0.09	0.73	0.89	0.796±0.03	0.95	-0.54
PLS (EZE)						
2.5	2.53 ± 0.01	1.19	1.02	2.55 ± 0.00	0.05	1.60
7.5	7.58 ± 0.01	0.16	1.00	7.60 ± 0.01	0.17	1.32
10.0	10.15±0.02	0.37	0.51	10.06±0.01	1.46	0.58
PLS (ATVC)						
2.5	2.54 ± 0.00	0.19	1.71	2.54± 0.01	0.68	1.65
7.5	7.58 ± 0.00	0.09	1.08	7.60 ± 0.00	0.06	1.25
10.0	10.09±0.04	1.00	0.98	10.10±0.03	0.60	1.01

[a]$\bar{x}$: Mean value, [b]RSD**:** Relative Standard Deviation,
[c]Bias**:** ((amount found - amount added)/amount found) x100

The developed methods were applied to the analysis of ATVC and EZE in pharmaceutical preparation. For this purpose, three different tablet formulations, Ezetec Plus 10/10, 10/20 and 10/40, were analyzed. The recovery values of ATVC and EZE were found to be in the range of 95.43% to 102.28% (mean) (Table 4). It was determined that the excipients did not interfere with the methods developed. In all recovery studies conducted using the PLS technique, the calculated standard error of prediction (SEP) values were found to be <0.8.

Table 4. Recovery values (%) obtained for Ezetec Plus 10/10, Ezetec Plus 10/20 and Ezetec Plus 10/40 preparations (mean values for n=6)

	First derivative zero-crossing		Ratio spectra first derivative		PLS		HPLC	
	EZE	**ATVC**	**EZE**	**ATVC**	**EZE**	**ATVC**	**EZE**	**ATVC**
10/10	98.9	100.2	102.0	98.5	101.7	101.6	99.0	99.6
10/20	100.1	99.8	99.1	101.9	98.8	101.7	99.7	100.8
10/40	98.1	101.8	95.4	100.4	99.7	99.2	99.1	102.3

A representative chromatogram obtained after the injection of Ezetec Plus 10/10 into the HPLC-DAD system is shown in Fig. 6. The ATVC, EZE, and IS peaks were well resolved, with no interfering peaks from the tablet observed, ensuring good separation.

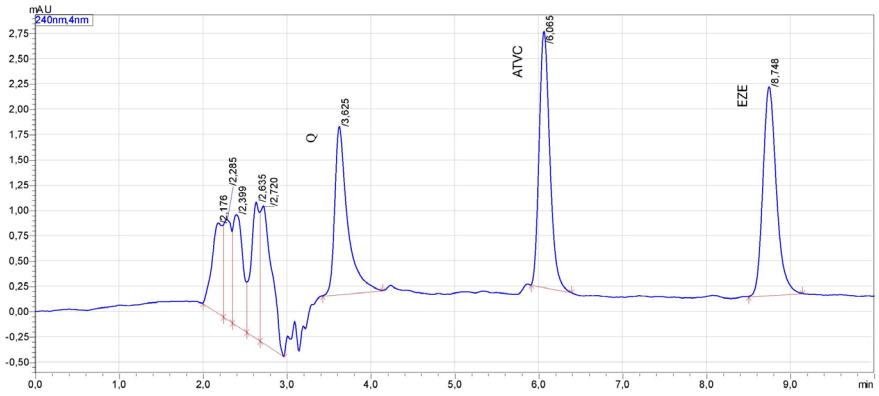


Figure 6. Chromatogram obtained for Ezetec Plus 10/10 tablet and spiked Q (IS)

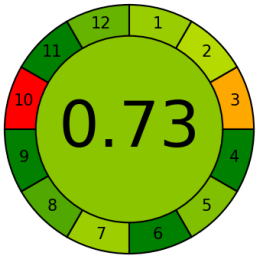
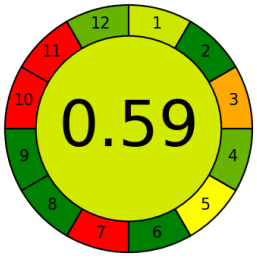
ANOVA was used to test whether there was a statistical difference between the developed methods and the results obtained after tablet analysis and no significant difference was observed ($p>0.05$).

The first derivative zero-crossing and ratio spectra first derivative spectrophotometric methods, commonly used as spectrophotometric analysis methods, is a practical and preferred analysis method that allows the separation of substances in a mixture without chemical pretreatment and separation procedure.

The selection of the appropriate wavelength for the derivative spectra of the active substances being spectrum scanned, especially for the analysis of substances in mixtures, is of great importance in quantitative calculations. As a chemometric approach, the PLS technique is more practical and environmentally friendly than many analytical methods in that it does not require excessive time for sample preparation, does not require complex liquid-liquid extraction processes, and does not require pre-treatment or any consuming process. There are various spectrophotometric methods for the analysis of ATVC and EZE in the literature. However, no studies have been conducted on the simultaneous analysis of ATVC and EZE in their binary mixtures using chemometric techniques with spectrophotometric data. Therefore, after the developed spectrophotometric methods, a quantification study was carried out with PLS, as a chemometric multicomponent technique. According to the Green Analytical Procedure Index (GAPI), the PLS technique

was evaluated based on the 12 criteria [26-28]. These criteria were listed in Table 5. By using these parameters, the Analytical Greenness Calculator (AGREE) metric and score were calculated. According to the metric results, the PLS technique (0.79 score) qualifies as a green chemistry technique, with all criteria classified as green. When other methods are evaluated using the AGREE metric, the chemometric-spectrophotometric approach emerges as the most environmentally friendly option (Table 5).

Table 5. AGREE Scores for methods

Score of AGREE Metric	Method Criteria
For PLS method 	1. Sample treatment 2. Sample amount 3. Device positioning 4. Sample pretreatment stages 5. Automation, miniaturization 6. Derivatization 7. Waste 8. Analysis throughput 9. Energy consumption 10. Source of reagent 11. Toxicity 12. Operator's safety
For Spectrophotometric methods 	
For HPLC method 	

Compared to previous studies, the developed HPLC method achieved nearly the same AGREE metric score of approximately 0.6 in terms of the organic component used in the mobile phase. However, the score obtained through the chemometric approach demonstrates a higher value, classifying it as green analytical chemistry. Thus, green analytical chemistry emphasizes analytical practices that reduce or eliminate the use or generation of substrates, solvents, or by-products that pose risks to the ecosystem or human health. One approach to minimizing the environmental impact of analytical procedures is the development of alternative direct analysis techniques that do not rely on organic solvents or substrates.

Within the scope of the studies to establish a new HPLC-DAD method, a simple and selective chromatographic method was developed for the simultaneous analysis of ATVC and EZE. The results obtained from other methods were then statistically compared with each other and with our newly developed HPLC-DAD method. Validation studies of the developed methods were performed according to ICH requirements; selectivity, sensitivity, linearity, accuracy, precision, recovery and reproducibility parameters were examined, and statistical evaluations were made. When the obtained LOD and LOQ values and working ranges for the developed methods are compared with previous studies, it can be said that the proposed methods are more precise and sensitive. In previous studies, for instance, the LOQ value for EZE using spectrophotometric methods was reported as 2 µg/mL [11]. In another study employing FTIR, the LOQ values for EZE and ATVC were found to be 1.839 µg/mL and 0.942 µg/mL, respectively [12]. An HPLC-based study reported the LOQ value for EZE as 0.19 µg/mL [14]. In the analysis of ATVC from pharmaceutical preparations, the LOD and LOQ values of the developed method were reported as 0.013 and 0.13 µg/mL, respectively [15]. In a method developed for the determination of ATVC in plasma, the LOQ values for ATVC and EZE were found to be 1.294 µg/mL and 1.384 µg/mL, respectively [16]. In another HPLC-based ATVC analysis, the LOD and LOQ values were found to be 0.31 µg/mL and 0.95 µg/mL, respectively [17]. Similarly, another HPLC method for ATVC reported LOD and LOQ values of 1 µg/mL and 3 µg/mL, respectively [18]. In our current study, the developed HPLC method achieved LOD and LOQ values of 0.01 and 0.03 µg/mL for ATVC and EZE, respectively, while the spectrophotometric methods yielded 0.05 and 0.15 µg/mL, indicating a higher sensitivity compared to those in the literature.

CONCLUSION

In this study, new easy, simple, selective, and validated first derivative zero-crossing and ratio spectra first derivative spectrophotometric, chemometric approach (PLS technique with spectrophotometry) and HPLC-DAD methods were developed for the simultaneous analysis of atorvastatin calcium (ATVC) and ezetimibe (EZE) in their binary mixture. All developed methods evaluated using the AGREE metric and PLS technique can be recognized as a green analytical technique in comparison to others. As a new method, it provides an innovative approach by being feasible, simple, and practical. First derivative zero-crossing, ratio spectra first derivative spectrophotometric, PLS (partial least squares regression), and HPLC-DAD methods were successfully applied for the analysis of ATVC and EZE in three commercially available tablets in Türkiye, yielding good recovery values. Additionally, in this study, four different methods were developed, and these methods were evaluated and compared in terms of active ingredient analysis from pharmaceutical preparations and their green analytical applications.

EXPERIMENTAL SECTION

Chemicals and reagents

Analytical standards of ATVC (USP standard) and EZE, (USP standard) kindly gifted from Neutec, Pharmaceutical Industries Inc, Quercetin (Q; internal standard, IS) was purchased from Merck, (Darmstadt, Germany). Acetonitrile (HPLC grade, CARLO ERBA, Italy), methanol (HPLC grade, CARLO ERBA, Italy), ethanol (analytical grade, CARLO ERBA, Italy), NaH_2PO_4, NaOH were purchased from Sigma-Aldrich (USA). ATVC, EZE and Q (IS) stock solutions were prepared with ethanol at 1 mg/mL and the solutions to be used in the analysis were prepared daily by diluting the stock solutions with ethanol:water (1:1, v/v) mixture to the desired concentrations.

Instrumentation and chromatographic conditions

For chemometric calculations, multivariate chemometric analysis was performed by using R.G. Brereton program. The current program was obtained directly from its developer R.G. Brereton and Multivariate analysis Add-ing for Excel v1.3 software [21].

Spectrophotometric analyses were performed by spectrophotometer (Jasco, V-730, C246261798). Used chromatographic system consisted of a Shimadzu liquid chromatography (LC-2030C 3D Plus) that was equipped with

a pump, a controller connected to a computer using a software, an autosampler, 10 µL injection loop and Diode Array Detector (DAD). In the HPLC system, the DAD detector allows for simultaneous analysis of drugs at different wavelengths, enabling analytical measurements at multiple wavelengths at once. This feature offers numerous advantages in analytical applications, making the DAD detector highly beneficial. The system was controlled through a system controller a personal computer workstation with a data processing system (Shimadzu, Kyoto, Japan) installed on it. The separation was performed on a XBridge, C18 (250 x 4.6 mm, particle size 5 µm) analytical columns (Waters, Milford, MA, USA). The column temperature was set to 24 °C. The mobile was phase composed of 20 mM NaH_2PO_4 (pH adjusted to 5.17 with NaOH):acetonitrile (50:50, v/v) at 1.0 mL/min. in isocratic mode and DAD detector wavelength was set to 240 nm. ATVC has a pKa value of 4.46 and the estimated pKa of ezetimibe is 9.7 [3,6]. Knowing the pKa of analytes allows for an effective selection of the mobile phase pH. The selection of mobile phase and pH has been adjusted to ensure optimal separation based on the pKa values of the analytes to be analyzed. 10 µL of sample solutions were injected into the HPLC. Quercetin was chosen as an internal standard (IS) because its peak was well separated from the peaks of the two drugs, with no interfering peaks observed. Type 1 water (Simplicity 185 Water System, Millipore Corp., Bedford, MA, USA) was used for all analytical applications. Before using of the mobile phase, it was filtered through a membrane filter with a pore diameter of 0.45 µm and kept in an ultrasonic bath for 15 min. After each analysis procedure analytical column was flushed with approximately 20 times of column volume of a mixture with HPLC grade water and methanol (50:50, v/v).

Analysis with PLS technique

For the chemometric approach, PLS technique was chosen, twenty different mixtures were prepared for calibration or regression matrix. These mixtures contain different amounts of ATVC and EZE (between 2 and 15 µg/mL concentration range). Zero-order absorbance values were recorded between 223 and 280 nm in the 4 nm wavelengths intervals. Matrix (20 concentration x 20 wavelength) (Table 6) was used as training set for prediction of unknown concentration. A matrix was prepared by recording absorbance values for 20 different solutions. Using this matrix, the active ingredient concentration in an unknown solution, for which 20 different absorbance values were similarly entered, and then it was estimated. For the prediction step, the prediction ability of this technique can be expressed in terms of the standard error of prediction (SEP) [20] (Brereton, 2003). Equation (1) defines the SEP equation. Where n is the total number of samples and C_i^{added} is the concentration of drug added.

$$SEP = \sqrt{\frac{\sum_{i=1}^{n}(C_i^{added} - C_i^{found})^2}{n}} \quad (1)$$

Table 6. Training set used in PLS technique for ATVC and EZE

Mixture No	ATVC (µg/mL)	EZE (µg/mL)
1	2	2
2	2	5
3	2	8
4	2	10
5	5	2
6	5	5
7	5	8
8	5	10
9	8	2
10	8	5
11	8	8
12	10	2
13	10	5
14	10	8
15	13	2
16	13	5
17	13	8
18	15	2
19	15	5
20	15	8

First derivative zero-crossing method

For the First Derivative zero-crossing method, the spectra analysis of 10 µg/mL containing standard solutions were recorded in the range of 200-600 nm. The first derivatives of the obtained spectrum graphs were taken and quantified by zero-crossing method in the range of 220-320 nm. Instrument parameters for the first derivative zero-crossing method: Response time: 0.015 s, scan rate: 1000 nm/min, spectral slit width: 1 nm, data spacing: 0.2 nm and Δλ = 4 nm.

Ratio spectra first derivative method

For the ratio spectra first derivative method, zero-order spectrums of the solutions that contain ATVC and EZE at various concentrations in ethanol:water (1:1, v/v) were recorded in the range of 200-600 nm. The

spectrums ratioed to the spectra of standard solutions of ATVC and EZE at various concentrations. The concentration of the solution of division spectra is very important and various concentrations of ATVC and EZE were tried as divisor for finding the optimum value. For this purpose, 5 µg/mL EZE solution was used as divisor for ATVC analysis and 5 µg/mL ATVC solution was used as divisor for EZE analysis. The first derivatives of these spectra were then recorded.

The results obtained by our new developed HPLC method were compared with the results obtained by chemometric technique and derivative spectrophotometric methods. In addition, ANOVA was used to compare the methods statistically with each other.

Method validation

The developed methods were validated according to International Conference on Harmonization (ICH) requirements ("Validation of Analytical Procedures:Text and Methodology Q2 (R1)", 2014; "validation of analytical procedures Q2(R2)", 2022). For validation, the data gathered from the HPLC method was used to determine the retention time, capacity factor, tailing factor, and theoretical number of plates for each active ingredient. Specificity, linearity, accuracy, limit of quantification (LOQ), limit of detection (LOD), and intra-day and inter-day precision parameters were investigated for spectrophotometric, chemometric, and HPLC techniques for validation studies [22-24].

The system suitability test of chromatographic method was performed with six repeated analyses of the standard solution (containing 1 µg/mL). Calibration curve was plotted against the ratio of the peak area under the analyte peak to the peak area under the IS peak against the concentration value of each drug. The r^2 values from the acquired curves were examined for the purpose of figuring out the linearity of the methods. Accuracy, sensitivity, selectivity, recovery values from tablets and precision studies were performed at three different concentration values (n=6). The limit of detection (LOD) was taken as the signal-to-noise (S/N) value equal to 3 and the limit of quantification (LOQ) value was taken as the signal-to-noise (S/N) value equal to 10 for repeated (n=6) different standard solution analyses [24,25].

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