Simultaneous Determination of Ezetimibe and Simvastatin in Pharmaceutical Preparations by MEKC

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Abstract

A micellar electrokinetic capillary chromatography method was developed and validated for the simultaneous determination of ezetimibe and simvastatin in pharmaceutical preparations. The influence of buffer concentration, buffer pH, sodium dodecyl sulphate (SDS) concentration, organic modifier, capillary temperature, applied voltage, and injection time was investigated, and the method validation studies were performed. The optimum separation for these analytes was achieved in less than 10 min at 30°C with a fused-silica capillary column (56 cm x 50 µm i.d.) and a 25mM borate buffer at pH 9.0 containing 25mM SDS and 10% (v/v) acetonitrile. The samples were injected hydrodynamically for 3 s at 50 mbar, and the applied voltage was +30.0 kV. Detection wavelength was set at 238 nm. Diflunisal was used as internal standard. The method was suitably validated with respect to stability, specificity, linearity, limits of detection and quantification, accuracy, precision, and robustness. The limits of detection and quantification were 1.0 and 2.0 µg/mL for both ezetimibe and simvastatin, respectively. The method developed was successfully applied to the simultaneous determination of ezetimibe and simvastatin in pharmaceutical preparations.

Introduction

Ezetimibe (EZE) (Figure 1A), is the first in a new class of cholesterol absorption inhibitors that blocks the intestinal absorption of dietary and biliary cholesterol without affecting the uptake of triglycerides or fat soluble vitamins. Simvastatin (SIM) (Figure 1B) is a competitive inhibitor of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the last regulated step in the synthesis of cholesterol (1). The administration of a new agent with a novel mechanism of action, EZE, with a well-characterized and effective statin, SIM, in a single tablet now appears to provide enhanced treatment without compromising safety. EZE/SIM has also been associated with other beneficial effects on lipids, and it achieves greater efficacy than monotherapy with the use of lower, safer doses of the statin (2). Recently, a single-tablet combination of EZE and SIM has become available.

Literature survey reveals that several methods for the determination of EZE in pharmaceutical preparations or in biological fluids including liquid chromatography (LC) (3) and liquid chromatography–tandem mass spectrometry (LC–MS–MS) (4–6). Several methods have also been described for the determination of SIM, such as LC (7–9), LC–MS–MS (10–13), micellar electrokinetic capillary chromatography (MEKC) (14), voltammetry (15), spectrophotometry (16), and gas chromatography (GC) (17). To our knowledge, only one study has been published recently for the determination of these two drugs from the combined dosage form based on LC (18).

As commercial capillary electrophoresis (CE) instruments have been available more than 15 years; CE has become a mature and well-established analytical tool. CE is extensively used for routine analysis as an alternative and complementary technique to LC because it offers several advantages, including highly efficient and fast separations, relatively inexpensive and long lasting capillary columns, small sample size requirements, and low reagent consumption.

In this article, a novel, simple, and rapid MEKC method has been developed for the simultaneous determination of EZE and SIM in pharmaceutical preparations. An optimization study of method variables, the influence of buffer concentration, buffer pH, surfactant concentration, organic modifier, capillary temperature, applied voltage, and injection time was carried out, and the method validation studies were performed. The validated method was successfully applied to the pharmaceutical preparations. The data obtained by the developed method were compared with the data of the LC method in the literature (18). No significant differences were found statistically.

Experimental

Apparatus

All CE experiments were performed using an Agilent ^{3D}CE (Waldbronn, Germany) system using ChemStation software, equipped with a diode array UV detector, an automatic sample injector, Peltier temperature controller, and 30 kV high voltage

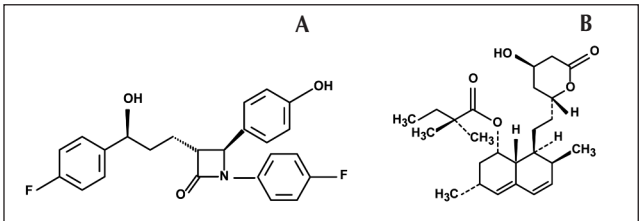

Figure 1. Chemical structures of Ezetimibe (A) and Simvastatin (B).

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power supply. Separation was carried out in a fused-silica capillary 56 cm × 50 µm i.d. (total length 64.5 cm) in normal mode, applying a voltage of 30 kV. Sample injections were made in a hydrodynamic mode over 3 s under a pressure of 50 mbar.

For pH measurements, a pH meter (Mettler Toledo MA 235, Schwerzenbach, Switzerland) was employed. Deionized water was prepared using a Barnstead Nanopure Diamond Analytical (Thermo Scientific, Waltham, MA) ultrapure water system. All solutions were degassed by ultrasonication (Sonorex, Bandelin, Germany).

Reagents

EZE and SIM reference standards were kindly supplied by Refik Saydam Hygiene Center (Ankara, Turkey) and Eczacıbası (Istanbul, Turkey), respectively. Pharmaceutical preparations containing EZE and SIM (Inegy containing 10 mg EZE and 20 mg SIM / tablet) were obtained from local pharmacies. All other chemicals were analytical-reagent grade.

Running buffer and samples were filtered through a 0.45-µm Titan syringe filters (Sri Inc., Eaton Town, NJ).

Standard and sample solutions

Standard solutions

Standard stock solutions (1000 µg/mL) of EZE, SIM, and diflunisal used as internal standard (IS) were prepared in acetonitrile. These solutions were stored at + 4°C.

Various aliquots of standard solutions were taken, the IS added, and then diluted to 1 mL with running buffer to give the desired final analyte concentration.

Running buffer

250 mM boric acid, 250 mM SDS, and acetonitrile were mixed in appropriate volumes and then adjusted with 0.1M NaOH to give the desired pH, surfactant concentration, and organic modifier percentage. The final optimal running buffer consisted of 25 mM borate buffer at pH 9.0 containing 25 mM SDS and 10% (v/v) acetonitrile.

Sample preparation

Ten tablets were weighed and finely powdered in a mortar. A quantity of the powder equivalent to one tablet was accurately weighed and transferred to a 25-mL volumetric flask. After adding 20 mL of acetonitrile, the flask was sonicated for 15 min and diluted to the mark with acetonitrile. Then an aliquot was centrifuged at 4000 rpm for 10 min, 1000 µL of clear supernatant was transferred to 5-mL flask and diluted to the mark with running buffer. 50 µL of this solution was transferred to a vial, 20 µL of 1000 µg/mL IS was added, and diluted with running buffer to 1 mL. This solution was analyzed by CE.

Synthetic tablet solutions were prepared by mixing inactive ingredients of tablet form (butylated hydroxyanisole, citric acid monohydrate, croscarmellose sodium, hydroxypropyl methylcellulose, lactose monohydrate, magnesium stearate, microcrystalline cellulose, and propyl gallate) with EZE and SIM standards in acetonitrile as mentioned in sample preparation.

All solutions were filtered through a 0.45-µm syringe filter and degassed with ultrasonic bath for 5 min before injection to the CE system.

Table I. Linearity parameters (n = 10)

	Ezetimibe	Simvastatin
Calibration range (µg/mL)	2.0–20.0	2.0–20.0
Slope*	0.0236 ± 0.0013	0.0302 ± 0.0014
Intercept*	0.0002 ± 0.0001	0.0056 ± 0.0002
Correlation coefficient (r)	0.9993	0.9988
Limit of detection (µg/mL)	1.0	1.0

* Mean ± standard error.

Electrophoretic procedure

Before the first use, the capillary was conditioned by flushing with 1.0 M NaOH for 30 min, then with water for 20 min. At the beginning of each working day, the capillary was rinsed with 0.1 M NaOH for 15 min, water for 10 min, and then the running buffer for 10 min. Before each injection, the capillary was preconditioned with 0.1 M NaOH (2 min), water (2 min), and running buffer (4 min) to maintain proper reproducibility of run-to-run injections. Injection was carried out under hydrodynamic pressure at 50 mbar for 3 s. A diode-array UV detector was set at 238 nm with a bandwidth of 10 nm. The capillary temperature was kept constant at 30°C, and a voltage of +30 kV was applied.

Results and Discussion

Optimization of electrolyte parameters

Capillary zone electrophoresis (CZE) is the simplest mode of CE and the most widely used. Therefore, before undertaking the MEKC experiments, preliminary studies using the CZE were attempted with buffer solutions in the pH range 3.0–9.0. None of these attempts were successful to separate EZE and SIM in this mode; then, MEKC was investigated in order to simultaneous determine the two analytes.

In MEKC, a detergent, such as SDS, is added to the running buffer at concentrations above its critical micelle concentration. Initial experiments using borate buffer containing SDS showed poor resolution between EZE and SIM. Even with the increase of

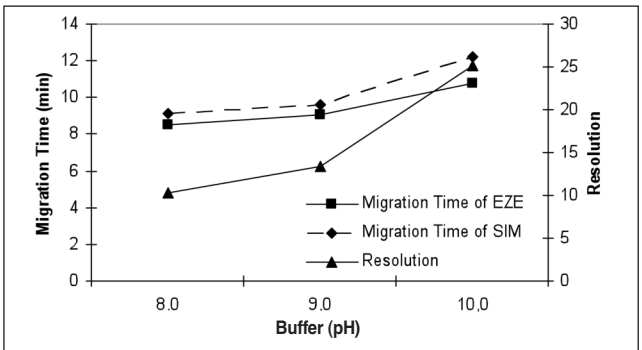

Figure 2. Effect of buffer pH on migration times and resolution. Operating conditions: 25 mM borate buffer containing 25 mM SDS and 10% (v/v) acetonitrile, hydrodynamic injection (3 s at 50 mbar), 30 kV, 30°C, 238 nm (bandwidth 10 nm). (EZE and SIM: 10 µg/mL).

the SDS concentration, change in pH, and concentration of running buffer, they coeluted. The introduction of organic modifier (acetonitrile) in the presence of SDS in the running buffer has played a key role on the separation of two analytes. Organic modifiers cause changes in the electroosmotic flow (EOF) because they change the zeta potential and the buffer viscosity. They cause changes in selectivity because they change the distributions of solutes between the buffer and the micelles (19). The separation between EZE and SIM increased with the increase in further levels of acetonitrile. 10% (v/v) acetonitrile was selected as a compromise between resolution and analysis time.

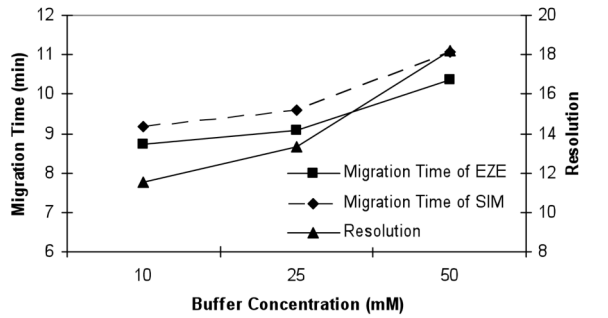

Figure 3. Effect of buffer concentration on migration times and resolution. Operating conditions: pH 9.0 borate buffer containing 25 mM SDS and 10% (v/v) acetonitrile, hydrodynamic injection (3 s at 50 mbar), 30 kV, 30°C, 238 nm (bandwidth 10 nm). (EZE and SIM: 10 µg/mL).

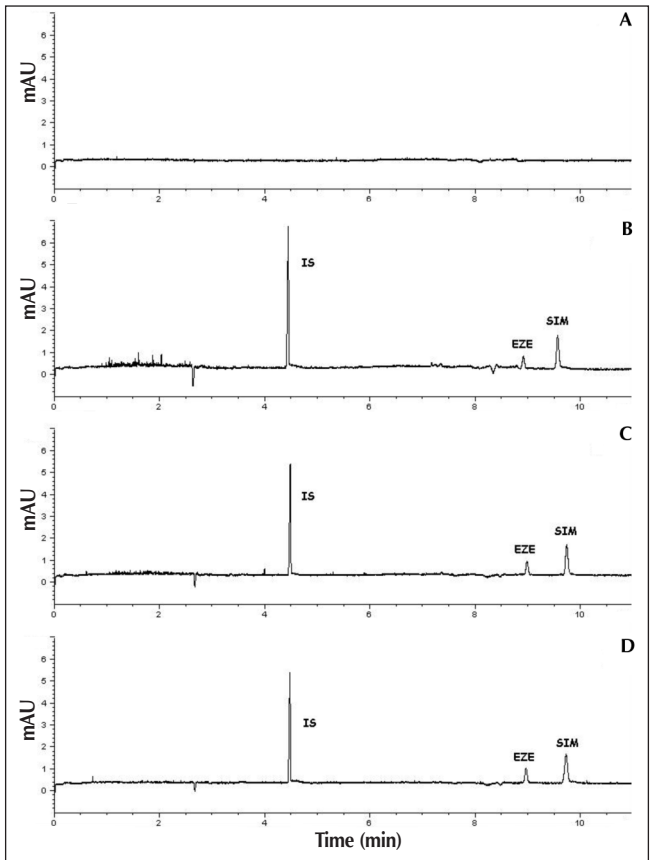

Figure 4. The electropherograms of Tablet excipients (placebo) (A), tablet excipients after being spiked with standards (B), commercial pharmaceutical preparation (C), standard solutions (D). Operating conditions: 25 mM borate buffer at pH 9.0 containing 25 mM SDS and 10% (v/v) acetonitrile, hydrodynamic injection (3 s at 50 mbar), 30 kV, 30°C, 238 nm (bandwidth 10 nm). (EZE: 4.0 µg/mL, SIM: 8.0 µg/mL, and IS: 20.0 µg/mL).

For concentrations lower than 20 mM of SDS, the peak shapes of the studied compounds deteriorated. On the other hand, the increase of SDS concentration remarkably increased the migration times, and the optimized value was chosen as 25mM.

The effect of buffer pH was investigated from pH 8.0 to 10.0 in the presence of 25 mM boric acid, 25 mM SDS, and 10% (v/v) acetonitrile. Considering both resolution and migration time, pH 9.0 was selected as optimum (Figure 2).

Buffer concentration also has a significant effect on the separation performance through its influence on the EOF, micelle, and the current produced in the capillary. The effect of borate concentration of running buffer was examined by varying the concentration from 10 to 50 mM. The results demonstrated that with an increasing borate concentration, both resolution and migration times increased. A 25 mM concentration of borate buffer was chosen in order to reduce the analysis time while maintaining good resolution (Figure 3).

The effect of applied voltage from 5 to 30 kV was investigated under the conditions described earlier. As expected, increasing the applied voltage increases the EOF, leading to shorter analysis time and higher efficiencies. However, higher voltages also exhibited higher currents and increased Joule heating. To limit this heating inside the capillary, the maximum applied voltage was chosen from an Ohm's plot (current versus voltage). This voltage was 30 kV (current ca. 22 µA).

Injection time affects the peak width and peak height. Sample solutions were hydrodynamically injected at 50 mbar while the injection time was varied from 0.5 and 5.0 s. After 3 s, the peak widths of EZE, SIM, and IS were increased, and the peak shapes were deformed, so 3 s was selected as the optimum injection time.

The viscosity of the running buffer is dependent on capillary temperature, so changes in temperature cause changes in EOF, electrophoretic mobilities, and injection volume. The influence of the temperature on analysis was investigated at 20, 25, and 30°C. The selected temperature was 30°C because it provided the best resolution.

Table II. Repeatability of System (n = 10)*

	t_m (min)	Peak Area	PN	Ratio of PN	Ratio of Peak Area
Ezetimibe					
x ± SE	9.06 ± 0.03	4.10 ± 0.07	0.45 ± 0.01	0.26 ± 0.001	0.51 ± 0.01
SD	0.09	0.21	0.03	0.002	0.02
RSD %	0.99	5.12	6.67	0.77	3.92
Simvastatin					
x ± SE	9.76 ± 0.02	6.07 ± 0.06	0.62 ± 0.01	0.35 ± 0.001	0.75 ± 0.01
SD	0.07	0.19	0.02	0.003	0.02
RSD %	0.72	3.13	3.23	0.86	2.67

* x ± SE, mean ± standard error; SD, standard deviation;
RSD %, relative standard deviation; tm, migration time;
PN (peak normalization), peak area / migration time;
Ratio of PN, PN of EZE or SIM / PN of IS;
Ratio of Peak Area, Peak Area of EZE or SIM / Peak Area of IS.

Method validation

The benefit of using an IS to correct errors, which are introduced by variable injection volume, voltage, or EOF, has been reported (20); thus, to improve precision, an IS was used. Diflunisal was chosen as the IS because it gave a good peak shape and resolution.

Validation of the proposed method was performed with respect to stability, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and robustness according to the ICH Guidelines (21).

Stability

Stability of the standard solutions of EZE and SIM were evaluated when stored at ambient temperature for 12 h (short-term stability) and when stored at 4°C for one month (long-term stability). The concentrations of freshly prepared solutions and those aged solutions were calculated by the method developed, and the difference between them was found less than 2.0%. These solutions can, therefore, be considered stable during this interval of time.

Specificity

Specificity, described as the ability of a method to discriminate the analyte from all potential interfering substances, was evaluated by preparing the analytical placebo, and it was confirmed that the signals measured were caused only by the analytes. A solution of an analytical placebo (containing all the ingredients of the formulation except the analyte) was prepared according to the sample preparation procedure and injected. To identify the interference by these excipients, a mixture of the inactive ingredients (placebo), before (Figure 4A) and after being spiked with standards (Figure 4B), standard solutions (Figure 4C), and the commercial pharmaceutical preparations including EZE and SIM (Figure 4D), were analyzed by the proposed method. The representative electropherograms show no other peaks, which confirms the specificity of the method.

Linearity

Under the optimum analysis conditions, linearity was studied simultaneously in the concentration range of 2.0–20.0 µg/mL for EZE and SIM. In all cases, 20.0 µg/L of diflunisal was added as IS. The peak normalization ratios of EZE and SIM to the IS were plotted versus the nominal concentrations of the calibration standards. Linearity parameters were summarized in Table I.

LOD and LOQ

The limits of detection defined as signal-to-noise ratio of 3:1 were 1.0 µg/mL for EZE and SIM. The limits of quantification were determined as 2.0 µg/mL for EZE and SIM with acceptable precision (RSD = 6.83% for EZE and 8.30% for SIM, $n = 7$) and accuracy (bias % ≤ 15 $n = 7$) under the stated conditions.

Precision

The assay was investigated with respect to repeatability and intermediate precision. In order to measure repeatability of the system (while keeping the operating conditions identical), 10 consecutive injections were made with a standard solution containing 10 µg/mL of EZE, 10 µg/mL of SIM, and 20 µg/mL of IS. The results were evaluated by considering migration time, peak area, peak normalization (peak area/migration time), ratio of peak normalization, and ratio of peak area values of EZE and SIM. The precision values with their RSD are shown in Table II. The results in Table II indicate that the RSD may be reduced to ~ 1% using peak ratio normalization; therefore, this parameter was employed for the quantitative procedures during the study.

Three different concentrations of EZE and SIM (in the linear range) were analyzed in six independent series on the same day (intra-day precision) and six consecutive days (inter-day precision); within each series, every sample was injected three times. The RSD values of intra- and inter-day studies varied from 3.26 to 0.50%, showing that the intermediate precision of the method was satisfactory (Table III).

Table III. Accuracy and Precision Data for EZE and SIM

	Intra-day				Inter-day		
	Added (µg/mL)	Found* (µg/mL)	Precision (RSD %)	Accuracy† (Bias %)	Found (µg/mL)	Precision (RSD %)	Accuracy (Bias %)
Ezetimibe	4.0	3.97 ± 0.03	2.02	–0.75	4.05 ± 0.02	1.48	1.25
	10.0	9.96 ± 0.03	0.80	–0.40	10.04 ± 0.05	1.20	0.40
	18.0	17.82 ± 0.07	0.90	–1.00	17.97 ± 0.04	0.50	–0.17
Simvastatin	4.0	3.97 ± 0.03	2.02	–0.75	3.99 ± 0.05	3.26	–0.25
	10.0	9.95 ± 0.03	0.80	–0.50	9.98 ± 0.05	1.20	–0.20
	18.0	17.89 ± 0.04	0.56	–0.61	17.91 ± 0.07	0.95	–0.5

* Mean ± Standard Error. †Bias % : [(Found – Added) / Added] × 100.

Table IV. Data From the Analysis of Commercial Tablets by MEKC and Reference LC Method (n = 6)*

Ingredient	Labeled Claim (mg)	Found (mg) MEKC method $\bar{x}$ ± SE	RSD %	Bias %	Reference Method $\bar{x}$ ± SE	RSD %	Bias %	$t_{calc.}$	$F_{calc.}$
Ezetimibe	10.0	9.92 ± 0.09	2.12	–0.80	10.03 ± 0.05	1.20	0.30	0.42	3.06
Simvastatin	20.0	19.86 ± 0.13	1.56	–0.70	19.92 ± 0.06	0.75	–0.40	0.14	4.27

* No difference (Student's t-test and F-test, p > 0.05).

Accuracy and recovery studies

The accuracy of the method was determined by calculating the percent difference (bias %) between the measured mean concentrations and the corresponding nominal concentrations. Table III shows the results obtained for intra- and inter-day accuracy.

The accuracy of the proposed method was also tested by recovery experiments. Recovery experiments were performed by adding known amounts of EZE, SIM, and IS to the analytical

placebo solution. EZE and SIM were spiked according to the label claim in the pharmaceutical preparations. These synthetic samples were treated as described in the procedure for sample preparation. The obtained recoveries were 102.96 ± 1.06 for EZE (RSD % = 2.53) and 101.90 ± 0.58 for SIM (RSD % = 1.39) (n = 6).

Robustness

Robustness relates to the capacity of the method to remain unaffected by small but deliberate variations introduced into the method parameters (21). In order to evaluate the robustness of the developed method, a fractional factorial design was built by setting small changes in the studied parameters (22). The six factors, buffer pH (± 0.2), buffer concentration (± 2 mM), SDS concentration (± 2mM), acetonitrile percentage (± 1), capillary temperature (± 1°C), and detection wavelength (± 2 nm) were studied at three levels. An 11-run fractional factorial design, three experiments with the optimized conditions, was chosen to evaluate if a change in factor value produced a statistically significant variation of the observed response. The evaluation consisted in analysing a standard solution containing 10 µg/mL EZE, 10 µg/mL SIM, and 20 µg/mL IS. In each case, the quantitative determinations of EZE and SIM were evaluated from the calibration lines. An ANOVA test was applied to the experimental data, and it was found that the model assumed was not significant, indicating that no factor influenced the response. Consequently, the method could be considered robust for this response.

Analysis of pharmaceutical preparations

The developed and validated method was applied to the simultaneous determination of EZE and SIM in pharmaceutical preparations. Each pharmaceutical preparation was analyzed by performing six independent determinations. Satisfactory results were obtained for each compound and were found to be in agreement with label claims (Table IV). An LC method mentioned in the literature (18) was used as a comparison method to evaluate the validity of the method developed. According to this method, determination of EZE and SIM were carried out by the application of a dual-mode gradient system using a C_8 column and a diode-array detector set at 240 nm. Dual-mode gradient system includes programming of both mobile phase composition and flow rate. A comparison of the results obtained by both methods was carried out using the Student's t-test and F-test. It was indicated that there were no significant differences between them ($p > 0.05$).

Conclusion

A simple, fast, and reliable MEKC method was developed and validated for the simultaneous determination of EZE and SIM. The method shows a good performance with respect to stability, specificity, linearity, accuracy, precision, and robustness, and it offered a simple, fast, inexpensive, and precise way for the determination of EZE and SIM in pharmaceutical preparations. LC consumes a relatively large amount of organic solvent, which is expensive and harmful to the environment. The advantages of the proposed MEKC method over the LC method are its ease of use and lower running costs in addition to having a lower environmental impact. It can be concluded that MEKC is an alternative to existing LC method for the determination of EZE and SIM in pharmaceutical preparations

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