



Capillary Electrophoresis Method for Simultaneous Quantification of Hypcholesterolemic Drugs in Binary Mixture Formulation: Fast, Green, and Cost-Effective Alternative to HPLC

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Abstract

A fast and sustainable environment-friendly micellar electrokinetic chromatography coupled with a diode array detector was investigated and validated in accordance with ICH requirements for the simultaneous quantification of ezetimibe and simvastatin in a tablet binary mixture formulation. Drugs' separation from their internal standard and degradation products was achieved using the optimized electrophoretic conditions: 50 μm i.d. \times 30 cm fused-silica capillary, background electrolyte composed of 20 mmol L^{-1} sodium tetraborate buffer solution (pH 9, adjusted with *o*-phosphoric acid), 30 mmol L^{-1} sodium dodecyl sulfate, 12% acetonitrile; with detection at 238 nm and voltage of +30 kV. Drugs were separated in less than 6 min. The method was linear with determination coefficients 0.9956 and 0.9920 for ezetimibe and simvastatin, respectively; and precise (intra-day 1.05% and 0.38%; inter-day 1.20% and 0.69%) for ezetimibe and simvastatin, respectively. Accuracy was tested through recovery and mean values ($101.0 \pm 0.77\%$ for ezetimibe and $100.4 \pm 0.35\%$ for simvastatin) were obtained. The proposed method is the first that determined ezetimibe and simvastatin in presence of their degradation products. This method can be successfully used in quality control laboratories as an alternative to HPLC technique for the simultaneous quantification of ezetimibe and simvastatin in their combined drug products and could also be used as a stability-indicating method. The proposed method greenness profile was confirmed using Green Analytical Procedure Index and Analytical GREENness tools with a final score of 11 and 0.81, respectively.

Keywords Hypercholesterolemia · Micellar electrokinetic chromatography · Ezetimibe · Simvastatin · Binary mixture formulation · Greenness evaluation

Introduction

Cardiovascular disease appears to be the principal cause of death in the world involving hypercholesterolemia as a major risk factor [1]. It should be preferred to prescribe efficacious doses of statins to minimize low-density lipoprotein

as per international treatment guidelines on cardiovascular disease prevention [2]. Contrarily, 2nd or 3rd lipid-lowering drugs, i.e., fibrates, bile acid sequestrants, and ezetimibe are preferred over statin monotherapy for the management of high cardiovascular risk patients with hypercholesterolemia [3]. Since 2004, Food and Drug Administration approved ezetimibe/simvastatin combined tablet dosage form with ezetimibe fixed dose (10 mg) and simvastatin variable dose (10/20/40 mg) for both primary and secondary forms of hypercholesterolemia therapeutic management [4]. Last decade, the combination therapy of these drugs has been prescribed to maintain low-density lipoprotein in patients resistant to statin monotherapy or statin dose related side effects that showed successful clinical outcome [5].

Chemically, ezetimibe (Fig. 1A) named as (3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*R*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidino-2-one [6]

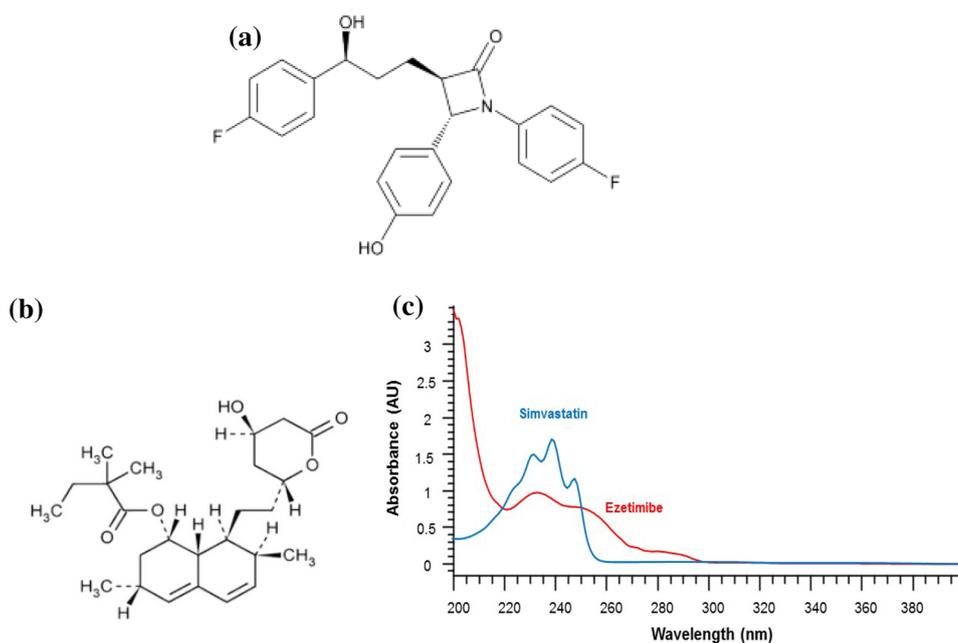
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Fig. 1 Chemical structure of ezetimibe **A**, simvastatin **B**, and spectra overlap **C**



which inhibits cholesterol absorption and decreases excessive accumulation in blood vessels [7]. This distinct mechanism of action results in a synergistic cholesterol lowering effect when used together with statins that inhibits cholesterol synthesis in the liver [5].

Simvastatin (Fig. 1B) named as [(1*S*,3*R*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl] 2,2-dimethylbutanoate [6] which inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase for the treatment of hyperlipidemia.

The literature review reveals that ezetimibe and simvastatin were officially listed in the USP [6]. Furthermore, several analytical approaches have been recommended for the simultaneous determination of both drugs in combined pharmaceutical formulations and/or biological fluids by TLC [8], HPLC [8–12], UPLC [13], UPLC–MS/MS [14], UPLC–Q–TRAP/MS [1], LC–MS [15], LC–ESI–MS/MS [16], and HPLC–MS/MS [17]. Some of these methods require extensive instrumentation and time-consuming sample pretreatment methodologies, hence not ideal for routine use. The literature also reveals that the pharmaceutical drugs under investigation were simultaneously analyzed by UV spectrophotometric [18, 19] and capillary electrophoretic [20] methods.

The capillary electrophoresis is rapidly gaining importance as an alternative green analytical separation technique and used in several pharmaceutical and biopharmaceutical analysis [21]. It has various advantages over conventional chromatographic methods such as short analysis time, low reagent consumption, without organic solvents, small sample volume, long-lasting capillary columns, high separation

efficiency (millions of theoretical plates), and faster sample development [22], consequently diminishing the impact on environment [23].

At the present, only one capillary electrophoresis method has been described for simultaneous determination of ezetimibe and simvastatin in tablets, and the analytes were determined in a long time, i.e., approximately 10 min [20], this might be due to longer capillary column. Therefore, in this work, we proposed a validated micellar electrokinetic chromatography method, mode of capillary electrophoresis, for the simple and fast simultaneous determination of ezetimibe and simvastatin in binary mixtures under optimized analytical conditions. Moreover, the stability-indicating potential of the proposed method was first time investigated under stress degradation conditions (thermal, oxidative, photolytic, alkaline and acidic hydrolysis) for both ezetimibe and simvastatin, while highlighting the use of green background electrolyte. The proposed method greenness was also determined by using Green Analytical Procedure Index and Analytical GREENness approaches.

Materials and Methods

Chemicals, Reagents and Samples

The active pharmaceutical ingredients ezetimibe and simvastatin with purities of 100.0% and 99.8%, respectively, were donated by Merck Sharp & Dohme (Sao Paulo, Brazil), internal standard losartan potassium was obtained from Sigma-Aldrich (Sao Paulo, Brazil), and Vyturin® (Merck Sharp & Dohme, Sao Paulo, Brazil) tablet (ezetimibe 10 mg

and simvastatin 20 mg) were procured from community pharmacy. Analytical grade methanol, hydrogen peroxide, hydrochloric acid, acetonitrile was brought from Merck (Sao Paulo, Brazil); while *o*-phosphoric acid, sodium dodecyl sulfate, sodium tetraborate-10-hydrate, dibasic sodium phosphate, and sodium hydroxide were procured from Sigma-Aldrich (Sao Paulo, Brazil). Deionized water was obtained from Milli-Q[®] (Millipore Corp., Bedford, MA, USA) ultrapure purification system.

Instrument

P/ACE[™] MDQ (Beckman Coulter, Inc., Fullerton, CA, USA) capillary electrophoresis system equipped with diode array detector and 32 Karat[™] software (ver. 8.0) was employed for data acquisition.

Condition of Electrophoretic Separation

50 μm internal diameter \times 42 cm total (30 cm effective) length uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used by adjusting temperature (30 °C) and voltage (+30 kV) with detection at 238 nm to perform the electrophoretic separation. The samples were injected by applying pressure of 0.3 psi for 3 s (1 psi = 6894.76 Pa) at cathodic side.

Background Electrolyte Preparation

Aqueous stock solutions (100 mmol L⁻¹) of sodium tetraborate buffer (pH 9, adjusted with *o*-phosphoric acid) and sodium dodecyl sulfate were separately prepared. The pH was measured using Gehaka[®] Mettler Toledo pH meter (Sao Paulo, Brazil). The optimized background electrolyte (30 mmol L⁻¹ sodium dodecyl sulfate, 20 mmol L⁻¹ sodium tetraborate buffer solution pH 9 and 12% acetonitrile) was prepared by transferring sodium tetraborate (5 mL) and sodium dodecyl sulfate (7.5 mL) stock solutions into a 25 mL volumetric flask, deionized water (8 mL), pH 9 adjusted with *o*-phosphoric acid solution, then acetonitrile (3 mL) was added, and deionized water was used to complete the volume. This mixture was sonicated for 10 min and filtered through a 0.45 μm polyvinylidene fluoride filter before injected on the capillary electrophoresis instrument.

Capillary Conditioning

Before using, new capillary was conditioned with NaOH 1 mol L⁻¹ for 30 min, followed by deionized water for 20 min and finally with background electrolyte for 30 min. At the beginning of each working day, the capillary was rinsed with NaOH 1 mol L⁻¹ for 15 min, deionized water for 10 min and then 20 min with background electrolyte.

Between runs, the capillary was washed with NaOH 1 mol L⁻¹ (1 min) and with the running buffer (1.5 min) to ensure reproducibility of the assay. Finally, at the end of the day, the capillary was flushed with NaOH 1 mol L⁻¹ for 10 min and water for 10 min.

Preparation of Stock Standard and Test-Mixture Solutions

Internal standard, ezetimibe and simvastatin stock standard solutions (1000 $\mu\text{g mL}^{-1}$) were separately prepared in diluent methanol–water (25:75, v/v) by weighing 10 mg of each one into three 10 mL amber volumetric flasks. The solutions were sonicated for 3 min to aid dissolution then filtered through 0.45 μm polyvinylidene fluoride filters and finally stored at 4 °C. The solutions were found stable for up to 15 days.

For the test-mixture, aliquots from each stock standard solution were transferred into a 2 mL amber volumetric flask and diluted to volume with diluent. The final concentrations were 100 $\mu\text{g mL}^{-1}$ for ezetimibe and internal standard, while 200 $\mu\text{g mL}^{-1}$ for simvastatin. The test-mixture was sonicated for 3 min, filtered in a 0.45 μm polyvinylidene fluoride filter and injected on the capillary electrophoresis instrument.

Pharmaceutical and Placebo Stock and Working Preparation

Twenty tablets of Vitoryn[®] containing 10/20 mg of ezetimibe/simvastatin were weighed and finely powdered. An amount of powder corresponding to 10 mg of ezetimibe and 20 mg of simvastatin was transferred into 10 mL amber volumetric flask and added 5 mL of methanol, the mixture was sonicated for 15 min to aid dissolution with occasional shaking, and diluted to volume with the same solvent. The solution was filtered through a 0.45 μm polyvinylidene fluoride filter. The obtained concentration was 1000 $\mu\text{g mL}^{-1}$ for ezetimibe and 2000 $\mu\text{g mL}^{-1}$ for simvastatin.

Placebo matrix was prepared according to the following formula: microcrystalline cellulose (46.8%), lactose monohydrate (45%), magnesium stearate (2%), propyl galate (0.1%), citric acid (1%), sodium croscarmellose (3%), hydroxypropyl methylcellulose (2%) and butylhydroxyanisole (0.1%).

An aliquot of 200 μL from sample stock solution was volumetrically transferred into a 2 mL amber volumetric flask, and 200 μL of internal standard stock solution was added and the volume was completed with diluent to prepare the working sample solution. The final concentrations

were $100 \mu\text{g mL}^{-1}$ for ezetimibe and internal standard, while $200 \mu\text{g mL}^{-1}$ for simvastatin. The solution was filtered in a $0.45 \mu\text{m}$ polyvinylidene fluoride filter before being injected.

Method Validation

System Suitability

The system suitability test was carried out to ensure the system resolution and reproducibility. The test was performed by injecting six replicates of working mix standard solutions of simvastatin ($200 \mu\text{g mL}^{-1}$), ezetimibe and internal standard ($100 \mu\text{g mL}^{-1}$). The assessed parameters were theoretical plates (N), peak area, migration time, resolution (Rs) and tailing factor.

Specificity/Selectivity

The specificity of proposed method for the determination of ezetimibe and simvastatin was assessed by injecting the Vitoryn[®] sample, placebo, standard solutions and diluent into the capillary electrophoresis system.

Selectivity and forced degradation stability indicating studies for ezetimibe and simvastatin were applied under hydrolytic (alkaline and acidic), photolytic, thermolytic and oxidative stress conditions according to ICH Q1A(R2) requirements [24]. Ezetimibe and simvastatin standards solutions, Vitoryn[®] sample solution and placebo were submitted for each forced degradation condition.

The peak purity for both drugs in the obtained electropherograms was evaluated with diode array detector. The interference from tablet excipients was performed by subjecting the placebo to the same stress conditions as Vitoryn[®] samples.

Alkaline and Acidic Hydrolysis

The concentration of 1 mg mL^{-1} for ezetimibe and 2 mg mL^{-1} for simvastatin of Vitoryn[®] sample was carried out for alkaline (0.1 N NaOH) and acidic (0.1 N HCl) hydrolysis. The solutions were placed at room temperature and under light protection for 7 days.

Thereafter, $200 \mu\text{L}$ of each solution was transferred volumetrically to 2 mL amber volumetric flask, internal standard stock solution ($200 \mu\text{L}$) and diluent (1.6 mL) were added, obtaining final concentrations of $100 \mu\text{g mL}^{-1}$ of ezetimibe and internal standard, while $200 \mu\text{g mL}^{-1}$ of simvastatin. The solutions were sonicated for 5 min then, filtered in a $0.45 \mu\text{m}$ polyvinylidene fluoride filter before being used. The same procedure was made with placebo tablets.

Thermal and Photolytic Degradation

500 mg of powder from Vitoryn[®] sample was transferred separately to two Petri plates, one of these was placed in a hot oven at $60 \text{ }^\circ\text{C}$ (thermal degradation) and the other one, was exposed to direct sunlight (photolytic degradation). After 7 days, an appropriate amount of each powder equivalent to 10 mg of ezetimibe and 20 mg of simvastatin was transferred into two 10 mL amber volumetric flasks. The contents were diluted with 5 mL of methanol and sonicated for 15 min. The volume was completed with the same diluent. The mixtures were submitted at the same condition as in *section alkaline and acidic hydrolysis (second paragraph)*.

Oxidative Degradation

Appropriate amount of Vitoryn[®] sample powder equivalent to 10 mg of ezetimibe and 20 mg of simvastatin were transferred into a 10 mL amber volumetric flask. The content was diluted with 5 mL of methanol, and 2.5 mL of 3% hydrogen peroxide. The mixture was sonicated for 15 min and submitted at the same condition as in *section alkaline and acidic hydrolysis (second paragraph)*.

Linearity and Range

Linearity was verified by preparing five concentrations by diluting adequate amounts of internal standard, ezetimibe, and simvastatin stock standard solutions in diluent. The concentration ranges from 80 to $120 \mu\text{g mL}^{-1}$ for ezetimibe, 160 to $240 \mu\text{g mL}^{-1}$ for simvastatin, and fixed concentration of internal standard ($100 \mu\text{g mL}^{-1}$) were obtained. Three analytical curves for ezetimibe and simvastatin were constructed by plotting the peak area ratio (ezetimibe or simvastatin peak area/internal standard peak area) against ezetimibe or simvastatin concentrations, respectively. Electropherograms were recorded for the solutions injected in triplicate. The method of least squares regression and analysis of variance were used to determine method linearity ($p = 0.05$). The data homoscedasticity and linearity were analyzed through an *F* test.

Detection and Quantification Limits

The limit of detection and quantification for ezetimibe and simvastatin were measured using the following equations according to ICH guidelines:

$$\text{Limit of detection} = 3.3(\sigma / \alpha), \quad (1)$$

$$\text{Limit of quantification} = 10(\sigma / \alpha), \quad (2)$$

where σ is the standard deviation of the intercept of the mean of three analytical curves, determined by a linear regression model and α is the slope of the corresponding analytical curve [25].

Accuracy

Accuracy was determined in terms of percent recovery. Placebo matrix was spiked with ezetimibe and simvastatin standard solutions at three different concentration levels 80, 100 and 120 $\mu\text{g mL}^{-1}$ for ezetimibe and 160, 200 and 240 $\mu\text{g mL}^{-1}$ for simvastatin, equivalent to 80, 100 and 120% of the analytical concentrations, respectively. Each determination was performed in triplicate to obtain the mean and relative standard deviation percentage.

Precision

Method precision was evaluated by repeatability (intra-day) and intermediate (inter-day) analysis. Six determinations from sample solution, on the same day by a single analyst, were performed to study the repeatability. Intermediate precision was evaluated in the same way as repeatability but by different analyst in a different day. Sample Vytorin[®] (ezetimibe 10 mg and simvastatin 20 mg) solution with constant concentration of internal standard (100 $\mu\text{g mL}^{-1}$) were used. The results were expressed in terms of relative standard deviation percentage for ezetimibe and simvastatin content in the tablets.

Robustness

The robustness was performed by evaluating slight deliberate changes of the optimal analytical conditions such as hydrodynamic injection time (0.1 s and 0.5 s, optimal 0.3 s) and % of organic modifier (10% and 14% acetonitrile, optimal 12%). The responses were evaluated in terms of system suitability i.e., tailing factor, resolution, and efficiency (theoretical plates).

Results and Discussion

For quality control laboratories, it is very important to develop separation methods for simultaneous quantitation of drugs in combined dosage forms that do not demand pretreatment. Capillary electrophoresis is an alternative technique for this purpose. It offers wide applications [22] and several benefits such as fast analysis, good resolution, low sample consumption, high efficiency and get over several disadvantages of HPLC technique [26]. It is also considered an eco-friendly analytical technique since it uses very small volumes of organic solvents or sometimes does not use them,

preventing or reducing the generation of hazardous waste. In addition, it uses long lasting and versatile capillary columns, the same capillary can be used to analyze from small ions to macromolecules. This feature also makes it an economical technique [23].

Method Development and Optimization

Different trials were carried out to optimize the simultaneous separation of ezetimibe and simvastatin within a short analytical time with good resolution. The electrolyte pH adjustment is very important to keep the electroosmotic flow and the migration velocities of weak electrolyte components constant. Thus, a stable and reproducible migration behavior of the analytes can be achieved. The effective mobility of cationic and anionic analytes is strongly dependent on their pK_a value related to background electrolyte pH. However, it is worth mentioning that even for substances without effective mobility, it can move through the capillary due to electroosmotic flow, which in turn, is also dependent on background electrolyte pH [27]. In the separation of neutral substances, the pH adjustment has little influence on selectivity; however, a pH value must be selected for the system in which the surfactant micelles are ionized in such a way to allow their migration at a different rate than electroosmotic flow [28].

As ezetimibe and simvastatin drugs are water insoluble, this problem can be overcome by using micelles as additives [29]. Solutes interact with a migrating micellar phase and this mechanism allows separation of neutral and charged species [22]. Additives act as a pseudo-stationary phase for the separation of closely compounds and other unresolved compounds in capillary zone electrophoresis [30].

Then micellar electrokinetic chromatography method was performed by addition of sodium dodecyl sulfate surfactant above its critical micellar concentration. This provides a chromatographic partition between pseudo-stationary phase (micelles), formed when the surfactants are above their critical micellar concentration, and the aqueous background electrolyte. To obtain a good separation, effects of pH, buffer concentration, sodium dodecyl sulfate concentration, organic modifier, applied voltage, injection time and detection wavelength were studied.

Effect of pH Value

The effect of pH was performed from 7 to 9 since pK_a values of ezetimibe and simvastatin were between 9.75 and 14.91 [31, 32]. For optimum resolution between analytes, the background electrolyte pH should be in the range of the corresponding pK values of the analytes [22]. The initial sodium tetraborate buffer concentration was set at 20 mmol L^{-1} as background electrolyte, containing 30 mmol L^{-1} sodium dodecyl sulfate, the buffer pHs were modified by

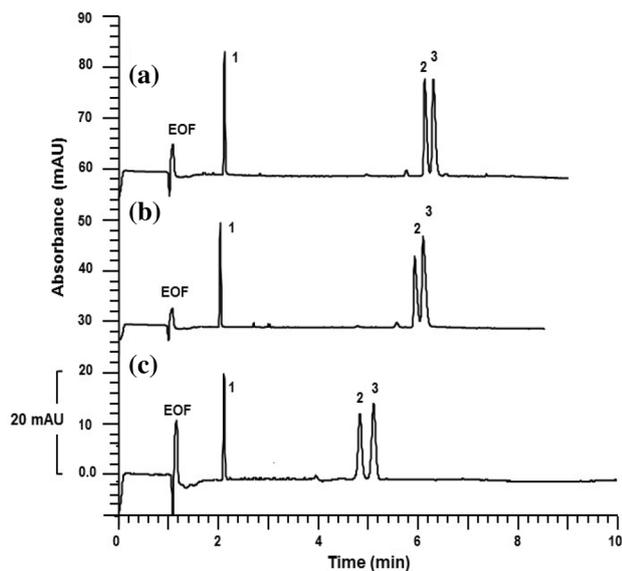


Fig. 2 Electropherograms of the effect of pH. **A** pH 7, **B** pH 8, and **C** pH 9 of sodium tetraborate buffer background electrolyte on the separation of standard mixture of ezetimibe, simvastatin and internal standard ($100 \mu\text{g mL}^{-1}$). Electrophoretic conditions: uncoated fused-silica capillary with $50 \mu\text{m i.d.} \times 42 \text{ cm}$ total (30 cm effective) length; electrolyte: 20 mmol L^{-1} sodium tetraborate buffer solution, and 30 mmol L^{-1} sodium dodecyl sulfate; hydrodynamic injection: $0.3 \text{ psi}/3 \text{ s}$; applied voltage: $+30 \text{ kV}$; temperature: $30 \text{ }^\circ\text{C}$; UV detection at 238 nm . Peaks: (1) losartan potassium (internal standard), (2) ezetimibe, (3) simvastatin, and (EOF) electroosmotic flow

using *o*-phosphoric acid and NaOH solutions. As sodium tetraborate buffer pH increased, the electroosmotic flow was also increased which resulted in shorter migration time (pH 9), but the analytes could not be well separated (Fig. 2C). To obtain complete separation, organic modifier was added to the background electrolyte (Fig. 3B). Here, pH was set at 9 under constant conditions, temperature $30 \text{ }^\circ\text{C}$, injection time $0.3 \text{ psi}/3 \text{ s}$ and voltage $+30 \text{ kV}$.

Effect of Organic Modifier

In micellar electrokinetic chromatography, the presence of water-miscible organic solvents such as methanol, 2-propanol and acetonitrile usually improve resolution and/or variation in the selectivity. They reduce capacity factor of highly hydrophobic solutes to within or near the ideal range because of altering the retention mechanism by changing the polarity of the aqueous phase, electrolyte viscosity and zeta potential. The organic solvents contribute to reduce the electroosmotic velocity and expand the migration time window and increase resolution [33]. However, these solvents in high concentrations cannot be used as they can breakdown micellar structure. Generally, the maximum content of the organic solvent that can be used is 20% or so [30]. The influence of acetonitrile and methanol in background electrolyte

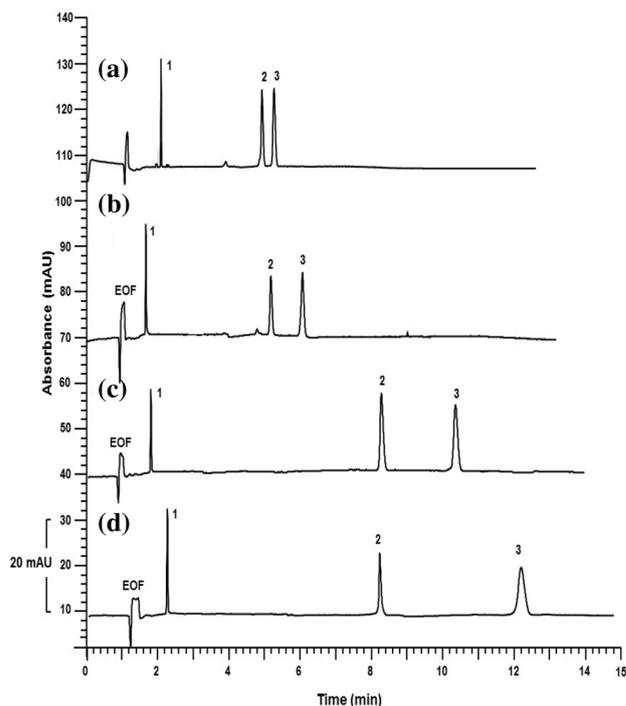


Fig. 3 Electropherograms of the effect of sodium dodecyl sulfate concentration. **A** 20 mmol L^{-1} , **B** 30 mmol L^{-1} , **C** 40 mmol L^{-1} , and **D** 50 mmol L^{-1} on the separation of standard mixture of simvastatin ($200 \mu\text{g mL}^{-1}$), ezetimibe and internal standard ($100 \mu\text{g mL}^{-1}$). Electrophoretic conditions: uncoated fused-silica capillary with $50 \mu\text{m i.d.} \times 42 \text{ cm}$ total (30 cm effective) length; electrolyte: 20 mmol L^{-1} sodium tetraborate buffer solution (pH 9), $x \text{ mmol L}^{-1}$ sodium dodecyl sulfate and 12% acetonitrile; hydrodynamic injection: $0.3 \text{ psi}/3 \text{ s}$; applied voltage: $+30 \text{ kV}$; temperature: $30 \text{ }^\circ\text{C}$; UV detection at 238 nm . Peaks: (1) losartan potassium (internal standard), (2) ezetimibe, (3) simvastatin, and (EOF) electroosmotic flow

was performed in concentration range 5 to 15% (v/v). The results showed a decrease of electroosmotic flow mobility giving longer analysis time when used 15% of solvent, especially when methanol was used, since methanol is more viscous than acetonitrile. Therefore, acetonitrile was chosen for further analysis. Ezetimibe and simvastatin were nearly co-eluted in a low amount of acetonitrile $\leq 8\%$. The separation between the analytes under study was getting increased with the increase in the further levels of acetonitrile. Hence, 12% (v/v) acetonitrile was chosen as the optimum concentration for the complete separation of ezetimibe and simvastatin (Fig. 3B), under constant conditions of pH 9, temperature $30 \text{ }^\circ\text{C}$, injection time $0.3 \text{ psi}/3 \text{ s}$ and voltage $+30 \text{ kV}$.

Effect of Surfactant Concentration

Sodium dodecyl sulfate is the most used surfactant in micellar electrokinetic chromatography. Surfactants are added to background electrolyte in concentrations above their critical micellar concentration to form micelles. Analytes distribute

according to their hydrophobicity in and outside of the micelles and they are separated by their different affinity to the micelles [30].

In the present work, the effect of surfactant concentration on the migration times of ezetimibe and simvastatin was studied. Sodium dodecyl sulfate concentration was evaluated in the range of 20 to 50 mmol L⁻¹. The obtained results shown that sodium dodecyl sulfate concentration had high effect in the mobility of the two analytes. The higher sodium dodecyl sulfate concentration the higher migration time. Hence, 30 mmol L⁻¹ sodium dodecyl sulfate was chosen for this study because it gave sharp peaks, short analysis time (approximately 5.8 min) (Fig. 3) compared with Yardimci and Özaltın work [20], they determined the analytes in approximately 10 min.

Effect of Borate Buffer Concentration

The influence of borate buffer concentration on the separation of ezetimibe and simvastatin was analyzed with concentration 20, 30 and 40 mmol L⁻¹ at pH 9. It was observed that migration time increased with increasing borate concentration. The higher buffer concentration the higher background electrolyte viscosity and the lower electroosmotic flow mobility. Furthermore, the current increased ($\approx 82 \mu\text{A}$) with increasing borate concentration, above 30 mmol L⁻¹. No change of the retention order was observed. Therefore, 20 mmol L⁻¹ borate buffer was chosen for its good symmetric peaks and analysis time (Fig. 4C).

Effect of Applied Voltage, Injection Time and Capillary Temperature

Regarding the applied voltage, values from 20 to 30 kV were tested using the optimized background electrolyte. The results showed that by increasing voltage, migration times decreased, it is owing to increase in electroosmotic flow, since the magnitude of electroosmotic flow is directly proportional to the strength of the electric field [33]. Hence, a voltage of 30 kV was chosen which producing best peak shape with short migration time of the analytes.

Another instrumental parameter evaluated was the injection time. The volume injected depends essentially on the injection time and it affects area, width, and height of the peaks. In this way, tests were carried out with times of 1, 3 and 5 s with hydrodynamic injection at 0.3 psi. It was observed that above 3 s there was a widening at baseline peaks, causing their distortion and deformation. Good peak shapes for ezetimibe and simvastatin (Fig. 4C) were observed with 3 s injection time. Therefore, this injection time was used for further experiments.

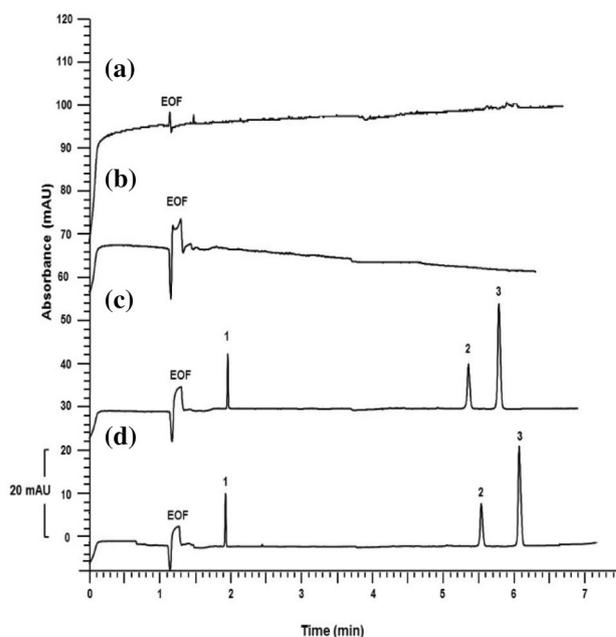


Fig. 4 Electropherograms at the finally optimized micellar electrokinetic chromatography method. **A** background electrolyte, **B** Vitoryn[®] placebo sample, **C** standard mixture of 100 $\mu\text{g mL}^{-1}$ ezetimibe, 200 $\mu\text{g mL}^{-1}$ simvastatin and 100 $\mu\text{g mL}^{-1}$ internal standard, **D** Vitoryn[®] sample (100 $\mu\text{g mL}^{-1}$ ezetimibe and 200 $\mu\text{g mL}^{-1}$ simvastatin) and 100 $\mu\text{g mL}^{-1}$ internal standard. Electrophoretic conditions are the same as in Fig. 3 with 30 mmol L⁻¹ sodium dodecyl sulfate. Peaks: (1) losartan potassium (internal standard), (2) ezetimibe, (3) simvastatin, and (EOF) electroosmotic flow

Capillary temperature was also investigated in the range from 25 to 40 °C. Best results were observed at 30 °C in terms of current generated in the capillary, resolution and run time. Increasing the temperature above 30 °C resulted in shorter run time but poor resolution and high current. Therefore, temperature of 30 °C was selected for further analysis.

Selection of Detection Wavelength

Using diode array detector, absorption spectra of ezetimibe and simvastatin showed maximum absorbance at 238 nm. Figure 1C shows an overlap of ezetimibe and simvastatin spectra. In addition, samples of the tablet matrix do not showed interference at this wavelength (Fig. 4D). Thus, we thereafter used a 238 nm detection wavelength for all experiments.

Internal Standard

Internal standard improves the quantitative performance of capillary electrophoresis methods in terms of linearity, precision, and recovery data. It eliminates error introduced by variability in voltage, injection volume or electroosmotic

flow [34]. Some substances such as clozapine, promethazine and losartan potassium were tested as internal standard candidates. Losartan potassium ($100 \mu\text{g mL}^{-1}$) was the most suitable due to good absorbance in the method wavelength. Complete separation of internal standard, ezetimibe and simvastatin were obtained with migration times of 2.0, 5.4 and 5.8 min, respectively (Fig. 4).

Optimized Electrolyte Composition

The best results were obtained by using the electrolyte composed of 20 mmol L^{-1} sodium tetraborate buffer solution (pH 9, adjusted with *o*-phosphoric acid), 30 mmol L^{-1} sodium dodecyl sulfate, and 12% acetonitrile. The quantitative determination of the analytes was performed at +30 kV and 30°C . Under these conditions, ezetimibe and simvastatin were separated below 6 min (Fig. 4).

Method Validation

The validation of an analytical method ensures credibility during routine use, being sometimes referred to as a process which provides documented evidence that the method is suitable for its purpose. The proposed method was fully validated as per ICH guidelines [25].

System Suitability

The obtained results of six replicate injections showed that the parameters tested were within the acceptable range. Ezetimibe and simvastatin were well separated at 5.4 and 5.8 min, respectively, expressing excellent resolution (mean resolution 7). The tailing factor for both ezetimibe and simvastatin peaks never exceeded 1.5 indicating good peak symmetry (acceptance limit is <2). The theoretical plates were always $>10,000$ in all electropherograms which ensured good efficiency throughout the developed separation process [25]. The proposed method offers high sensitivity, and both drugs can be detected accurately. The analytes were well separated from the degradation products. Results are presented in Table 1.

Table 1 System suitability results

Parameter	Ezetimibe \pm RSD% ^a	Simvastatin \pm RSD% ^a
Migration time (min)	5.40 ± 0.7	5.80 ± 0.9
Peak area (mAU)	$14,556 \pm 4.8$	$33,996 \pm 4.1$
Theoretical plates (N)	$12,507 \pm 13.4$	$101,066 \pm 10.5$
Tailing factor	1.1 ± 0.2	1.2 ± 0.3
Resolution (Rs)	7.0	

^aMean of six replicates

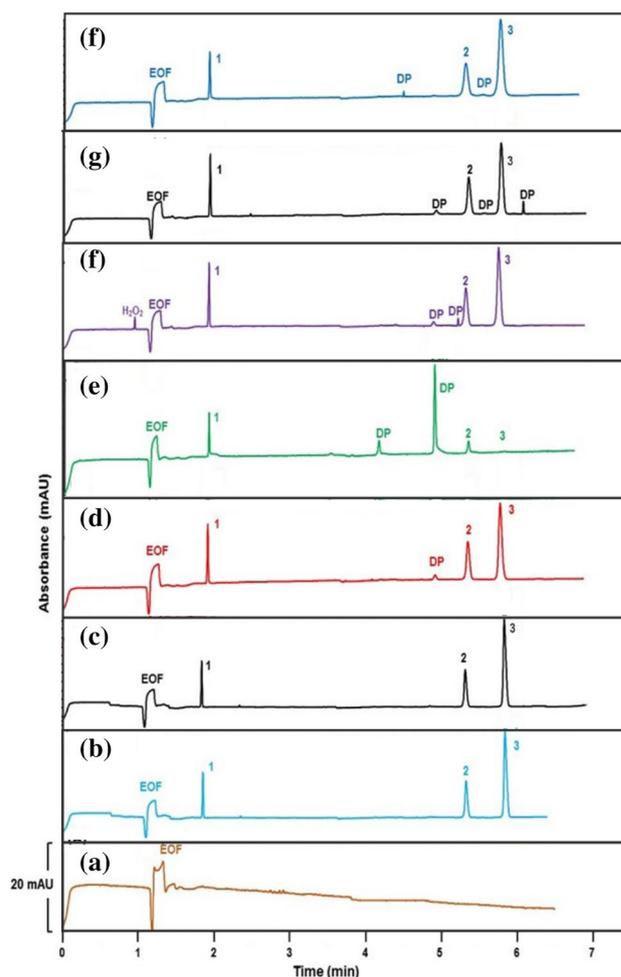


Fig. 5 Electropherograms of forced degradation study. **A** placebo, **B** standard mixture of $100 \mu\text{g mL}^{-1}$ ezetimibe, $200 \mu\text{g mL}^{-1}$ simvastatin and $100 \mu\text{g mL}^{-1}$ internal standard, **C** Vitoryn[®] sample ($100 \mu\text{g mL}^{-1}$ ezetimibe and $200 \mu\text{g mL}^{-1}$ simvastatin, **D** acidic hydrolysis (0.1 mol L^{-1} HCl); **E** alkaline hydrolysis (0.1 mol L^{-1} NaOH), **F** oxidative conditions (3% hydrogen peroxide), **G** thermal conditions (60°C), and **H** photolytic conditions (visible light) for 7 days for all above conditions. Electrophoretic conditions are the same as in Fig. 2 except pH 9. Peaks: (1) losartan potassium (internal standard), (2) ezetimibe, (3) simvastatin, and (EOF) electroosmotic flow

Specificity/Selectivity and Forced Degradation Stability Indicating Capability

The electropherograms of background electrolyte, standards (ezetimibe and simvastatin), placebo and Vitoryn[®] sample were compared to evaluate the specificity of the proposed method. The specificity was demonstrated by the absence of interference among ezetimibe, simvastatin and excipients from Vitoryn[®] samples (Fig. 4).

Selectivity and stability indicating capability of the analytical method was performed through forced degradation study. Peak purity was assessed using diode array detector.

The analysis was carried out to ensure that the proposed method was able to separate ezetimibe and simvastatin from the degradation products generated during the forced degradation study. The samples were subjected to stress conditions by acidic and alkaline hydrolysis, oxidative, thermal (60 °C) and photolytic degradation for 7 days. It was observed that sample was sensitive to acidic hydrolysis, with the formation of one degradation product in 5.1 min (Fig. 5D). In alkaline hydrolysis, it was observed degradation of ezetimibe and almost total degradation of simvastatin, showing two degradation products at 4.3 and 5.1 min (Fig. 5E). In oxidative condition, it was observed the formation of two degradation products at 5.1 and 5.3 min (Fig. 5F). Under heating test, it was observed the formation of three degradation products at 5.1, 5.7 and 6.2 min (Fig. 5G). Under photolytic stress, there were observed two degradation products at 4.5 and 5.7 min (Fig. 5H). In all cases, ezetimibe, simvastatin and internal standard peaks showed purity factor greater than 99%. In addition, resolution between the analyte peaks and the possible degradation product was greater than 1.5, which shows acceptable degree of method specificity.

Linearity and Range

The data obtained from plotting the peak area ratios (ezetimibe or simvastatin/internal standard) versus drug concentrations confirmed the method linearity in the concentration range between 80 and 120 $\mu\text{g mL}^{-1}$ for ezetimibe; while 160 and 240 $\mu\text{g mL}^{-1}$ for simvastatin. The correlation equations obtained for the linear model were $y=0.0373x-1.1003$ and $y=0.0417x-1.7465$ with determination coefficient of 0.9956 and 0.9920 for ezetimibe and simvastatin, respectively. Analysis of variance at 95% was performed to verify the good fitting of the micellar electrokinetic chromatography method, to evaluate its linearity and the validity of linear regression. From the obtained data (Table 2) we can see there was significant regression since calculated F was greater than critical F .

Therefore, analysis of variance showed that the adjustment of the linear model is appropriate for the two curves constructed, since the p values found were lower than F_{critical} values, indicating that the variances of the response in Y are independent of the concentration levels in X . The adjustment to the linear model was verified by normal and random distribution of the residuals (Fig. 6A and 6B), thus showing that there is no dependency between the values. Therefore, the homoscedasticity assumption is satisfied indicating a good fit for the linear model.

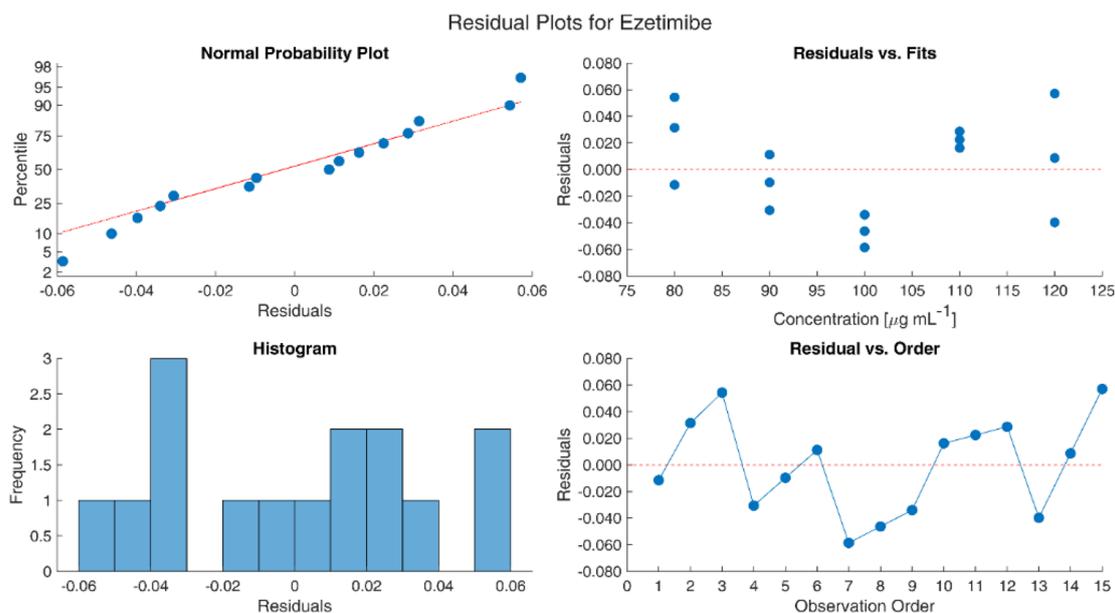
Table 2 Linearity, detection, and quantification limits

Parameter	Analyte							
	Ezetimibe	Simvastatin						
Range (%)	80–120	80–120						
Concentration range ^a ($\mu\text{g mL}^{-1}$)	80–120	160–240						
Intercept (b)	-1.1003	-1.7465						
Slope (a)	0.0373	0.0417						
Determination coefficient (R^2)	0.9956	0.9920						
Intercept standard deviation (S_b)	0.0694	0.2101						
Slope standard deviation (S_a)	0.0006	0.0010						
Probability (p)	7.04×10^{-10}	1.47×10^{-6}						
Significance (F)	1.05×10^{-16}	5.23×10^{-15}						
Detection limit ($\mu\text{g mL}^{-1}$)	6.14	16.63						
Quantification limit ($\mu\text{g mL}^{-1}$)	18.63	50.40						
Ezetimibe and simvastatin analysis of variance (analytical curve)								
	DF EZT	SVT	SS EZT	SVT	MS EZT	SVT	F_{cal} EZT	SVT
Regression	1	1	4.169581	20.857873	4.169581	20.857873	2938.07	1605.96
Residual	13	13	0.018449	0.168841	0.001419	0.012987		
Total	14	14	4.188030	21.02671				

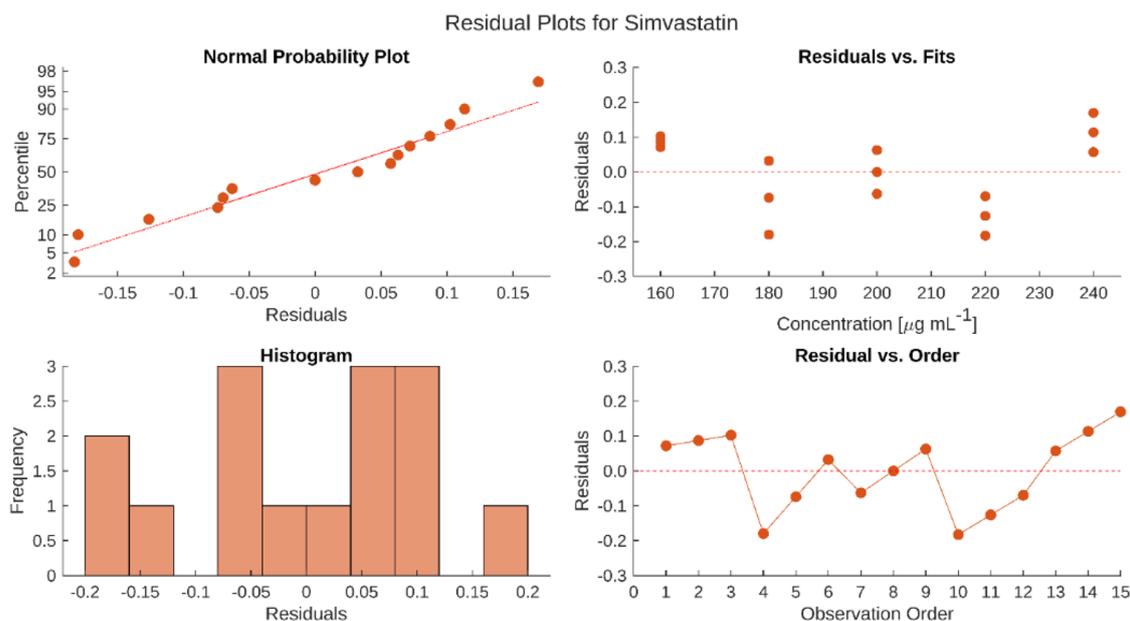
^aFive data points, triplicate at each concentration level

F_{critical} : 2.48, $\alpha=0.05$

DF degrees of freedom, SS sum of squares, MS mean squares, EZT ezetimibe, SVT simvastatin



(a) Residual plots for ezetimibe for linear regression model.



(b) Residual plots for simvastatin for linear regression model.

Fig. 6 **A** Residual plots for ezetimibe for linear regression model. **B** Residual plots for simvastatin for linear regression model

Limits of Detection and Quantification

Limits of detection and quantification was obtained by using the equations specified in section detection and quantification limits, demonstrate that the method is sensitive and viable to be applied for the determination

of ezetimibe and simvastatin in combined dosage forms (Table 2).

Accuracy

The proposed analytical method accuracy was evaluated by determining the mean percent recoveries of ezetimibe

Table 3 Recovery results for ezetimibe and simvastatin

Analyte	Theoretical (claimed) concentration ($\mu\text{g mL}^{-1}$)	Concentration found ($\mu\text{g mL}^{-1}$)	Recovery ^a (%)	Mean recovery ^b (%)
Ezetimibe	80	80.1	100.1 \pm 1.6	101.0 \pm 0.77 RSD% 0.76
	100	101.6	101.6 \pm 0.3	
	120	121.4	101.2 \pm 1.2	
Simvastatin	160	160.6	100.4 \pm 1.6	100.4 \pm 0.35 RSD% 0.35
	200	200.2	100.1 \pm 1.5	
	240	241.9	100.8 \pm 1.1	

^aMean of three replicates \pm SD^bMean of all recovery levels \pm SD ($n=9$)

and simvastatin standards added to the placebo matrix using three replicate determinations at three different levels (80, 100 and 120% of the working concentration). The obtained mean recoveries were 101.0% for ezetimibe and 100.4% for simvastatin with RSD% < 1, demonstrating the method accuracy (Table 3). Recovery values in the range of $100 \pm 2\%$ of the target value are considered acceptable [35].

Precision

The repeatability and intermediate method precision (intra- and inter-day, respectively) were assessed. The repeatability ($n=6$) was evaluated as the RSD% of six independent samples, performed in the same day and by the same analyst. The intra-day mean assay was 9.95 ± 0.10 mg/tablet (RSD% 1.05) for ezetimibe and 19.68 ± 0.08 mg/tablet (RSD% 0.38) for simvastatin. The intermediate precision was assessed by the analyses of other six independent samples by a different analyst, which showed mean assay of 10.07 ± 0.12 mg/tablet (RSD% 1.20) for ezetimibe and 19.77 ± 0.14 mg/tablet (RSD% 0.69) for simvastatin. Both repeatability and intermediate precision levels showed low RSD% values (< 2) which confirmed the good method precision. These results also indicated that the mean of the assay 10.01 ± 0.12 mg/tablet (RSD% 1.24) for ezetimibe and 19.73 ± 0.11 mg/tablet (RSD% 0.58) for simvastatin was in good agreement with the label claim for Vitorin[®] tablets. The RSD% was < 2, demonstrating that the precision of the analytical method is satisfactory (Table 4).

Robustness

The proposed method robustness test showed deliberate changes in the injection time and in the proportion of the organic solvent that can interfere in the instrumental responses. It was observed broad peaks at baseline for both

Table 4 Precision analysis for the quantification of ezetimibe and simvastatin

Analyte	Labeled value	Intra-day Found		Inter-day ($n=12$)
		I. Analyst	II. Analyst	
Ezetimibe	10	9.9	9.9	
	10	10.1	10.2	
	10	10.0	10.1	
	10	9.8	10.2	
	10	9.9	10.0	
	10	10.0	10.0	
	Mean	9.95	10.07	10.01
	SD	0.10	0.12	0.12
	RSD%	1.05	1.20	1.24
Simvastatin	20	19.7	20.0	
	20	19.7	19.8	
	20	19.6	19.6	
	20	19.7	19.7	
	20	19.8	19.7	
	20	19.6	19.8	
	Mean	19.68	19.77	19.73
	SD	0.08	0.14	0.11
	RSD%	0.38	0.69	0.58

SD standard deviation, RSD% relative standard deviation percent

analytes when injection time decreased (symmetry > 2), on the other hand, when the injection time increased no broad peaks was observed (symmetry < 2). Contrarily, decreasing the proportion of organic solvent makes the asymmetry factor better as compared to increased organic solvent. While increasing the theoretical plates makes the injection time and proportion of organic solvent decreased, on the other hand, when the injection time and proportion of organic solvent increased then the theoretical plates decreased. Resolution was unaffected with deliberate change in injection time and proportion of organic solvent (Table 5).

Table 5 Robustness analysis for the quantification of ezetimibe and simvastatin

Condition	Ezetimibe			Simvastatin	
	Injection time (s)	Tailing factor*	N*	Rs*	Tailing factor*
0.1	2.6	303,936	8	2.6	215,398
0.3	1.1	222,227	7	1.7	105,749
0.5	0.9	129,372	7	1.3	54,074
Acetonitrile (%)					
10	1.2	176,390	13	1.2	56,821
12	1.1	222,227	7	1.7	105,749
14	2.1	142,235	7	1.3	55,378

*Mean of three determinations

N Efficiency (theoretical plates)

Stability of Solutions

The stability of sample solutions even as standards working solutions in the diluting solvent (methanol and ultrapure water) was checked, no changes were detected within 8 h at room temperature. On the other hand, the standard stock solutions were stored in refrigerator at 4 °C, they were stable for at least two weeks. Peaks areas and migration time of the drugs seemingly remained unchanged (RSD% < 2). No significant degradation was observed during that period.

Application of the Proposed Method to the Commercial Pharmaceutical Preparation

Ezetimibe and simvastatin analysis in their combined tablets (Vitoryn[®]) was performed using the developed and validated micellar electrokinetic chromatography method. The assay of ezetimibe and simvastatin in commercial pharmaceutical formulation indicated a mean value of 10.10 ± 0.77 mg/tablet (101.0%, RSD% 0.76) for ezetimibe and 10.04 ± 0.35 mg/tablet (100.4%, RSD% 0.35) for simvastatin. There was good agreement between the obtained results and the label claim for ezetimibe and simvastatin tablets (Table 3). The results were satisfactory, and they are within tolerance limits (95 to 105% label claim) [6].

Greenness Assessment of the Proposed Method

Green Analytical Procedure Index [36] and Analytical GREENness [37] were employed in this study to evaluate the method greenness.

According to Green Analytical Procedure Index, three different colored (green, yellow and red) pictograms (five-pentagram) allowed us to determine the method greenness in which green color is considered to be more eco-friendly as compared to other two colors (yellow and red). The result showed that 10 regions are shaded green, 2 yellow, 2 red and 1 white indicating the proposed method greenness (Table 6A). A numerical estimation was also made

with HPLC [10] which revealed that 4 regions are green, 4 yellow, 6 red and 1 white making the reported method less green as compared to the proposed micellar electrokinetic chromatography method.

Analytical GREENness found to be comprehensive, convenient, reliable and straightforward evaluation approach which gathers both quantitative and qualitative aspects of comparison. According to this approach, each of the 12 parameters has a score from 0.0 to 1.0 which is indicated by a specific pictogram color ranging from red (0.0) to dark green (1.0). In this work, the final score was 0.81 indicating the greenness of the proposed micellar electrokinetic chromatography method (Table 6B). A numerical estimation was also made with HPLC [10] which revealed less green (0.67) as compared to the proposed method.

Therefore, both are highly recommended approaches that produces an easily interpretable and informative results for the greenness assessment [36, 37].

Conclusion

A fast, sustainable and green micellar electrokinetic chromatography method has been investigated for the simultaneous quantification of ezetimibe and simvastatin. The method was fully optimized and validated as per ICH guideline and was successfully applied for the analysis of ezetimibe/simvastatin in binary mixture tablet dosage form and in the presence of their degradation products after submitted to different forced degradation conditions (oxidative, thermal, photolytic, alkaline and acidic hydrolysis). The proposed method was the first to be considered stability indicating since it enabled the determination of the active pharmaceutical ingredients without any interference under different forced degradation conditions. To our knowledge, there is no work with this intend, on the other hand there is only one research in which ezetimibe and simvastatin were determined simultaneously using

Table 6 Greenness assessment

Parameter	Response
(A) Green analytical procedure index	
<i>Sample Preparation</i>	
(1) Collection	In-line
(2) Preservation	None
(3) Transport	None
(4) Storage	Under normal conditions
(5) Method type	Simple procedures
(6) Extraction scale	Not applicable
(7) Reagents/solvents used	Non-green reagents/solvents
(8) Additional treatments	None
<i>Solvents/reagents</i>	
(9) Amount	< 10 mL (< 10 g)
(10) Health hazard	Slightly irritant and toxic; National Fire Protection Association (NFPA) score 1
(11) Safety hazard	Highest NFPA stability/flammability score 1
<i>Instrumentation</i>	
(12) Energy	< 0.1 kWh/sample
(13) Occupational hazard	Hermetic sealing of analytical procedure
(14) Waste	< 1 mL (< 1 g)
(15) Waste treatment	No treatment
<i>Method type</i>	
Type of analysis	Qualitative and quantitative
(B) Analytical GREEnness	
(1) Sample treatment	In-field sampling and direct analysis
(2) Sample amount	0.025 g
(3) Device positioning	In-line
(4) Sample preparation stages	3 or fewer
(5) Miniaturization and automation	Miniaturized and automatic
(6) Derivatization	None
(7) Waste	1 mL
(8) Analysis throughput	7 analytes; 70/h
(9) Energy Consumption	0.4 kWh
(10) Reagents and source	Some reagents are bio-based
(11) Toxic solvent/reagent amount	0.3 mL
(12) Operator's safety	Toxic to aquatic life, highly flammable, explosive, corrosive
Green analytical procedure index and analytical GREEnness evaluation result	

capillary electrophoresis in 10 min. In this work, we determined the analytes in less than 6 min. The proposed micellar electrokinetic chromatography method has several advantages like specific, accurate and cost-effective. Therefore, it can be successfully applied in quality control laboratories for determining ezetimibe and simvastatin in

their combined binary mixture. Furthermore, the method consumes less solvent with high separation efficiency. Green Analytical Procedure Index and Analytical GREEnness metrics were used to demonstrate the greenness of the proposed method.

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Author Contributions AMCS and FAGF: investigation, methodology, formal analysis, data curation, software, writing—original draft. MFMT: conceptualization, visualization, and resources. AR and MSAP: conceptualization, validation, resources, writing—review and editing, supervision, and project administration.

Data Availability The data that support the findings of this study is available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest All contributing authors declare no conflict of interest.

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