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Article

Simultaneous determination of atorvastatin and ezetimibe from combined pharmaceutical products by micellar electrokinetic capillary chromatography

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A rapid and sensitive micellar electrokinetic capillary chromatography method with UV photodiode-array detection was developed for the simultaneous determination of atorvastatin and ezetimibe in fixed dose drug combination. Experimental conditions such as buffer concentration and pH, surfactant concentration, system temperature, applied voltage, injection parameters were optimized in order to improve the efficiency of the separation. The best results were obtained when using fused silica capillary (48 cm length X 50 µm ID) and 25 mM borate buffer electrolyte at pH 9.3 containing 25 mM SDS, + 30 kV applied voltage, 20 ºC system temperature. The separation was achieved in approximately 2 minutes, with a resolution of 7.02, the order of migration being atorvastatin followed by ezetimibe. The analytical performance of the method was verified with regard to linearity, precision, robustness and the limit of detection and quantification were calculated.

Uniterms: Micellar Electrokinetic Capillary Chromatography. Fixed Dose Drug Combination. Atorvastatin. Ezetimibe.

INTRODUCTION

Ezetimibe (EZE) and atorvastatin (ATO) are two lipid-lowering compounds with complementary mechanisms of action (Ballantyne *et al.*, 2003). ATO, (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid, lowers plasma cholesterol and lipoprotein levels by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and cholesterol synthesis by increasing the number of hepatic low-density lipoprotein (LDL) receptors on the cell-surface to enhance uptake and catabolism of LDL (Malhotra, Goa, 2001). EZE, (3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidin-2-one, acts by decreasing cholesterol absorption in the small intestine, leading to a decrease in the delivery of intestinal cholesterol to the liver. This causes a reduction of hepatic cholesterol stores and an increase in clearance of cholesterol from blood (Ara *et al*., 2008). The chemical structures of the two analytes ATO and EZE are presented in Figure 1.

Combining the different mechanisms of action of these agents appears to provide substantial reductions in LDL cholesterol, with additional favorable changes in total cholesterol. Clinical studies have shown that co-administration of ATO and EZE was significantly more effective at reducing LDL cholesterol concentrations than ATO and EZE alone. EZE co-administered with an HMG-CoA reductase inhibitor is indicated as adjunctive therapy to diet for use in patients with primary hypercholesterolaemia who are not appropriately controlled with a statin alone (Gagné, Gaudet, Bruckert, 2002).

Taking into consideration the great prevalence and importance of fixed dose combination in modern therapy, the elaboration of new methods of analysis for the simultaneous determination of combined substances represents a necessity and also a challenge for analyst.

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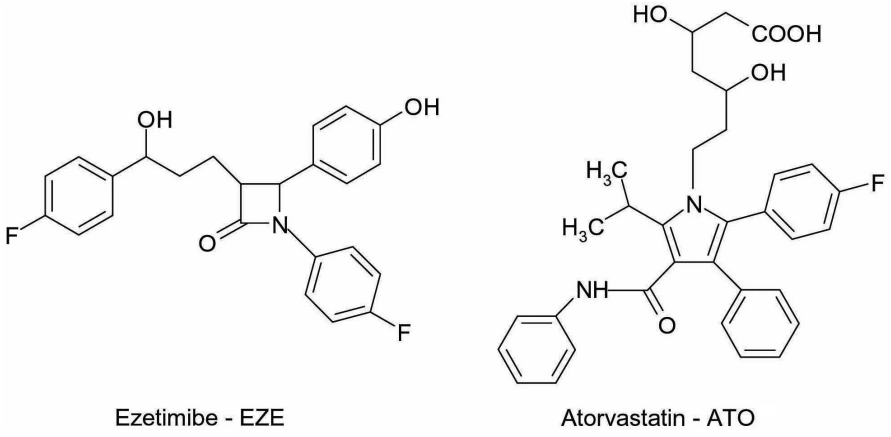

FIGURE 1 - Chemical structures of EZE and ATO.

Capillary electrophoresis (CE) can be considered as an alternative and also a complementary separation technique to the more frequently used high performance liquid chromatography (HPLC) methods, with advantages related to the fast analysis time, high separation efficiency and especially to the low consumption of analytes and reagents (Morzunova, 2006).

ATO in combination with EZE can be determined by UV spectrophotometry, thin layer chromatography (TLC) – densitometry (Baghdady *et al.*, 2013), HPLC (Sechachalam, Kothapally, 2008; Patel *et al*., 2012) or liquid chromatography – mass spectrometry (LC-MS) methods (El-Bagary *et al*., 2014). Only one CE method was found in literature for simultaneous determination of ATO and EZE by applying a capillary zone electrophoresis (CZE) technique using a background electrolyte solution consisting of phosphate buffer (2.5 mM, pH 6.7): methanol (70:30 v/v) (AlShehri, 2012). However, a few articles were also published describing simultaneous determination of another HMG-CoA reductase inhibitor, simvastatin and EZE from their combination drug products by CE (Dalmora *et al*., 2008; Yardimci, Özaltin, 2010).

Our aim was to develop a simple, rapid and efficient CE method for the simultaneous determination of EZE and ATO under optimized analytical conditions and to verify the applicability of the newly developed method for determination of the two analytes from fixed dose combination product.

MATERIAL AND METHOD

Chemicals

Pharmaceutical grade samples of atorvastatin calcium (Morepen Laboratories, New Delhi, India) and ezetimibe (MSN Laboratories Ltd., India) were used.

Sodium tetraborate, sodium dodecyl sulphate (SDS) were purchased from Merck (Germany) while methanol from LachNer (Czech Republic). All reagents were of analytical purity. Purified water was provided by a Milli-Q Plus water purification system (Millipore, USA).

The pharmaceutical dosage form used in this study was Liptruzet (MSD, UK) containing an EZE/ATO ratio of 10/40 mg.

Equipment

The measurements were performed on an Agilent 1600 CE system equipped with a photodiode array (DAD) detector and ChemStation software for data handling. Separations were carried out in an uncoated fused-silica capillary with a total length of 48 cm (40 cm effective length), having an internal diameter of 50 µm (Agilent, Germany). Buffer pH was determined using a Terminal 740 pH–meter (Inolab, Germany).

Electrophoretic procedure

Conditioning of new capillaries was conducted by flushing with 0.1 M NaOH for 60 minutes and water for 30 minutes. Prior to all runs the capillary was preconditioned by flushing with 0.1 M NaOH, water and background electrolyte (BGE) each for 2 minutes.

Stock solution containing 1 mg $mL^{-1}$of each analyte were prepared in methanol and diluted prior to use with the same solvent to the appropriate concentration. Both BGE and sample solutions were filtered through a 0.45 µm pore size membrane filter and sonicated in an ultrasonic bath for 5 minutes prior to use.

In the preliminary analysis we applied some "standard" electrophoretic conditions for a CE analysis: 25 mM buffer concentration, temperature 20 ˚C, applied

voltage + 20 kV, injection pressure x time 50 mbar x 3 sec, sample concentration 25 μg mL^{-1}. The samples were introduced in the system at the anodic end of the capillary by hydrodynamic injection. Detection was performed at 210, 230 and 250 nm, and full spectra of the analytes were also stored to facilitate peak identification.

Preparation of pharmaceutical samples

When preparing samples from commercial formulations, average weight was calculated by weighing 10 tablets, the tablets were crushed, mixed and powdered in a mortar into a homogenous powder; an amount of powder equivalent to the weight of one tablet was dissolved in 100 mL methanol by sonication for 5 minutes with intermittent shaking. The solution was filtered through a 0.45 μm syringe filter, centrifuged at 3500 rpm for 10 minutes and diluted with methanol to the appropriate concentration. The same procedure was applied as in the separation from standard solutions.

RESULTS AND DISCUSSION

Optimization of the analytical conditions

The pH value is always a critical selectivity parameter for the determination of acidic and basic compounds, as it determines the ionization degree of the analytes, their electrophoretic mobility and the magnitude of the electroosmotic flow (EOF). The effective mobility of weak anionic and cationic analytes is strongly dependent on their pKa values related to the pH of the running buffer.

Preliminary CZE measurements were attempted with different phosphate and borate buffer solutions in a pH range between 3 and 11; ATO can be determined over a relatively large pH range (5-11) being completely ionized in anionic forms in neutral and alkaline pH environments, EZE can be determined only in an acidic environment while over a pH range between 5 -11 presents no own electrophoretic mobility and will be carried by the EOF.

Consequently micellar electrokinetic capillary chromatography (MEKC) was applied for the separation of the two analytes by adding an anionic surfactant, sodium dodecyl sulfate (SDS), to the buffer electrolyte. MEKC is based on the addition to the buffer solution of a micellar "pseudostationary" phase, which interacts with the analytes according to partitioning mechanisms, just like in a chromatographic method where the "pseudostationary" phase is composed by the surfactant added to the buffer solution in a concentration above its critical micellar concentration (CMC) and EOF acts like a chromatographic "mobile phase". The separation principle of MEKC is based on the differential partition of the analytes between micelles and water, as analytes which are highly retained by the micelle will have longer migration times (Hancu *et al*., 2013).

The optimization of the electrophoretic separation was based on the production of acceptable peak shape, resolution and separation time. Optimization of the developed method was performed in an univariate manner, changing one factor at a time, while keeping the others constant, in terms of BGE concentration (25-100 mM), BGE pH (8-11), SDS concentration (20-50 mM), applied voltage (15-30 kV), capillary cassette temperature (15°C-25 °C) and injection parameters (25-50 mbar x 1-5 seconds).

The effect of buffer pH was investigated in the range 8 – 11 using a 25 mM sodium tetraborate, 25 mM SDS buffer; the best correlation between migration times, resolution and peak shapes were obtained at pH 9.3.

The effect of running buffer concentration was examined by varying it between 25 and 100 mM at a constant pH of 9.3. An increase in buffer concentration generated higher currents and increased migration time, consequently a concentration of 25 mM was chosen in order to reduce analysis time.

The effect of surfactant concentration was assessed in the range of 20 – 50 mM, the increase of SDS concentration resulted in increased migration times, consequently the optimum SDS concentration was set to 25mM.

The effects of organic modifiers like acetonitrile or methanol in the concentration range of 5 – 20%, were also evaluated, but only an increase in migration times was obtained and no improvement on the electrophoretic separation was achieved.

The effect of the separation voltage in the range of 15–30 kV was examined, high voltage reduces analysis time while low voltage enhances separation, a voltage of + 30 kV was selected as the optimum.

The influence of capillary temperature (15-25ºC) was evaluated; when temperature increased migration times decreased; a temperature of 20ºC was chosen as working temperature for the analysis.

In order to determine the optimal injection parameters, the influence of injection time (1-5 s) and injection pressure (25-50 mbar) were studied. An injection pressure of 50 mbar and an injection time of 1 second proved to be appropriate to attain low detection limit without affecting the peak shape, migration time and resolution.

The best results for the simultaneous determination of ATO and EZE were obtained when using 25 mM borate

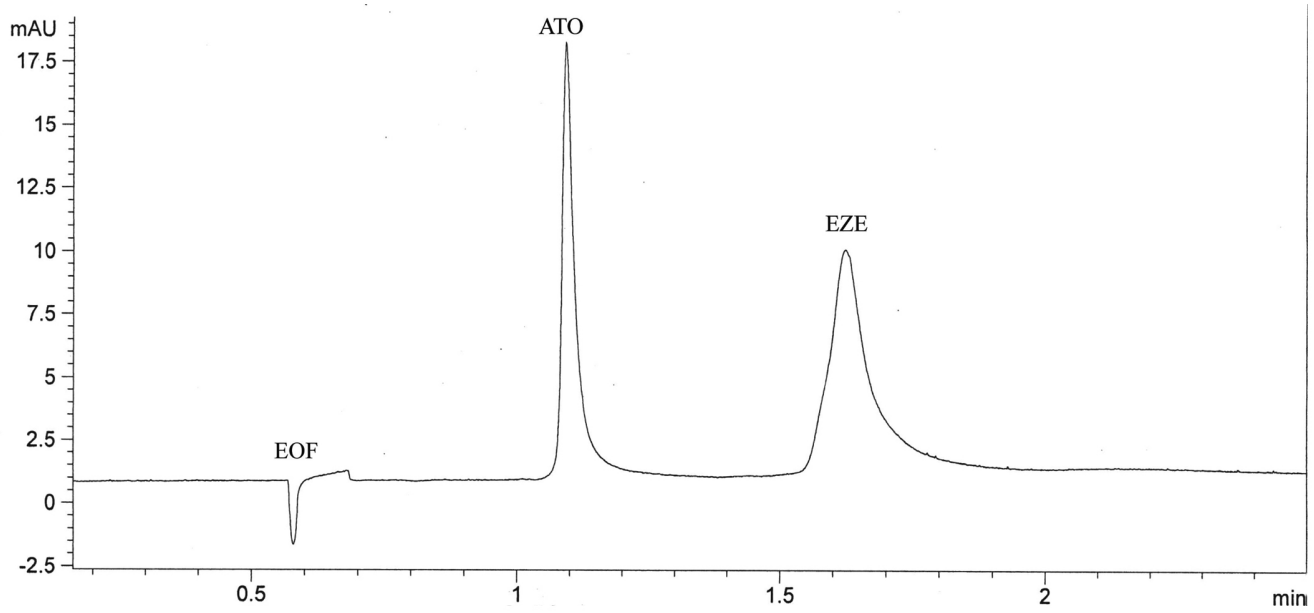

FIGURE 2 - Capillary electrophoretic separation of atorvastatin (ATO) and ezetimibe (EZE), (experimental conditions: 25 mM borate buffer electrolyte at pH 9.3 containing 25 mM SDS, +30 kV applied voltage, 20 ºC system temperature, 50 mbar/s injection parameters, UV detection 230 nm).

buffer containing 25 mM SDS at pH 9.3, + 30 kV applied voltage, 20ºC system temperature, injection pressure 50 mbar, injection time 1 second, UV detection at 230 nm. The two analytes migrated in less than 2 minutes, the order of migration was ATO followed by EZE; the resolution of the separation was 7.02 with a selectivity factor of 1.49 (Figure 2).

Analytical performance

Linearity solutions were prepared from stock solution at six concentration levels and three replicates per concentration. The linear regression analysis of ATO and EZE were constructed by plotting the peak area of the analytes (y) versus analytes' concentration in (x) axis. The calibration curves were linear in the studied range (5-100 µg mL^{-1}) with correlation coefficients above 0.99. The regression equation and correlation coefficients are presented in Table I.

The approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection (LOD) and quantification (LOQ) limits. LOD and LOQ values were estimated as [(standard deviation of repeatability)/(slope of the regression equation)] by multiplying with 3.3 and 10 respectively. The values obtained are given in Table I.

The intra-and inter-day variability or precision are summarized in Table II and were assessed by using standard solutions prepared to produce solutions of three different concentrations of each drug. Repeatability or intra-day precision was investigated by injecting six replicate at of each of the samples of three different concentrations. Inter-day precision were assessed by injecting the same three samples over three consecutive days.

To demonstrate the robustness of the method, minor changes in the experimental conditions were performed; as pH of the buffer was varied in the range ± 0.5 pH unit, separation temperature in the range ± 2 °C while applied voltage in the range ± 2 kV. None of the modifications caused significant changes in the resolution. The RSD for migration times and peak areas was under 3%.

TABLE I - Analytical parameters for the simultaneous determination of ATO and EZE (n = 6)

Analyte	Concentration range (µg mL^{-1})	Regression equation	Correlation coefficient	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
ATO	5-100	y = 0.8279x + 8.3137	0.9965	0.27	0.89
EZE	5-100	y = 0.9114x + 8.0645	0.9981	1.29	4.31

TABLE II - Intra and inter-day precision for the simultaneous determination of ATO and EZE

Analyte concentration (μg mL^{-1})	RSD (%)			
	Migration time		Peak area	
	ATO	EZE	ATO	EZE
Intra-day precision (n=6)				
10	0.95	1.05	1.27	2.17
20	0.94	1.06	1.22	2.18
30	0.95	1.06	1.14	2.02
Inter-day precision (n=18)				
10	1.04	1.51	2.55	2.75
20	1.03	1.48	2.42	2.68
30	1.05	1.53	2.47	2.73

TABLE III - Assay results of ATO and EZE simultaneous determination in pharmaceutical formulation

Pharmaceutical preparation	Declared amount (mg)		Found amount (mg)		RSD (%)	
	ATO	EZE	ATO	EZE	ATO	EZE
Liptruzet 10/40	40	10	40.8	9.9	0.85	1.62

The solution stability of ATO and EZE was carried out by leaving the test solution in tightly capped volumetric flask at room temperature for 24 hours. The sample solution was assayed against freshly prepared standard solutions, the RSD of the assay of ATO and EZE were within 5% and it indicates that both standard and test preparation were stable for 24 hours on benchtop at room temperature.

The electropherograms obtained from tablets were similar to those obtained from standard solutions. There were no interfering peaks of excipients with the analytes. The % recovery was found to be in the range of 98 - 102% indicating high degrees of accuracy of the proposed method (Table III).

CONCLUSION

A MEKC method for the simultaneous determination of ATO and EZE was successfully developed. A conventional method optimization based on changing one variable at a time, while keeping the other variables constant was applied in order to establish the optimal analytical conditions. Under the optimized conditions, baseline separation of the two analytes was obtained in approximately 2 minutes. In comparation with the results published by AlShehri (2012) much shorter migration times and comparable resolution were obtained by applying a MEKC technique instead of CZE (2 minutes versus 15 minutes).

Good analytical performance with regards to linearity, precision, reproductibility and robustness was achieved. Furthermore, the proposed method was successfully applied for the determination of the analytes in their co-formulated tablets.

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