ORIGINAL ARTICLE

SIMULTANEOUS DETERMINATION OF FIBRATES BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

ELEONORA MIRCIA[1], GABRIEL HANCU[2], OANA NIȚU[2], HAJNAL KELEMEN[2]*, MELANIA CÂRCU-DOBRIN[2], ŞERBAN ANDREI GÂZ[1], TEODORA BALACI[3]

[1]*Department of Organic Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy, Târgu Mureş, Romania*
[2]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy, Târgu Mureş, Romania*
[3]*Department of Pharmaceutical Technology and Biopharmacy, Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*

**corresponding author: hajnal.kelemen@umftgm.ro*

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Abstract

Fibrates are effective hypolipidemic agents used for a large range of metabolic disorders, mainly hypercholesterolemia, with peroxisome proliferator–activated receptor agonist activity. Even though fibrates are not used in combination with other fibrates during therapy, the development of a rapid general analytical procedure that will not be limited to the analysis of only one fibrate could be very useful. Four fibrates - bezafibrate, clofibrate, fenofibrate and gemfibrozil were analysed by capillary electrophoresis. Migration time and selectivity were examined in different separation buffers at different pH values, varying the separation voltage, the system temperature and the injection parameters. While bezafibrate and gemfibrozil are ionizable analytes and can be determined by capillary zone electrophoresis, clofibrate and fenofibrate are neutral from the electrophoretic point of view and can be determined only using micellar electrokinetic capillary chromatography. The four analytes were determined simultaneously with high resolution and fast migration times. The analytical performance of the method was verified regarding precision, linearity, robustness and limits of detection and quantification were calculated. The method was applied for the determination of fenofibrate from pharmaceutical preparations.

Rezumat

Fibrații sunt agenți hipolipemianți utilizați în tratamentul unui număr ridicat de tulburări metabolice, în special în hipercolesterolemie, activând receptorii proliferării peroxizomilor. Chiar dacă fibrații nu sunt utilizați în combinații cu alți fibrați în terapie, dezvoltarea unei proceduri analitice rapide care nu este limitată la analiza unui singur fibrat poate fi o variantă utilă. Patru fibrați - bezafibrat, clofibrat, fenofibrat și gemfibrozil au fost analizați prin metoda electroforezei capilare. Timpii de migrare și selectivitatea separării au fost evaluate utilizând diferite soluții tampon la diferite valori de pH, variind voltajul, temperatura sistemului și parametrii de injectare. În timp ce bezafibratul și gemfibrozilul sunt analiți ionizabili și pot fi determinați prin electroforeză capilară zonală, clofibratul și fenofibratul sunt compuși neutri din punct de vedere electroforetic și pot fi determinați doar prin electroforeză capilară în soluție micelară. Cei patru analiți au fost determinați simultan cu rezoluție ridicată și timpi de migrare rapizi. Performanța analitică a metodei a fost verificată pe baza preciziei, liniarității, robusteții și s-au calculat limitele de detecție și cuantificare. Metoda a fost aplicată pentru determinarea fenofibratului din formulări farmaceutice.

Keywords: fibrates, capillary electrophoresis, simultaneous determination

Introduction

Fibrates are a group of amphipathic carboxylic acids, used for the treatment of metabolic disorders, mainly hypercholesterolemia, which have agonist activity for peroxisome proliferator–activated receptor (PPAR) α, nuclear receptors involved in the transcription of genes involved in fatty acid oxidation, apolipoprotein production, and cholesterol transport, with additional functions in inflammation, endothelial function, and vascular remodelling [1, 2]. Fibrates effectively decrease plasma triglycerides (TG) levels and increase high-density lipoprotein cholesterol (HDL-C) levels, with variable effects on low-density lipoprotein cholesterol (LDL-C) levels [3].

Structurally fibrates are fibric (2-phenoxy-2-methyl-propanoic) acid derivatives, and include among others: bezafibrate (2-[4-[2-[(4-chlorobenzoyl)amino]-ethyl]phenoxy]-2-methylpropanoic acid), clofibrate (2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester), fenofibrate (2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoic acid 1-methylethyl ester) and gemfibrozil (5-(2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid).

The structural characteristics of the four substances mentioned above are presented in Figure 1.

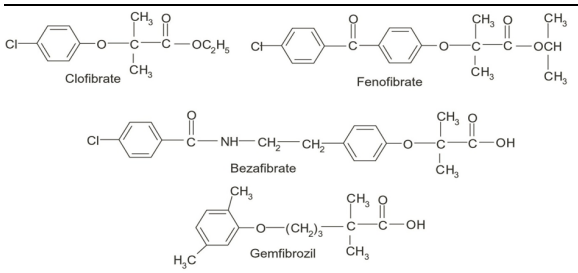

Figure 1.
Chemical structures of the studied analytes

Fibrates can be determined from pharmaceutical preparations using UV spectrophotometric [4-7], high performance liquid chromatographic (HPLC) [8], RP-HPLC [9], LC-MS and differential pulse polarographic and square wave voltametric techniques [11].
Capillary electrophoresis (CE) is a relatively new analytical technique based on the separation of charged analytes through a small capillary under the impact of an electric field, which is gaining more and more adepts in the field of drug analysis, its advantages being related with the high separation efficiency, rapid method development, quick migration times and low consumption of analytes and reagents [10, 12].
Surprisingly, the articles which describe determination of fibrates using CE are scarce. Komsta *et al.* [13] describe a CE method for the determination of bezafibrate, ciprofibrate, gemfibrozil from pharmaceutical preparation using clofibric acid as internal standard; the determination of clofibrate and fenofibrate was not resolved because the analytes migrated together with the electroosmotic flow (EOF). A micellar electrokinetic capillary chromatographic (MEKC) method was also published by Shihabi [14] for the determination of fenofibrate and its metabolite, fenofibric acid in both in capsules and in serum.
Analytical methods for determination of fibrates are developed individually as expected, because fibrates are not used in combination with other fibrate molecules during therapy. However, the development of a rapid analytical procedure that will not be limited to the analysis of only one analyte could be a very useful and interesting approach. Therefore, the purpose of this study was to develop a universal CE method for the rapid determination of four of the most representative fibrate derivatives bezafibrate, clofibrate, fenofibrate and gemfibrozil in a single run without the need for developing a separate and distinct method for each analyte.

Materials and Methods

Chemicals and Reagents
Bezafibrate, clofibrate, fenofibrate and gemfibrozil were purchased from Cayman Pharmaceuticals (USA). The analytes were of pharmaceutical grade, complying with the stipulations of European Pharmacopoeia. All reagents were of analytical grade quality: phosphoric acid (Chimopar, Bucharest, Romania), methanol, sodium hydroxide (LachNer, Neratovice, Czech Republic), sodium tetraborate, disodium hydrogenophosphate, sodium didydrogenophosphate, sodium dodecyl sulfate - SDS (Merck, Darmstadt Germany). Purified water was provided by a Milli-Q Plus water purification system (Millipore, Milford, MA, USA).
Lipivim® (Vim Spectrum Târgu Mureş, Romania) capsules containing 200 mg fenofibrate was used as pharmaceutical dosage form for the determination of fenofibrate.
Instrumentation
Experiments were performed using an Agilent (Waldbronn, Germany) 1600 CE system equipped with a diode-array (DAD) detector. Separations were performed on an uncoated fused-silica capillary with a total length of 38.5 cm (effective length 30 cm) and an internal diameter of 50 µm (Agilent). Electropherograms were recorded and processed by use of Chemstation 7.01 software (Agilent). Buffer solution pH was determined with a Terminal 740 pH-meter (Inolab, Germany).
Sample preparation
Sample stock solutions were prepared by dissolving the analytes in methanol in a concentration of 1 mg/mL and later diluted with the same solvent to the appropriate concentration. All samples and buffers were filtered through a 0.45 µm syringe filter and sonicated for 5 minutes before use. The samples were introduced in the system at the anodic end of the capillary by hydrodynamic injection.
To prepare samples from commercial formulations the content of ten capsules were weighed and powdered in a mortar; an accurately weighed powdered sample equivalent to the average weight of the content of one capsule was individually transferred into a 100 mL volumetric flask and dissolved in methanol by sonication for 5 minutes. After sonication both sample solutions were filtered through a 0.45 µm syringe filter, centrifuged at 3500 rpm for 10 min, and diluted with methanol to the appropriate concentration. The same electrophoretic procedure was applied as in the separation from standard solutions.
Electrophoretic conditions
Before first use, the capillary was flushed for 30 minutes with 0.1 M sodium hydroxide followed by a 30 minutes rinsing with water and a 15 minute conditioning with the background electrolyte (BGE) used in the analysis. Between runs the capillary was preconditioned with 0.1 M sodium hydroxide, ultrapure water and BGE each for 1 minute, in order to maintain proper reproducibility of run-to-run injections.
In the preliminary analysis we applied some "standard" electrophoretic conditions for a CE

analysis: temperature 25°C, applied voltage +20 kV, injection pressure x time 50 mbar x 1 sec, sample concentration 1 mg/mL.

Results and Discussion

Preliminary analysis

The first step in CE method development for optimizing the separation of the fibrates molecules was the selection of the buffer pH, which determines the extent of ionization and mobility of each analyte. Bezafibrate (pKa - 3.6) and gemfibrozil (pKa - 4.5) have in their structure a ionisable carboxyl group and can be determined over the whole examined pH range between 5 and 11 where both fibrates are in anionic forms; but clofibrate and fenofibrate are esters and not ionisable and have no own electrophoretic mobility. Consequently, capillary zone electrophoresis (CZE), a technique where the separation occurs due to the differences between the electrophoretic mobilities of the analytes, can be applied only for the determination of bezafibrate and gemfibrozil, while clofibrate and fenofibrate will migrate together with the electroosmotic flow (EOF).

In order to separate all four analytes, an anionic tensioactive substance (sodium dodecyl sulphate - SDS) was added to the BGE. When an anionic surfactant such as SDS is employed, the micelle migrates toward the positive electrode by electrophoresis; the EOF transports the bulk solution toward the negative electrode due to the negative charge on the surface of fused silica, but EOF is usually stronger than the electrophoretic migration of the micelle under neutral or alkaline conditions and, therefore, the anionic micelle also will travel toward the negative electrode at a retarded velocity. MEKC can separate both ionic and neutral substances while CZE typically separates only ionic substances; thus MEKC has a great advantage over CZE for the separation of mixtures containing both ionic and neutral compounds [15].

Borate buffer containing SDS at pH 9.3 as background electrolyte gave the best preliminary results, since at this alkaline pH, the apparent mobilities of the four analytes were the highest, and also the generated current was low. The observed increase of apparent mobilities with increasing the buffer pH was attributed to a predominant effect of pH on EOF.

Optimization of the analytical conditions

An increase in SDS concentration (25 - 100 mM) resulted in prolonged analysis time and high current in the capillary. The micellar solution generally has a higher conductivity and hence causes a higher current than the simple buffer does in CZE. Therefore a concentration of 25 mM was chosen as optimal for the separation.

The addition of an organic modifier (methanol) changes the selectivity and migration times due to the change in BGE viscosity, but resulted in a significantly increased migration times.

The influence of voltage (15 - 25 kV) on the migration time was investigated using the optimized BGE conditions. Increasing the voltage resulted in shorter migration times; thus a +20 kV voltage was selected as the optimum, because higher applied voltages lead to higher current and also generation of Joule heating which may limit the theoretical gain in resolution and efficiency.

An increase in temperature (15 - 25°C) caused a slight reduction in migration time because of the decrease in the distribution coefficient as well as the viscosity of the BGE. The temperature effect on selectivity was not significant; a temperature of 20°C was chosen as the working temperature for the analysis.

For the determination of the optimal injection parameters, the influence of injection time (1 - 5 seconds) and injection pressure (30 - 50 mbar) was studied in order to attain low detection limits without affecting the quality of the peak shapes and resolution; an injection pressure of 50 mbar for 1 second was selected as optimum.

The separation of all four fibrates was completed in a short 4 minutes analysis time, the order of migration being: bezafibrate, gemfibrozil, clofibrate and fenofibrate. In MEKC the migration velocity of the analyte depends on the distribution coefficient between the micellar and the non-micellar (aqueous) phase.

The electropherogram obtained in the simultaneous separation of the four studied fibrates using the optimized analytical conditions is presented in Figure 2.

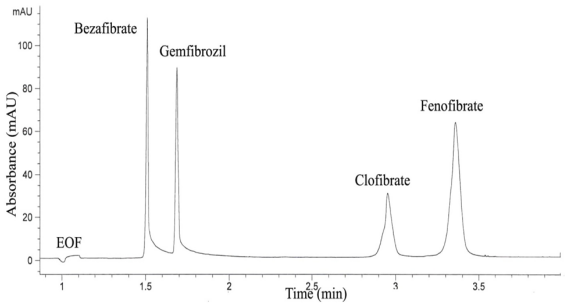

Figure 2.

Separation of fibrates under optimized analytical conditions (analytical conditions: 25 mM sodium tetraborate, 25 mM SDS, pH 9.30, +20 kV, 20°C, 50 mbar/1 sec, UV detection 210 nm, 1 mg/mL analytes concentration)

Analytical performance

After the method development, experiments were carried out to evaluate the analytical performance of the method for the simultaneous determination of fibrates under optimized conditions.

In order to measure repeatability of the method, six consecutive injections were made with a standard solution containing 0.25 mg/mL of each drug. Intermediate precision was evaluated over three consecutive days (Table I). Each day, newly prepared BGE and sample solutions were used.

Table I

Intra-day and inter-day precision of the migration times, peak areas and height for the proposed method (c = 0.25 mg/mL)

Substance	Intra-day precision (n = 6)			Inter-day precision (n = 18)		
	RSD (%) migration time	RSD (%) peak area	RSD (%) peak height	RSD (%) migration time	RSD (%) peak area	RSD (%) peak height
Bezafibrate	0.02	1.01	0.91	0.02	1.54	1.24
Gemfibrozil	0.02	0.99	0.95	0.02	1.42	1.32
Clofibrate	0.04	0.44	0.71	0.04	0.66	0.94
Fenofibrate	0.05	0.53	0.89	0.05	0.97	1.21

The linearity of the method was investigated over a specific concentration range 0.05 - 1 mg/mL on the basis of eight measurement points and three triplicate analyses (n = 3) per concentration (Table II). LOD and LOQ were estimated as (standard deviation of regression equation)/(slope of the regression equation) × 3.3 and 10, respectively (Table II).

Table II

Regression and correlation data for the calibration curves of fibrates (concentration range 0.05 - 1 mg/mL, n = 6)

Substance	Regression equation	Correlation coefficient	LOD (mg/mL^{-1})	LOQ (mg/mL)
Bezafibrate	y = 0,858x + 1,488	0,999	0.028	0.092
Gemfibrozil	y = 0,787x + 1,142	0,999	0.025	0.082
Clofibrate	y = 0,701x - 0,077	0,998	0.029	0.097
Fenofibrate	y = 1,752x + 0,385	0,999	0.022	0.073

The robustness of the method was demonstrated by studying the influence of the variation of several experimental parameters like buffer concentration (20 - 30 mM), applied voltage (18 - 22 kV) and temperature (18 - 22°C), changing only one of these variables each time, and monitoring variation of the migration times of the analytes. Only small acceptable deviations (RSD < 2%) were observed indicating that the robustness of the method was good.

The applicability of the proposed MEKC method to the assay of fenofibrate in commercial pharmaceutical products was tested and an example of electropherogram is shown in Figure 3.

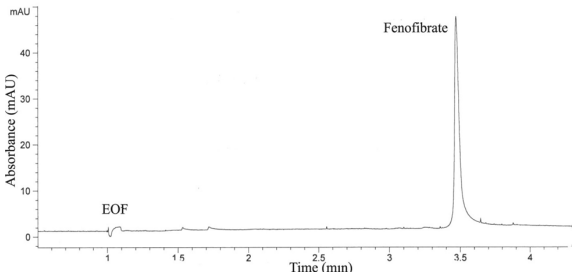

Figure 3.

Sample electropherogram obtained during analysis of fenofibrate from Lipivim® (analytical conditions: 25 mM sodium tetraborate, 25 mM SDS, pH 9.30, +20 kV, 20°C, 50 mbar/1 sec, UV detection 210 nm, 1 mg/mL analyte concentration)

No interference could be observed from the formulation excipients, which demonstrates the selectivity of the proposed method.

Recoveries of the drug from the matrix were in agreement with the labelled content (Table III).

Table III

Assay results of fenofibrate determination in pharmaceutical formulations (n = 6)

Pharmaceutical preparation	Declared amount (mg)	Found amount (mg)	RSD (%)
Lipivim®	200	198.05 ± 1.45	0.85

Conclusions

The developed method is the first report on the simultaneous analysis of four fibrates by MEKC. The good separation with high efficiency achieved in short analysis time is the main advantage of the newly developed method. The proposed new MEKC method provides lower sensitivity compared to that obtained by HPLC, however lower sensitivity is no problem for quantitation of the active ingredients in pharmaceutical preparations. This study highlights the benefits of using universal methods for rapid quantitation of related pharmaceutical substances without the need for development of separate and distinct method for each analyte.

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