Faculty of Pharmacy[1], Ss. Cyril and Methodius University in Skopje, North Macedonia; Department of Inorganic and Analytical Chemistry[2], Medical and Health Science Centre, University of Debrecen, Hungary; Department of Pharmacy[3], Faculty of Medicine, University of Prishtina "Hasan Prishtina", Prishtina, Kosovo

Chemometrics approach for optimization of capillary electrophoretic conditions for the separation of insulin analogues

B. Pajaziti[1], M. Andrási[2], D. Nebija[3,*], B. A. Pajaziti[3], L. Anastasova[1], R. Petkovska[1]

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**Corresponding author: Dashnor Nebija, University of Prishtina "Hasan Prishtina", Faculty of Medicine, Department of Pharmacy, 10000 Prishtina, Kosovo*
dashnor.nebija@uni-pr.edu

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Six insulin analogues with high degree of chemical similarity were separated using Capillary Electrophoresis (CE). In order to improve the method performance, optimization and development the Design of Experiment (DoE) approach has been used and this strategy provided related statistical models. This methodology delivered the information regarding the influence of main factors in the method development and explained the interaction of relevant factors in terms of shortening the analysis time. The response surface methodology (RSM) design employing Central Composite Face Centered (CCF) Design analyzed the effect of the most influencing factors, including background electrolyte pH and concentration, applied voltage and temperature. This study demonstrated that the approach of analyzing the influence of each parameter in the migration behavior of analyzed peptides was capable to assess the best electrophoretic conditions for the separation of insulin analogues.

1. Introduction

The analysis on human insulin and its analogues has been usually performed using numerous analytical methodologies and these peptides has been analyzed in different matrices, including determination of insulin in body fluids for clinical and forensic purposes, and the analysis in pharmaceutical formulations for quality control purposes (Thevis et al. 2005). In order to analyze complex rDNA derived products, such are insulin and its analogues, various analytical methodologies, including immunochemical methods (Webster et al. 1990; Simmons et al. 2019) and chromatographic methods, such as HPLC were used. Owning its performance HPLC has been commonly used as a method of choice for the quality control purposes of pharmaceutical formulations containing insulin and its analogues (Khaksa et al. 1998; Sawicka et al. 2006). In addition, HPCL has been applied as a standard pharmacopeia method for the quality determination of insulin and its related substances and it is routinely used in the quality control setting. Nevertheless, the common application of HPLC in this field potentially leads to substantial contamination of environment with organic solvents (Altria et al. 2006).

On the other hand, CE has already matured as a suitable method for determination of therapeutic peptides and proteins. This methodology is particularly important due to many advantages such as high sensitivity, selectivity, low sample consumption, low amount of buffers needed, shorter analysis time and high resolution (Kunkel et al. 1997; Yomota et al. 1997). These advantages make CE very suitable method for quality control of biological medicines, particularly for qualitative and quantitative analysis of peptide drugs, assessment of their purity and related substances, chiral analysis and the determination of enantiomeric purity (Watson 2007). Nevertheless, as an analytical technique, CE has several limitations, for instance in comparison to HPCL it demonstrates lower sensitivity and more parameters require optimization, it has lower robustness and reproducibility. These disadvantages primarily are caused form the adsorption of charged species to the capillary wall and the presence of Joule heating creating variances in the electroosmotic flow. These disadvantages might be very important in pharmaceutical industry setting where method development, and optimization including its performance are critical issues (Harris 2010).

To improve the separation process, CE coupled with chemometric optimization can provide a complete profile of separation, offering the useful information of factors influencing the separation as well as their interactions. The principle of DoE is arrangement of the experiments at changed combination of factors with the purpose of gaining the maximum information with minimal runs. This process is arranged by changing all the applicable factors simultaneously, according to a planned set of experiments which determine the effect of each factor and their significance (Aboul-Enein and Abdel-Megied 2019; Orlandini et al. 2014; Harnisch et al. 2018).

In order to overcome aforementioned drawbacks of CE, different modelling techniques have been developed with the aim to improve method development and optimization. Since for the development of a CE method more parameters are required to be optimized, the use of conventional technique (one-factor at a time) has proved to be time consuming, and after finishing such experiments the data do not represent the combined effect of many factors. The DoE approach helps to better understand the interactions between different factors is possible and the analysis of smaller number of data is required, which helps the process of method optimization and constructions of statistical models. The optimization and analysis of response of multivariate systems could be determined by RSM (Dutta and Basu 2011). CE permits numerous of these factors to be varied within a programmed sequence. Thereby the instrument can be programmed to use different background electrolytes operating at various voltages and temperatures. These automated services make the procedure of experimental designs in CE method optimization particularly attractive (Riggs et al. 2001). Literature data revealed that for the optimization of a chiral separation of anti-Alzheimer and antifungal drugs by CE full factorial experimental design has been employed (Abdel-Megied et al. 2018). In this study, the influence of two parameters (buffer pH and concentration of cyclodextrins) was studied. The "main effect" of variation of each parameter was calculated for electrophoretic resolution for each drug. In other study, reported in the reference

Table 1: Literature references of CCD designs with CE methods

Studied analytes	Influencing factors	Number of experiments	Software used	References
DL-alanine, DL-aspartic acid, DL-glutamic acid, glycine, g-amino-n-butyric acid (GABA)	Buffer conc., SDS conc., voltage	19 experiments	Modde 5.0 and Codex program	(Wan et al. 2001)
sodium salt of Pentosan Poly-sulfate	Injection pressure, injection time, voltage	20 experiments	Minitab Inc. Release	(Prochazka et al. 2003)
d,l-tryptophan-methylester hydrochloride (TrMe), d,l-tyro-sine-methylester hydrochloride (TyMe) and methyl- -cyclodextrin (Me- CD)	Cyclodextrin concentration, crown-ether concentration and buffer concentration	15 experiments	Nemrod software	(Elek et al. 2005)
zofenopril calcium (ZOF) and hydrochlorothiazide (HCT) in presence of two major impurities	pH, buffer concentration, voltage	20 experiments	Design-Expert® Software	(Fayed et al. 2018)
chondroitin sulfate, dermatan sulfate, and hyaluronic acid	Buffer concentration, pH, applied voltage	20 experiments	MATLAB	(Chindaphan et al. 2019)
lansoprazole and rabeprazole	CD concentration, voltage, temperature	20 experiments	Design Expert 7.0	(Papp et al. 2019)

(Mamani et al. 2006) for the optimization of a CZE method for determination of tetracyclines, a fractional factorial design approach has been employed. The estimated factors were pH, temperature and sodium carbonate concentration.

As noted in the Table 1, the response surfaces are appropriate to be used for the optimization of the CE method development and optimization. This approach implies that the most influencing factors affecting the performance of the technique are recognized. Design methodologies of this type offer a graphical illustration of the data over the planned ranges, and they can be used to choose the optimal performance of the method. Response surfaces can be obtained by employing various designs such as central composites and Box-Behnken design. In Table 1 literature review information using the aforementioned design approaches in CE is presented.

Plackett-Burman experimental design was used for method optimization and robustness for the enantioseparation of dexmedetomidine and separation of mizolastine and its related impurities (Krait and Scriba 2018; Orlandini et al. 2007).

The structural variations of insulin analogues consist in small variation in the amino acid sequence, consequently the CE separation of insulin analogues represents challenging task. At the best of our knowledge, currently no papers address the application of this methodology for the optimization of CE method for the determination of insulin and its analogues. Hence, in this work, we present an investigation of the principal factors affecting migration behavior of insulin and its analogues. Application of response surface methodology for the optimization of a CE method with the aim to improve the separation of peptides with high degree of chemical similarity was used.

2. Investigations, results and discussion

Capillary Zone Electrophoresis (CZE) which is known as a simple and fast technique was employed for the separation of insulin and its analogues. Applying CZE method the migration times were influenced by several factors, which were analyzed with a RSM design called CCF Design. For each product a list of experiments, was performed and the selected factors were changed simultaneously.

The analysis for six insulin analogues was performed using this experimental design. Upon the completion of CE experiments ran in different conditions, the migration time for each drug has been calculated, and the influence of these factors on the migration behavior was studied.

As depicted in Fig. 1, using the coefficient plot the coefficients related to scaled and centered variables were analyzed. As seen from the plot, one factor varies from 0 to 1, in coded unit, while the other factors were kept in averages to investigate the change of response. In all cases the factor with most influence was the voltage; the voltage increase was inversely related to the migration time. The voltage influence was more significant than any other factor, or the combination of the factors (pH and BGE concentration, pH and voltage or BGE concentration and voltage). This can be explained by the fact that the increasing of voltage in CE results a higher field strength, which causes increase of Electro Osmotic Flow (EOF). Consequently, the migration time of analytes will decrease and their electrophoretic mobility will increase. Other influencing factor is pH of BGE, which also has an important effect on the separation time. Changes of the buffer pH affect separation by modifying the charge of the analyte and by changing the

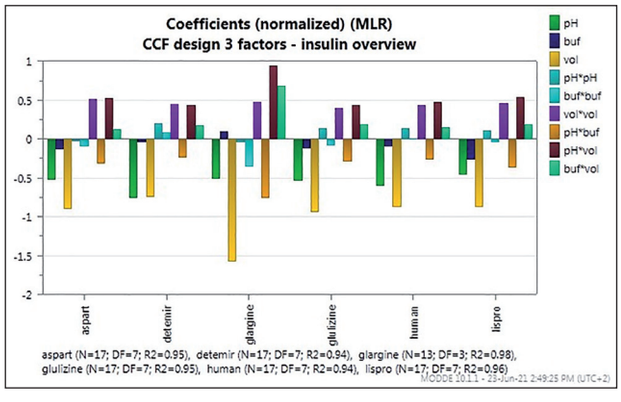

Fig. 1: Coefficient plot of the selected factors (separated and combination of factors) influencing the migration behavior of six insulin analogues. (*pH* = pH of BGE, *buf* = concentration of BGE, *vol* = voltage applied)

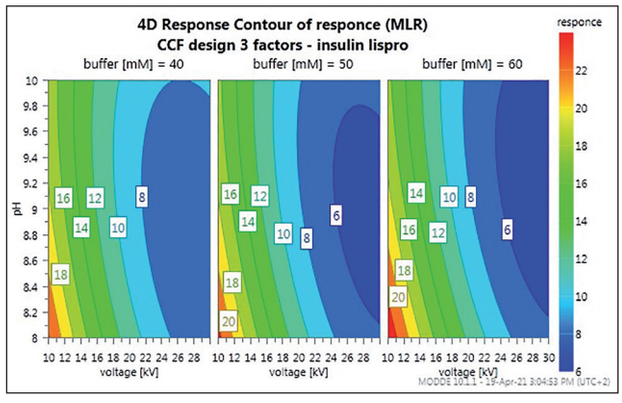

Fig. 2: Response surface plots for insulin lispro. Buffer strength has been kept between 40 mM and 60 mM.

electroosmotic flow. These findings suggest that the pH variation appears to be the most effective method of controlling a CE separation and consequently analysis time

The contour diagrams of response are shown in Fig. 2 where buffer concentration as the factor with the lowest and non-significant influence in response was kept as a fixed value. From the coefficient plots of each insulin analogue, it was assumed that the most influencing and significant factors were the applied voltage and pH of the buffer.

According to the contour diagrams the migration behaviors of each insulin analogue is very similar due to their high degree of chemical similarity. The applied voltage in the electrophoretic system is the factor with the most significant influence in the response. The voltage increase is inversely related to the migration time. Similar effect was noted for the pH of the buffer.

Using the diagrams above, is possible to find the optimal range of the most influential factors including pH and buffer concentration, in terms of analysis time reduction. Considering that, using CE for determining proteins and peptides a long condition time is needed to remove the adsorbed molecules in capillary wall, the proper analysis for this determination would need 8-10 min. Depending of each insulin analogue, the optimal range of selected factors to achieve these preferred analysis time would be: applied voltage 20-25 kV, the buffer pH: 8.5-9.5 for the buffer concentration 40 mM ammonium acetate.

In Fig. 3, the observed vs. predicted plot for insulin lispro is presented. It displays the observed *versus* predicted values of the response. This is additional proof of the good model where the points fall on the 1:1 line very closely.

In Fig. 4, the representative electropherogram of each insulin analogue in the chosen range for optimal conditions are shown.

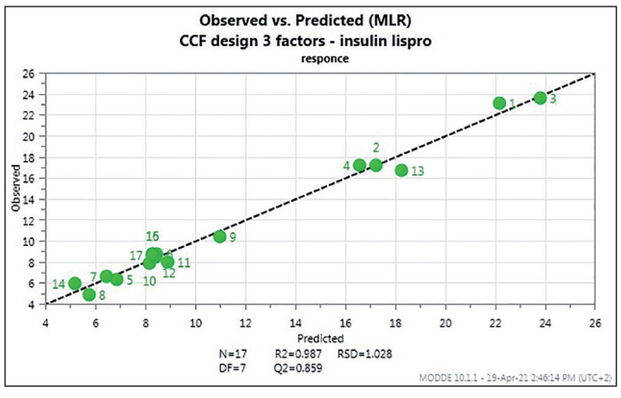

Fig. 3: CCF design 3 factors – insulin lispro.

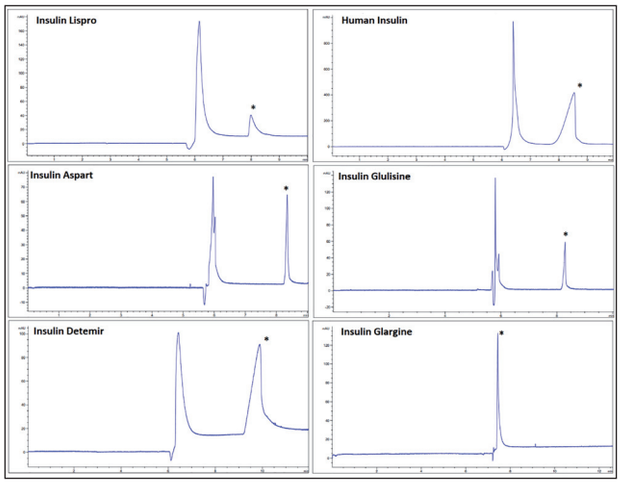

Fig. 4: Representative CE electropherograms of separation of insulin analogues. * = the main compound (insulin). Measurements conditions: BGE: 40mM ammonium acetate, pH 9, voltage 20 kV, temperature 25°C.

3. Experimental

3.1. Chemicals and reagents

Acetic acid, ammonium acetate, ammonia, hydrochloric acid and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents used were of analytical grade. Prior analysis, samples were filtered using the syringe membrane filter. Pharmaceutical formulations containing insulin and analogues - Humulin®R (Lilly) and Humalog® (Lilly), Lantus® SoloStar® (Sanofi), Apidra® SoloStar® (Sanofi), NovoRapid® Penfill® (Novo Nordisk), Levemir® FlexPen® (Novo Nordisk) were used.

3.2. Instrumentation

In this study we used the CE 7100 system (Agilent, Waldbronn, Germany). Electrophoretic separations were carried out using fussed silica capillaries (Polymicro Technology, Phoenix, AZ, USA) with an internal diameter of 50 µm and a total length of 65 cm in positive mode using constant voltage.

Before the first use the capillary was washed with 0.1 M NaOH (15 min), with water (15min) and with the buffer electrolyte (10 min). At the beginning of each working day, the capillary was flushed with 0.1M NaOH for 15 min. For CZE separations between each injection, the capillary was preconditioned for 18 min with 0.1M NaOH, 6 min with acetonitrile and 24 min with the running buffer. This strong preconditioning was used to remove all possible adsorbed materials. The samples were introduced at the cathodic end of the capillary; injections were performed using -50mbar pressure for 4s. Since the proteins tend to adsorb on the inner surface of the capillary, high pH was applied.

3.3. Experimental design

The factors that have the higher influence in the migration behavior of each insulin analogue were the pH and buffer concentration of the background electrolyte and the voltage applied. The traditional method optimization is based on varying one factor at a time, and is called "one-factor-at-a-time", while the other factors are kept constant. Using this technique, a large number of individual experiments is involved and such method is time consuming. Therefore, the effect of these factors individually and their interaction (combination of the factors) were analyzed by RSM design called CCF. This multivariate method delivers optimal conditions with reduced number of experiments. The total number of experiments was calculated by the formula $2^k + 2k + 1$, where k represents the number of factors (Asghar et al. 2014; Mangili et al. 2015). The statistical software MODDE® was used for the DoE.

The levels of factors selected for optimization were the following: BGE pH (8, 9, 10), BGE concentration (40, 50, 60), voltage (10, 20, 30). The number of total experiments calculated for these 3 factors was 15. Nevertheless, the center point was repeated with three different temperature values (15 °C, 22.5 °C and 30 °C) to consider the effect of this factor as well.

Conflict of interests: None declared.

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