CAPILLARY ZONE ELECTROPHORESIS STUDY OF *CIS/TRANS* ISOMERIZATION OF DELAPRIL

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ABSTRACT: The angiotensin-converting enzyme (ACE) inhibitor delapril is shown to exist in two conformational isomers, cis and trans, which interconvert around the amide bond. The two conformers were separated by capillary zone electrophoresis (CZE). The experimental conditions, such as buffer parameters (concentration, pH and nature), voltage, temperature and pH of sample solution were optimized. In order to improve selectivity, different organic modifiers were also investigated. The baseline separation of delapril conformers was accomplished within 6.5 min with a buffer consisting of 100 mM ammonium formate with pH of 3; voltage 25 kV; capillary temperature 15°C and sample pH of 1.6 with detection at 214 nm.

Keywords: Angiotensin-converting enzyme inhibitor; delapril; Capillary Zone Electrophoresis; conformer separation.

RESUME: Le délapril est un inhibiteur de l'enzyme de conversion, qui présente deux isomères de conformation, cis et trans, qui s'interconvertissent autour de la liaison amide. Les deux conformères sont séparés par électrophorèse capillaire de zone ECZ. Nous avons étudié l'effet d'un certain nombre de paramètres à savoir l'effet de la nature du tampon, du pH, de la force ionique, de la température du capillaire, du voltage et du pH de la solution de délapril sur l'évolution de l'équilibre conformationnel. Afin d'améliorer la sélectivité, l'ajout de quelques solvants organiques a été étudié. La séparation des deux conformères du délapril a été accomplie dans 6,5 min environ en utilisant un tampon formiate d'ammonium de concentration 100 mM et de pH 3, un voltage de 25 kV, une température du capillaire de 15°C, un pH de la solution de délapril de 1,6 et une longueur d'onde de détection de 214 nm.

Mots clés : Délapril, inhibiteur de l'enzyme de conversion, Electrophorèse Capillaire de Zone, équilibre conformationnel.

INTRODUCTION

Delapril, (N-[N-(S)-l-ethoxycarbonyl-3-phenylpropyl]-(S)alanyl-N-(indan-2-yl) glycine) (Fig. 1), is an active angiotensin-converting enzyme (ACE) inhibitor which belongs to the class of non-sulfhydryl dipeptides and is marketed as an antihypertensive drug. ACE is responsible for the enzymatic conversion of angiotensin I to angiotensin II and also for the degradation of the depressor hormone bradykinin [1,2].

Delapril is a highly lipophilic carboxyl-alkyl dipeptide with potent ACE inhibitory activity [3]. The molecule also exhibits potential cis/trans isomerism with respect to the conformation around the amide bond. Single-crystal X-ray diffraction analysis was carried out to determine the stereochemistry and conformation of delapril hydrochloride across the amide bond. Simultaneously, NMR was employed for establishing which rotational isomer predominates in solution, as the rate of interchange between the cis and trans forms is slow on the NMR time scale [1].

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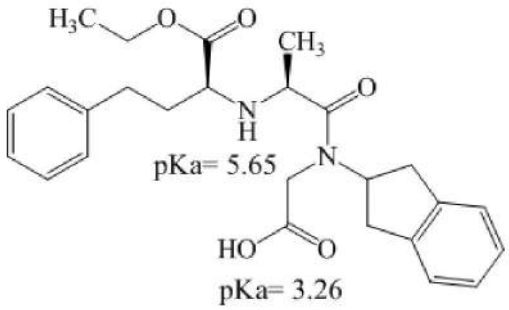

Figure 1: Chemical structure of delapril

Among the separation techniques, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are most often applied in stereoisomer analysis [4].

Capillary electrophoresis (CE) has been used for the identification and quantification of several ACE inhibitors [5-8]. Another study was limited to the determination of only four ACE inhibitors [9]. Other studies have been limited to the determination and rotamer separation of enalapril maleate [10,11], lisinopril [12] and cilazapril [13]. R. Lozano and al. have reported on the determination of fosinopril and its related impurities [14].

CE is a rapidly growing separation technique which has several advantages over other analytical methods, including simplicity, greater separation efficiency, shorter analysis time and lower consumption of reagents [15,16]. CZE is the simplest mode of CE and, therefore, the most widely used. During the past decade, high performance capillary zone electrophoresis (HPCZE) has become an attractive and powerful technique for the analytical separation of peptide [17,18].

The aim of this work is to develop a method for separation of delapril isomers using CZE in order to obtain the best compromise between resolution, analysis time and the quality of separation. A systematic investigation of instrument parameters such as: buffer parameters (concentration, pH and nature), voltage, temperature and sample solution pH were performed. The addition of various organic solvents to the buffer has also been studied to improve separation selectivity.

EXPERIMENTAL

1. Chemicals and reagents

All chemicals and reagents used in this study were of analytical grade. Distilled water is obtained from SIPHAT and used for the preparation of all solutions, sodium hydroxide is obtained from Agilent technologies (Wald-Bronn, Germany), orthophosphoric acid (85%), hydrochloric acid and formic acid were obtained from Prolabo (Fontenay-sous-Bois, France), acetic acid (99%) from Carloerba (Rodano, Italy), and acetone from Labscan (Dublin, Ireland). Ammonium formate, sodium acetate and disodium hydrogenphosphate obtained from Carloerba (Rodano, Italy) and potassium dihydrogenphosphate from Panreac (Barcelona, Espana) are buffers used in the analysis. Methanol and acetonitrile, obtained from Labscan (Dublin, Ireland), are organic modifiers added to the buffer. This study was conducted on a sample of delapril purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Instrumentation and electrophoresis procedure

The study of the cis/ trans isomerization of delapril was conducted with an Agilent, G1600A, capillary zone electrophoresis system equipped with an UV visible detector DAD with variable wavelength (190 to 600 nm), an automatic sampler, a variable pressure system (-50 to 50 mbar), a high voltage generator (0 to 30 kV) and a thermostated cassette of capillary (4 to 60°C).

The separation was performed in 50×50 µm I.D. fused-silica capillary (G1600-60211). The effective separation length up to the detection window was 40 cm and the width of the optical path was 150 µm. The detection wavelength was set at 214 nm.

Between runs the capillary was flushed with 0.1 M NaOH for 5 min, with water for 5 min and finally rinsed with buffer for 5 min.

Electrolytes were filtered through the Millipore 0.45 µm membrane filter before use.

Injection of sample was hydrodynamically performed for 15 s at 50 mbar.

3. Sample solution and electrolyte preparation

Sample solution was prepared by dissolving 5 mg of delapril in water to yield a concentration of 0.5 mg mL^{-1} and then the pH was adjusted to 1.6 (±0.02) with 0.1 M hydrochloric acid.

The running buffer was freshly prepared by dissolving 157.65 mg of ammonium formate in 100 mL of water and the pH was adjusted with formic acid (99%).

RESULTS AND DISCUSSION

1. Selection of the buffer

The electrolyte composition has an important effect on the mobility of the compounds and on electroosmotic flow within the capillary [19]. For the present study, four kinds of buffers namely ammonium formate, potassium dihydrogenphosphate, disodium hydrogenphosphate and sodium acetate were tested and ammonium formate was selected as buffer, because it is one of the most commonly used and easily available in analytical laboratories of CE. Moreover, this buffer presents low absorbance at working wavelength, does not require special preparation. As shown in Fig. 2, ammonium formate is the only buffer that allows separation of cis and trans conformers of delapril.

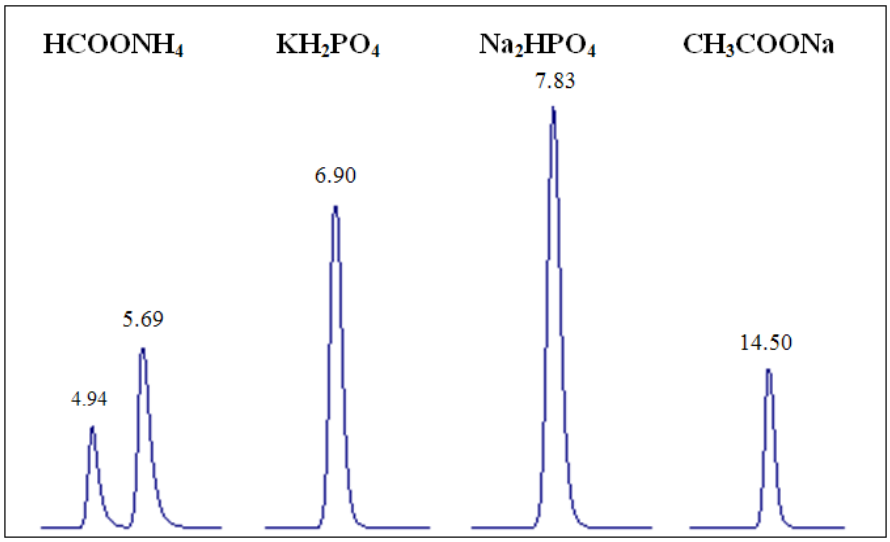

Figure 2: Effect of buffer nature on the conformer separation: electrolyte: 100 mM buffer at pH 3; potential +25 kV; temperature 15°C; sample solution pH 1.62; capillary 50×50 µm I.D.; injection time 15 s and detection wavelength 214 nm.

2. Choice of buffer concentration

It was shown that the buffer concentration influences the viscosity of the solution, the diffusion coefficient of the analytes and the zeta-potential of the inner surface of capillary tube. This affects not only the resolution and migration time of the analytes, but also the peak current [20].

Buffer concentration has also a significant effect on the separation performance through its influence on the electroosmotic flow EOF and the current produced in the capillary [21].

The effect of ammonium formate concentration on the separation was examinated with a concentration range from 25 to 150 mM, keeping other parameters constant, as shown in Fig. 3.

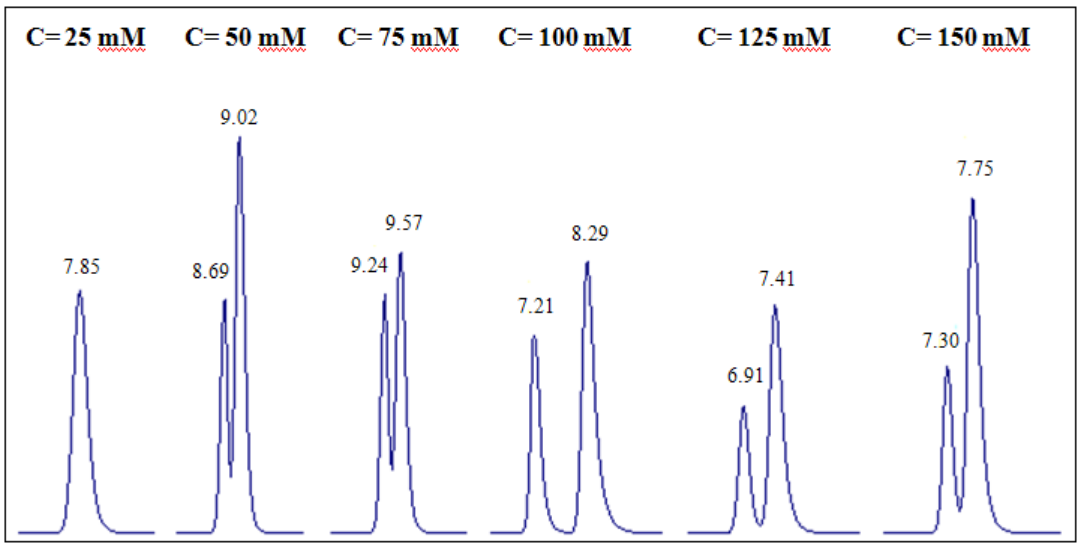

Figure 3: Effect of the buffer concentration on the conformers separation: electrolyte: ammonium formate at pH 3; temperature 15°C; potential +25 kV; sample solution pH 1.62; capillary 50×50 µm I.D.; injection time 15 s and detection wavelength 214 nm.

Figure 3 shows that when the buffer concentration was increased, the separation was improved and more resolved peaks were obtained.

For concentrations up to 100 mM, the resolution increased with ionic strength. Above this concentration, the resolution decreased; a phenomenon that could be attributed to the generation of higher current and Joule heating.

High buffer concentrations are useful in limiting coulombic interactions of solute with the walls by decreasing the effective charge at the wall. Indeed, increased buffer concentration would increase buffering capacity and also may lead to decreased solute-wall interaction. This could cause a decrease both in sample adsorption and migration time.

A concentration of 100 mM was therefore selected as the best buffer concentration.

3. Effect of buffer pH

Manipulation of buffer pH is a key strategy for optimizing the separation of ionizable analytes in CZE because buffer pH determines the extent of the ionization of each analyte and the magnitude of the electroosmotic flow (EOF) [21].

Because of the amphoteric character of ACE inhibitors, their retention is greatly influenced by pH. All ACE inhibitors have an ionisable carboxylic group. With the exception of fosinopril, they also possess a secondary amine in their structure. Depending on the pH of the medium, the inhibitors may be negatively or positively charged. This offers the possibility of using either an acidic or an alkaline running buffer. Most of the ACE inhibitors are esters for which stability problems occur in alkaline medium (above pH 8.5) [5]. Therefore, the measurements were performed at four pH levels (pH 3, 4.45, 6 and 8), covering a large pH range in which no stability problems occur.

The reported pka values for delapril are 3.26 for the carboxylic acid and 5.65 for the amine group [22]. The molecule can be positively or negatively charged or neutral depending on the pH value of the electrolyte (Fig. 4). At pH below 4.45, the molecule is positively charged and migrates to the negative end of the capillary at the detection window. At pH around 4.45, which corresponds to the isoelectric point, the molecule does not have a net charge, and thus the electrophoretic mobility is 0, but it is detected because it is drawn by the osmotic flow. At pH values above 4.45,

the molecule is negatively charged, however, the EOF dominates the electrophoretic mobility and therefore delapril migrates towards the negative end.

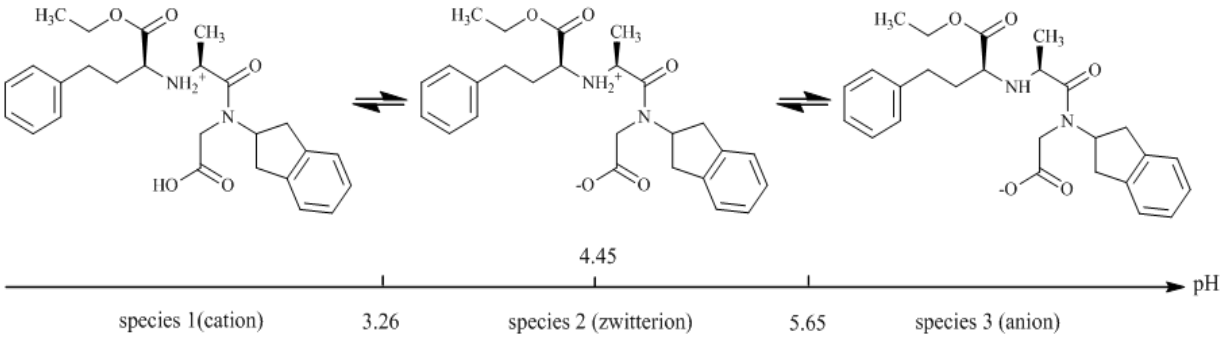

Figure 4: Different species of delapril in function of pH

In this work, the effect of the buffer pH was investigated using 100 mM ammonium formate buffer over the pH range 3-8, as shown in Fig. 5.

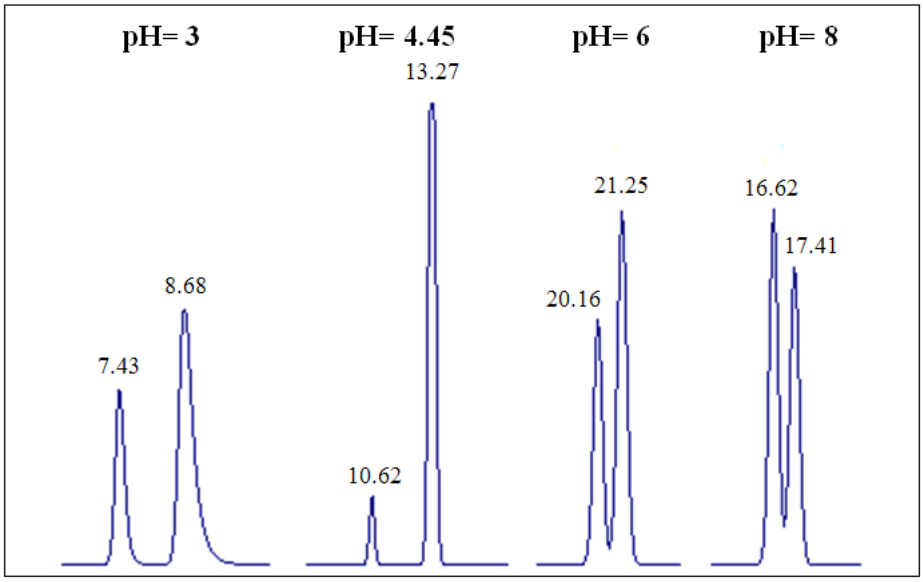

Figure 5: Effect of the buffer pH on the conformers separation: electrolyte: 100 mM ammonium formate; temperature 15°C; potential +25 kV; sample solution pH 1.62; capillary 50×50 µm I.D.; injection time 15 s and detection wavelength 214 nm.

Examination of Fig. 5 shows that resolution and time analysis increased with increasing pH.

At acidic pH, the capillary wall is uncharged. The positively charged molecule does not electrostatically interact with the wall, although hydrophobic interaction may still occur. Therefore, the two conformers are rapidly eluted. At moderate pH (pH above 4), the negatively charged wall can cause adsorption of cationic solute through coulombic interactions, leading to an increase in migration time. At high pH, where the silanol groups are predominantly deprotonated, the EOF is significantly greater than at low pH where they become protonated and may be too rapid resulting in a decrease in the migration time.

Considering the sensitivity, the time of analysis and the resolution, the pH 3 of buffer was found to be optimal and selected to perform the separation of delapril conformers.

4. Influence of the applied potential

The resolution depends strongly on electrophoresis voltage. For instance, high voltage increases EOF and analysis time is therefore reduced. In addition, high voltage results in Joule heating leading to bandbroadening which decreases resolution. On the other hand, very low voltage results in high migration times and bandbroadening due to increased diffusion [23].

Although the most commonly used method when working with CZE is to maintain the voltage constant during analysis, in this study the applied voltage is maintained constant instead, favoring the reproducibility of the migration times [24].

To study the effect of this parameter on the separation of the isomers, the voltage was progressively increased from 10 to 30 kV so as to obtain a good compromise between good separation and analysis time (Fig. 6).

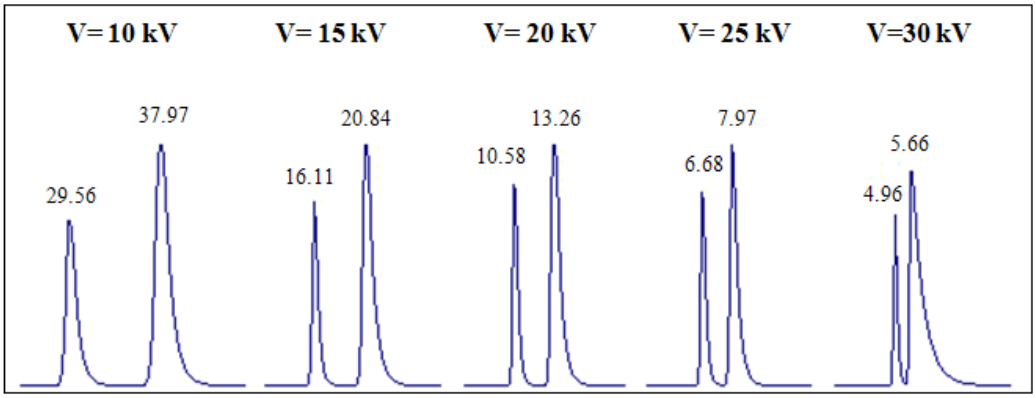

Figure 6: Effect of the voltage on the conformers separation: electrolyte: 100 mM ammonium formate at pH 3; temperature 15°C; sample solution pH 1.62; capillary 50×50 µm I.D.; injection time 15 s and detection wavelength 214 nm.

The analysis time is, as expected, reduced and a voltage of 25 kV which produced a rapid (about 8 min) and complete separation was selected as the best voltage.

5. Effect of capillary temperature

The effect of capillary temperature was studied in the range 5-60°C.

Below 10°C the sample was precipitated in the running buffer and at higher temperatures (above 50°C) the phenomenon of coalescence was observed and the sample was eluted as a single large peak.

In the range 10-45°C, temperature does not affect the mobility of the isomers (Fig. 7).

Therefore, during the analysis the capillary temperature was kept thermostated at 15°C.

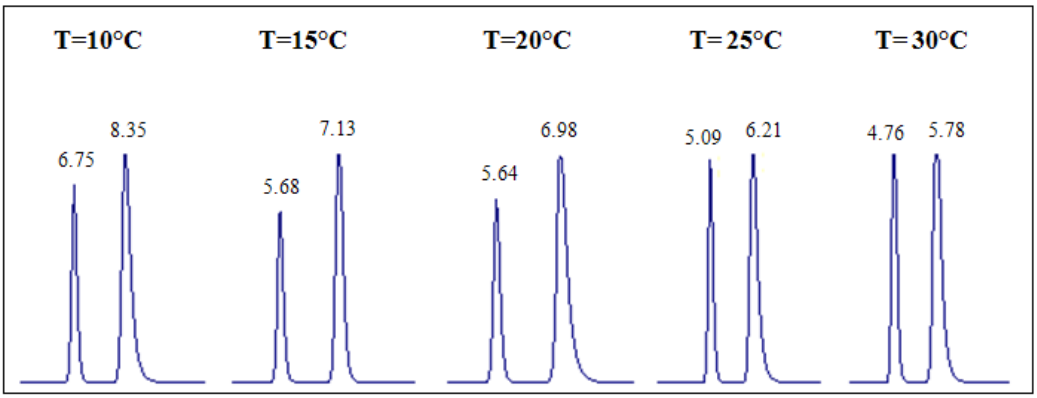

Figure 7: Effect of capillary temperature on the isomers separation: electrolyte: 100 mM ammonium formate at pH 3; potential +25 kV; sample solution pH 1.62; capillary 50×50 µm I.D.; injection time 15 s and detection wavelength 214 nm.

6. Effect of analyte pH

E. Stellwagen and al. have reported on the analysis of the isomeric composition of enalapril using CE. This study shown that a pH of 1.6 allows a complete separation of the two conformers [25].

The effect of the pH of sample solution of delapril was investigated over the pH range 1-3.

The results are depicted in Fig. 8 which shows that a pH of 1.6 produces complete separation.

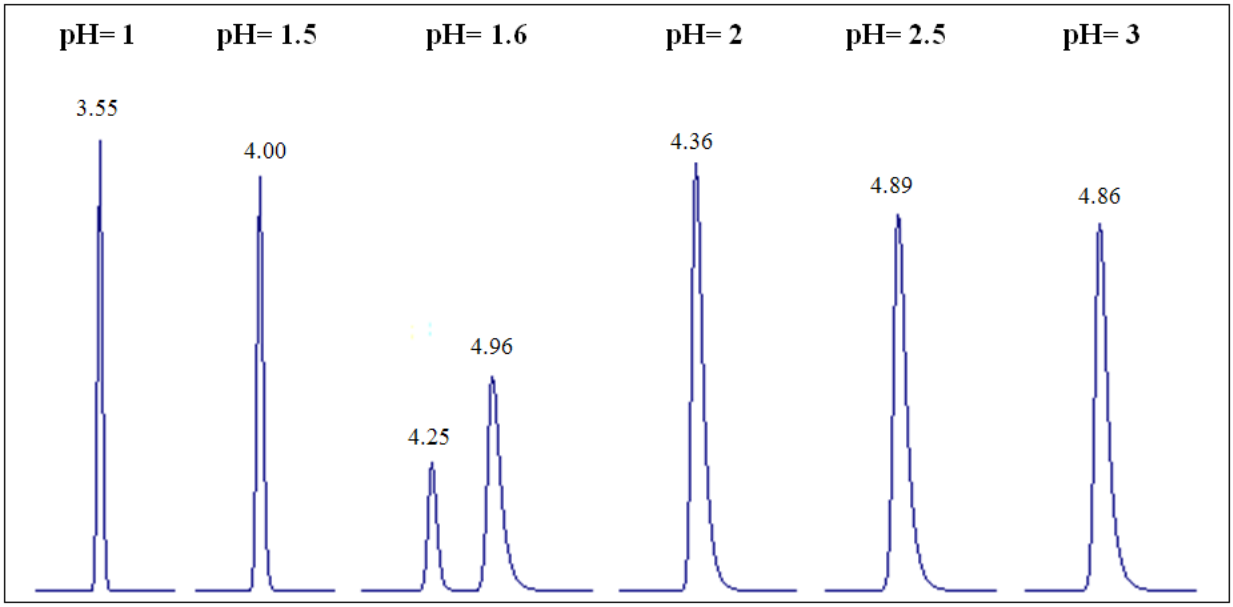

Figure 8: Effect of pH of sample solution on the conformers separation: electrolyte: 100 mM ammonium formate at pH 3; potential +25 kV; temperature 15°C; capillary 50×50 µm I.D.; injection time 15 s and detection wavelength 214 nm.

7. Influence of organic modifiers on selectivity

A variety of modifiers are used in capillary electrophoresis to change the physico-chemical nature of the separation system. Organic solvents added to the buffer electrolyte alter the polarity and viscosity of the mobile phase. As a consequence, both the electroosmotic flow and electrophoretic mobility of the analytes are affected [26].

In CE, methanol, 2-propanol, or acetonitrile are most frequently used. They usually constitute no more than 10-20% of the run buffer volume. Greater percentage leads to slow migration and sometimes to the separation deterioration [27]. In this work, the influence of different organic solvents such as methanol and acetonitrile on the separation of isomers has been studied. For this, various amounts of solvent were added systematically.

7.1. Methanol

Reductions in electroosmotic flow as well as in electrophoretic velocities are caused by viscosity variations of the buffer solutions [28].

Fig. 9 shows the effect of the addition of 5 and 10 % (v/v) methanol to the buffer (100 mM ammonium formate), under the same experiment condition. The addition of organic modifiers had a negative influence on peak symmetry and selectivity was not improved [7]. In this case, addition of methanol does not affect the mobility of isomers.

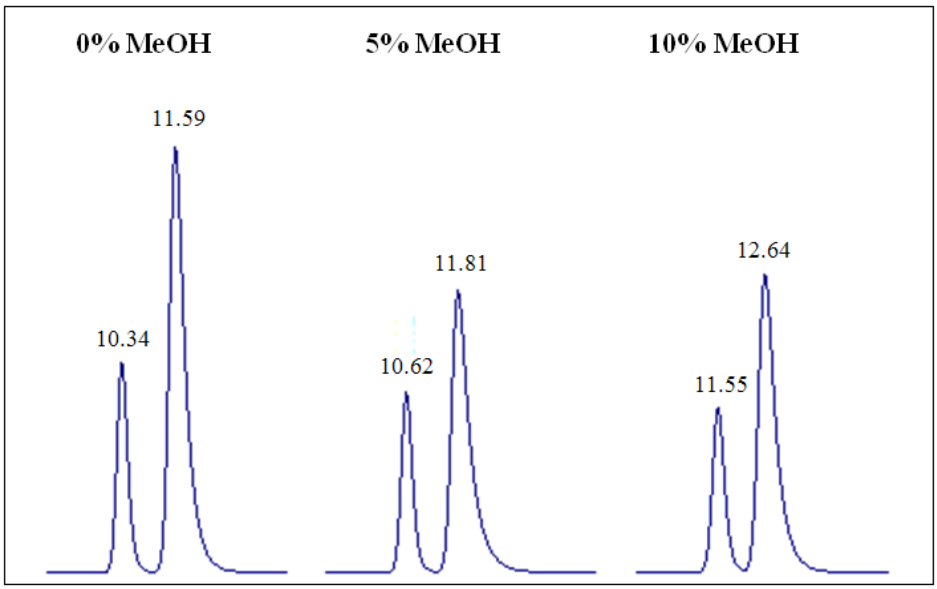

Figure 9: Effect of methanol addition on the isomers separation: electrolyte: 100 mM ammonium formate at pH 3; potential +25 kV; temperature 15°C; sample solution pH 1.62; capillary 50×50 µm I.D.; injection time 15 s and detection wavelength 214 nm.

7.2. Acetonitrile

Acetonitrile is an organic solvent frequently employed to improve selectivity of various electrophoretic systems [29]. With 10% acetonitrile, higher and narrower peaks were obtained and resolution was improved, whereas with greater percentage, migration time was increased and peak shape was deteriorated (see Fig.10).

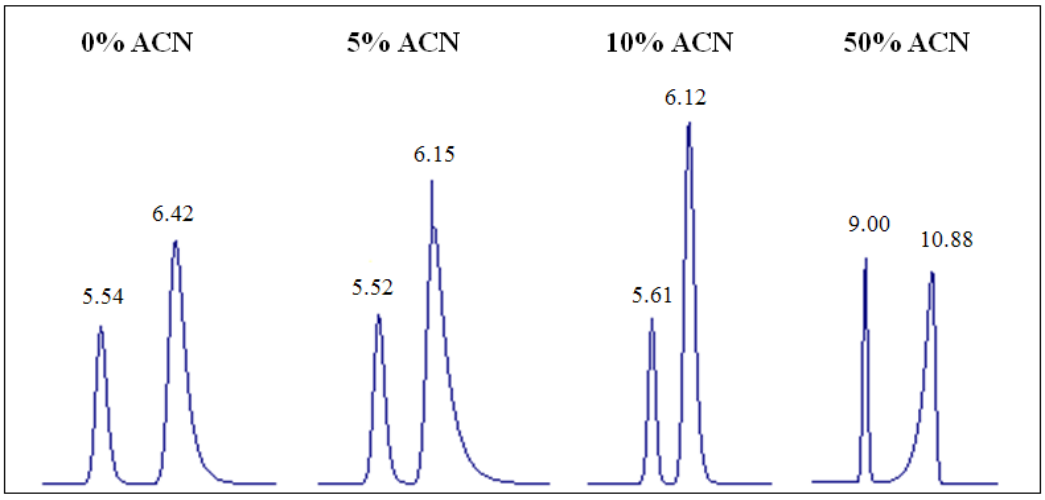

Figure 10: Effect of acetonitrile addition on the isomers separation: electrolyte: 100 mM ammonium formate at pH 3; potential +25 kV; temperature 15°C; sample solution pH 1.62; capillary 50×50 µm I.D.; injection time 15 s and detection wavelength 214 nm.

CONCLUSION

A simple, selective and fast CZE method for the separation of cis and trans conformers of delapril was developed. Successful separation of the two conformers was achieved in a short analysis time (6.5 min) at a temperature of 15°C using an electrolyte composed of acetonitrile and

ammonium formate (pH 3; 100 mM) (10/90 v/v) with sample (pH 1.6) injected at a voltage of 25 kV. The results also show that the use of the electrophoretic system allows a better qualitative analysis with lower consumption of reagents and solvents as compared to the chromatographic system.

It is shown that migration time reproducibility is dependent on the condition of capillary wall, the nature of the sample and quality of instrumentation. Thus, variations in temperature due to changes in ambient or from Joule heating, interaction of the running buffer with the surface (that is, equilibration) can alter EOF but generally do not affect selectivity. Unlike in our case, the use of an internal standard can considerably improve relative migration time reproducibility [30].

The investigation of the effect of other parameters such as capillary length, sample concentration, surfactants, additives and chiral selectors could also be important in such a study and is underway in our laboratory.

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