PALACKÝ UNIVERSITY IN OLOMOUC

Faculty of Science

Department of Analytical Chemistry

CAPILLARY ELECTROPHORESIS SEPARATION OF PHYSIOLOGICALLY ACTIVE COMPOUNDS

Ph.D. THESIS

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I herby declare that this Ph.D. thesis has been written solely by myself. All the sources quoted in this work are listed in the “Reference” sections. All published results included in this work are approved by co-authors.

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SUMMARY

This work mainly deals with application of the capillary electrophoresis (CE) for determination and separation of physiologically active compounds. The principal determinant of methodology choice, mainly in the field of pharmaceutical industry as well as for any clinical purposes, is speed and simplicity of analysis. Thus, preferred approaches include automated methods with high sensitivity and specificity, which is characteristic for CE. Simply, the motto: "time is money" rules.

Capillary electrophoresis was found to be useful as a verification and confirmation tool in the screening analysis for amphetamines, opiates, benzodiazepines, cocaine and their metabolites for toxicological applications.

The other thesis chapter is focused on the determination of drugs belonged to the antiandrogen class (nilutamide and flutamide), employing the micellar electrokinetic chromatography (MEKC). The proposed method allowed their quick separation by addition of sodium dodecyl sulfate to the BGE. Moreover including of on-line preconcentration step to the analytical procedure enabled the determination of drug therapeutic level in serum.

The next electrophoretic approach describes antidiabetic drugs determination (rosiglitazone and metformin) using the hyphenation of CE with mass spectrometry (MS). The utilization of the method is applied on the patient serum.

Generally, CE is a very promising tool for clinical analysis at the present time and it could be a simple and time saving alternative technique to HPLC and GC. The selectivity of CE can be also utilized for separation of complicated mixtures of target analytes from matrix without multistep sample preparation.

Table of content

1 INTRODUCTION

The beginnings of the capillary electrophoresis (CE) in its modern form date back to 1980', when the first demonstration of CE separation was presented by Jorgenson and Lukacs [1]. In the following decades this method was used e.g. for genetic analysis [2], analysis of pharmaceuticals [3], enantiomers [4, 5], protein characterization [6] or carbohydrate analysis for the determination of post translational modifications [7] as well as for analysis of biopolymers [8]. Over the years the CE was developing to enhance the sensitivity, selectivity or efficiency of the method.

Capillary electrophoresis gives broad variety of possibilities how to influence the separation. The separation could be affected by composition of background electrolyte, its concentration, pH, ionic strength, additives – type and concentration, mode of injection, temperature, etc. Important force of the electrophoretic process is the electroosmotic flow (EOF) which could be modified by e.g. addition of ionic liquids or polymers to the background electrolyte (dynamic coating [9]) or covalent modification of the capillary surface. These chemical or physical changes of the inner capillary wall greatly influence the separation, leading to e.g.: better resolution, lower detection limits, or better reproducibility.

Apart from above mentioned applications and advancements the pharmaceutical industry still mostly use the HPLC, however many laboratories increasing the presence of CE instrumentation due to substantial advantages. To predominantly commented (repeated by experts in many publications) belong rapidity of separation and method development, selectivity, high efficiency, low sample and buffer consumption as well as minimal sample pretreatment [10]. Moreover the possibility to choose between different modes of CE is significant and helps to solve many analytical problems in the field of pharmaceutical

industry, such as separation of closely related protein species using the capillary isoelectric focusing (cIEF) [11]. The other is the separation of analytes moving in nearly the same velocity or uncharged molecules, which could be achieved by using the micellar electrokinetic chromatography (MEKC) [12] or microemulsion electrokinetic capillary chromatography (MEEKC) mode [13]. Addition of cationic or anionic surfactants to the background electrolyte alone (MEKC) or with an oil component (MEEKC) enables the separation due to different distribution of analytes between micelles and bulk phase of running electrolyte.

Unfortunately the CE suffers from some disadvantages, which with some effort could be overcome. The experimental problems include the separation of adsorbing compounds, or species that have low solubility in water and similar pK_a values in water based electrolytes as well as the analysis reproducibility. Solutions can, be approached by coating of the capillary wall thus modifying the EOF, employing nonaqueous capillary electrophoresis (NACE) [14].

One case is the separation step but also important is to reach the low enough quantification and detection limits. The lowest LOD and separation efficiencies are usually achieved by using narrow capillaries (5 – 50 μm I.D.); by including the preconcentration step [15] to the method or coupling the CE to sensitive and selective detector such as laser induced fluorescence (LIF) detector [16] or mass spectrometry (MS) [17], which offers additional information about analytes.

2 AIMS OF THE THESIS

Aims of the present thesis were to develop new analytical methods for clinical purposes:

- toxicological applications of the capillary electrophoresis for immunochemical drug screening, used as a verification and confirmation tool for common abused drugs such as amphetamines, opiates, benzodiazepines and cocaine and their metabolites,
- to determine antiandrogens nilutamide and flutamide by micellar electrokinetic chromatography in plasma samples, including the online preconcentration step,
- to study the determination of rosiglitazone and metformin by capillary electrophoresis with electrospray mass spectrometry and to apply the method on plasma samples.

The PhD study was focused on acquaintance and use of capillary electrophoresis with different detection techniques and its application for separation of physiologically active compounds. During study the results were successfully published in impacted scientific journals. The content of presented work is based mainly on the results published in following journals:

J. Znaleziona, V. Maier, V. Ranc, J. Ševčík, Determination of rosiglitazone and metformin in human serum by capillary electrophoresis with electrospray mass spectrometry, J. Sep. Sci. (under revision)

J. Znaleziona, V. Maier, J. Petr, J. Chrastina, J. Ševčík, Separation and determination of antiandrogen drug nilutamide in human serum by high salt sample stacking - micellar electrokinetic chromatography, Chromatographia (under revision).

J. Znaleziona, J. Petr, V. Maier, R. Knob, J. Horáková, D. Smetanová, J. Ševčík, Capillary electrophoresis as a verification tool for immunochemical drug screening. Biomed. Pap. Med. Fac. Univ. Palacký Olomouc Czech Repub. 151 (2007) 31.

J. Znaleziona, J. Petr, R. Knob, V. Maier, J. Ševčík, Dynamic coating agents in capillary electrophoresis. Chromatographia, 67 (2008) S5.

THEORETICAL PART

3 THEORETICAL BACKGROUND OF CAPILLARY ELECTROPHORESIS

3.1 The electroosmotic flow and its modification

Separation in capillary electrophoresis is based on different ions' mobility, being a consequence of differences in the charge-to-size ratio of the ions. Effective ion mobility depends mainly on the degree of analyte dissociation and is described by equation (1):

$$\mu_{eff} = \mu_{app} \cdot \alpha \qquad (1)$$

Where α is the degree of dissociation, μ_{eff} is the effective ion mobility and the μ_{app} is the apparent ion mobility. The effective mobility is equal zero when the analyte is completely undissociated, and maximum when is fully ionized [18]. Thus this parameter is strongly influenced by pH of the electrolyte. The ion mobility also depends on different ion interactions during the analysis, such as: ion - ion interactions, ion - dipole interactions with solvent molecules, ion - solvent interactions or electrostatic interactions [19].

The apparent mobility, μ_{app} of an ion is a function of both the ions effective mobility and the electroosmotic mobility μ_{EOF} The relation between effective mobility, apparent mobility and electroosmotic mobility described equation (2) [20].

$$\mu_{app} = \mu_{eff} + \mu_{EOF} \qquad (2)$$

The EOF origin is connected with the formation of an electric double layer on the contact of the inner surface of the capillary and solution when voltage is applied. Structurally the double layer consists of the stationary (Stern's layer) and diffuse layer and is characterized by the zeta potential, which affects the magnitude and direction of the EOF. The zeta potential

is namely determined by dissociation of silanol groups of the capillary wall (-SiOH, $pK_a \sim 5.3$ [21]), the charged density in Stern layer and the thickness of diffuse layer. The detail description was presented by Stern-Gouy-Chapman model [22].

The electroosmotic mobility can be expressed by the Smoluchowski equation (3):

$$\mu_{EOF} = -\frac{\varepsilon \cdot \zeta}{\eta} \qquad (3)$$

Where ε is the permittivity, η is the dynamic viscosity of the BGE and ζ is the zeta potential. The values deviated from those in bulk liquid, due to the orientation of dipolar molecules near to the charged surface.

To easily determine the EOF mobility the addition of a neutral marker to the sample is needed. The EOF mobility is calculated then from migration time of the marker. In practice the mostly used markers are: dimethyl sulfoxide, methanol, formamide, acrylamide [23]. The ideal EOF marker should be easily miscible with the BGE, gives a high detector response and must not interact with analytes and other BGE's components e.g. micelles or chiral additives. Moreover, the price and impact on the environment have to be also taken under consideration.

Unfortunately the conventional EOF measurement described above, is not suitable for weak EOF (as low as 0.5×10^{-9} $m^2 \cdot V^{-1} \cdot s^{-1}$) due to time-consuming analysis. Therefore, there was a need to develop other approaches for determination of EOF velocity. They include measurement of the change in solution level in the receiving vial [24], measurement of streaming potential [25], on-line measurements using conductivity cell [26] or current-monitoring measurement [27].

Very common is the Williams and Vigh method [28], which is based on the formation of three bands of the neutral marker, in set order, in the capillary and measuring of migration times. Ermakov *et al.* made a statement, that the amount of injected neutral marker is proportional to the EOF velocity [29]. The marker is electrokinetically injected into the

capillary what corresponds to the injection done by EOF, thus the EOF velocity is proportional to the amount of a neutral marker.

Changing the separation parameters such as ionic strength of the background electrolyte, its pH or temperature, the EOF is modified, what influence the separation (Table 3.1). In some cases the separation could be impaired by irreproducible EOF or analytes adsorption on the capillary wall. Cationic analytes have tendency to interact with silanol groups of the capillary wall, thus leading to peak broadening, peak distortion, what decrease the separation efficiency and sensitivity. The adsorption effect can be minimized by the use of low sample concentration, using capillaries with large inner radius, changing the pH and ionic strength of electrolyte or chemical or physical modification of the capillary wall [9].

Table 3.1 Parameters influencing the EOF [19, 30 - 35]

Parameter	**Result**	**Description**
pH	The EOF decreased at low pH, increased at high pH.	Simple method to control EOF.
Ionic strength	The reduction of EOF mobility. Decreases ζ and EOF with increasing buffer concentration.	The double-layer is compressed what leads to the ζ changes. High ionic strength means high current and Joule heating.
Temperature, electrolyte viscosity	Increase of the EOF, shortening the analysis time.	When temperature increases, the viscosity decreases.
Nature of the solvent (dielectric constant, permittivity)	The permittivity affects the magnitude of the ζ, and ionization of the analytes.	Influence of the electrostatic interactions.

Parameter	Result	Description
BGE composition (additives)		
Neutral hydrophilic polymers	Adsorbs to capillary wall via hydrophobic interactions.	Decreases EOF by shielding surface charge, also increases viscosity.
Surfactants	Adsorbs to capillary wall through hydrophobic or ionic interactions.	Cationic surfactants decrease the EOF anionic surfactants can increase it, changes in separation selectivity.
Amines, oligoamines	Adsorbs to capillary wall through hydrophobic or ionic interactions.	Decreases EOF by shielding surface charge.
Ionic liquids	Adsorbs to capillary wall through hydrophobic or ionic interactions.	Can effectively reduce or reverse the EOF in the capillary.
Organic modifiers	Decreases ζ and EOF with increasing modifier concentration.	Complex effects.
Chemical nature of the capillary wall	Chemical change of the inner capillary wall.	Many possibilities. Limitation is the stability coating under the extremely high or low pH.

The change of separation selectivity is briefly described in the chapter devoted the separation of antiandrogens, while the next section is focused on basic theory of micellar elektrokinetic chromatography (MEKC).

3.2 Micellar electrokinetic chromatography

The MEKC method allowed the neutral analytes separation by addition of an ionic surfactant to the BGE, such as cationic cetyltrimethyl ammonium bromide (CTAB) or anionic sodium dodecyl sulfate (SDS) over their critical micellar concentration (CMC). This provides distribution of analytes between micelles and bulk solution thus making the separation

possible. There are known three types of mechanism (1) incorporation of analyte into hydrophobic core (usually highly hydrophobic and nonpolar analytes), (2) interactions of analyte with the surface layer and (3) incorporation of analyte as a cosurfactant. The micelles formation is not only dependent of CMC value but also on aggregation number, micellar size, polarity or microviscosity [36].

The neutral analytes which are incorporated to micelles migrate at the velocity of micelles, while the analytes which stain in bulk solution, migrate at EOF velocity. The migration time t_M of analyte is then given by the equation (4):

$$t_M = \frac{1+k}{1+(t_0 / t_{mc})k} t_0 , \qquad (4)$$

The variable k represents the retention factor. It is defined by the ratio of the analyte amount incorporated into the micelle over that in the aqueous phase. The t_0 and t_{mc} are the migration time of the EOF marker and that of the micelle marker, respectively [37]. The k factor could be easily manipulated by micelles concentration to which is related to, according to $k = KV_{mc}/V_{aq.}$ Where K is the distribution coefficient, V_{mc} and V_{aq} are volume of the micelle and the aqueous phase, respectively. The equation means that k is linearly proportional to the surfactant concentration what was also confirmed by experiments [38].

Mostly used surfactant in the MEKC mode is anionic sodium dodecylsulfate (SDS), which CMC is 8.1 mmol·L^{-1} in pure water at 25 °C [39]. To its advantages belong: high stability, low Krafft point (minimum temperature at which surfactants forms micelles), low UV absorbance and high solubilizing capability. According to its charge SDS migrates toward anode. Under the neutral and basic conditions the EOF is strong enough to forced SDS micelles in direction of cathode, and in this case we have so-called restricted elution mode [36].

The preconcentration step utilized in a study of antiandrogens was high-salt stacking

method. Its mechanism makes use of the MEKC mode attractive for biological samples, which are usually high salt concentrated. This preconcentration approach did not required the time-consuming and labor-intensive procedure, because is the part of the separation step. The accumulation effect is obtained due to the high salt concentration of the sample, higher conductivity, and difference in mobilities of the anion in the sample zone and anionic micelles. The salt concentration manipulates the relative field in the sample zone and in the buffer. According to the Palmer *et al.* [40] the conductivity of the sample should be two or three times greater than the conductivity of the buffer. Besides the anion mobility of the sample has to be higher than the anionic micelles (usually chloride), thus avoid the entrance of the micelles to the sample zone. Palmer *et al.* proposed the stacking as a physical mechanism. The analytes in the sample zone have velocity of the EOF, which is reduced at the analyte/micelles boundary, leading to the accumulation.

Including the preconcentration step to electrophoretic procedure, in many cases, enable the determination of low drugs concentration in biofluids (studies on nilutamide and flutamide).

3.3 Capillary electrophoresis and electrospray ionization mass spectrometry

The development of electromigration techniques and its commercial use is depends on the development of better and more sensitive detector systems. One of the most powerful techniques for analytes detection seems to be mass spectrometry (MS). First hyphenation of

MS with CE was reported in 1987 [41] and from that time the great progress was made. That is greatly described e.g. in the special issue of Electrophoresis [42].

The mass spectrometry detection enables the characterization of complex samples, is independent of presence of chromophore and fluorophore and allows achieving the low detection limits comparing to UV detection. According to my best knowledge, the most widely used ionization technique is electrospray ionization (ESI) allowing the transfer of analytes ions from the liquid phase to the gas phase. The major advantages of it are simplicity and high ionization efficiency.

To obtain the coupling of CE with MS, two major problems had to be overcome. One considered the increase of the liquid flow from the CE part, from $nL \cdot min^{-1}$ to $\mu L \cdot min^{-1}$. The second considered use of volatile components of the background electrolyte to form stable spray in the MS part. There are two main types of interfaces for CE and ESI-MS: sheath-flow (coaxial, liquid junction interface, and pressurized liquid junction interface) and sheathless arrangements [43]. The CE-ESI-MS we are working with, in our laboratory exploits coaxial sheath liquid interface and is presented on the Fig. 3.1.

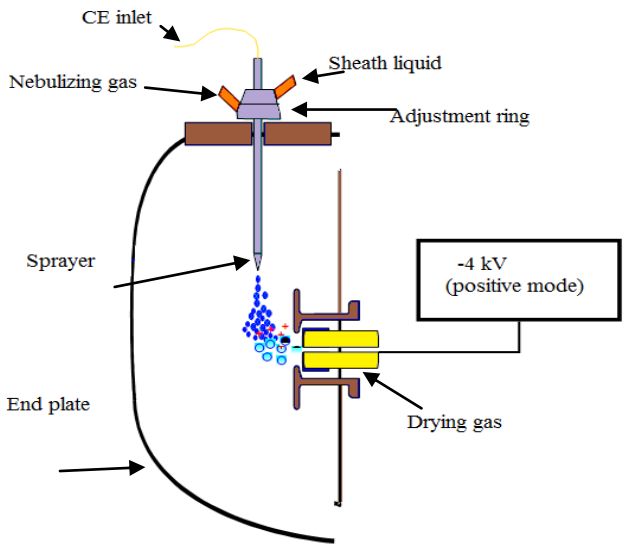


Fig. 3.1 Coaxial sheath-liquid interface CE-ESI-MS [44]

The point of the hyphenation between capillary electrophoresis and mass spectrometric detector is crucial in the system due to its significant effect on the stability and intensity of the signal. Demanding task is the proper cut of the capillary and its adjustment within the sprayer needle as well as the positioning of the sprayer needle with respect to the MS inlet. Problematic is also robustness of the system. Those are evident difficulties, but at the moment, the coaxial sheath liquid coupling is commercially available and relatively easy to implement and use.

Typically the sheath liquid consists of 50 % (v/v) of solvent and appropriate electrolyte solution. Mostly used is formic acid (at low pH) or ammonium acetate (at high pH). Sheath liquid provides electrical contact of the CE with MS and stable ion production. Unfortunately the sheath liquid dilute the samples thus influence the signal sensitivity. Moreover the ions presented in the sheath liquid could be a source of chemical noise.

The ESI technique is the most suitable for compounds that exist as ions in solution or molecules that can be ionized at a certain pH in solution or can associate with small ions like Na^+. Basic analytes are preferably analysed in the positive mode, at low pH, when the acidic compounds under the opposite conditions. Typical ions formed by ESI are presented in the Table 3.2.

Table 3.2 Ions formed by ESI [45]

Analytes	**Positive mode**	**Negative mode**
Non-polar	$[M+H]^+$, $[M+alkali]^+$ if any	$[M-H]^-$, $[M+A]^-$
Medium to high polarity	$[M+H]^+$, $[M+alkali]^+$ *e.g.: exchange* $[M-H_n+alkali_{n+1}]^+$ {*clusters* $[2M+H]^+$, $[2M+alkali]^+$ *adducts* $[M+solv+H]^+$, $[M+solv+alkali]^+$}	$[M-H]^-$, $[M+A]^-$ *e.g.: exchange* $[M-H_n+alkali_{n-1}]^-$ {*clusters* $[2M-H]^-$, *adducts* $[M+solv-H]^-$}
Ionic	C^+, $[C_n+A_{n-1}]^+$	A^-, $[C_{n-1}+A]^-$

*C - cation, A - anion

The signal intensity depends on analyte ability to leave the droplet. This is the effect of analyte specific properties like surface activity, solubility, polarity and solvent properties such as surface tension and ionic strength [46].

Table 3.3 Parameters affecting the ionization efficiency [47]

Solution properties	**Effect on electrospray performance**
Decreasing surface tension	Unstable spray.
Increasing conductivity	Increasing spray current, electric discharge in consequence unstable spray. Decrease signal intensity of analyte, due to competition with other ions.
Decreasing viscosity	Decreasing droplet size, thus increase evaporation from droplet.
Decreasing dielectric constant	Increasing ion escape from the droplet.
Higher content of organic solvent	Reducing the surface tension, easier evaporation form droplet.
pH adjustment, addition of protonation agent	Facilitate protonation of analytes.

The CE-MS combination provides a powerful system for analysis of complex biological mixtures. The separation before mass analysis gives a few advantages. What means: analytes can be concentrated, component identification is simplified and accurate quantification is achieved [48].

EXPERIMENTAL PART

4 IMMUNOCHEMICAL DRUG SCREENING BY CAPILLARY ELECTROPHORESIS

J. Znaleziona, J. Petr, V. Maier, R. Knob, J. Horáková, D. Smetanová, J. Ševčík: Capillary electrophoresis as a verification tool for immunochemical drug screening. Biomed. Pap. Med. Fac. Univ. Palacký Olomouc Czech Repub. 151 (2007), 31-36.

4.1 Introduction

Nowadays the misuse of abuse drugs is a wide spread problem being a subject for clinical toxicology, forensic science, drug testing at the work place or doping control. The aim of screening tests is to reveal which drug or combination of drugs the patient/employee/sportsmen may have use, and thus to apply the proper treatment or to call to account. Screenings are usually done by using the speed and easy immunochemical approaches available in a kit form [49, 50], Fig. 4.1. Most immunoassays are competitive binding assays in which drug and labeled drug molecules competitively interact with the binding sites of an antibody raised against the drug [51]. These simple tests allow determination of common drugs in small sample volumes (saliva, urine, plasma, sweat etc.) and can be used directly on-site. On the other hand, in some cases they lack specificity and sensitivity, besides they are unsuitable for simultaneous monitoring of several drugs and metabolites. Thus, positive results of immunoassay demand confirmation by a second independent analytical method. Techniques include chromatographic methods such as GC-MS, HPLC-MS, HPTLC as well as CE, techniques which offer high level of specificity and selectivity [52 - 58].

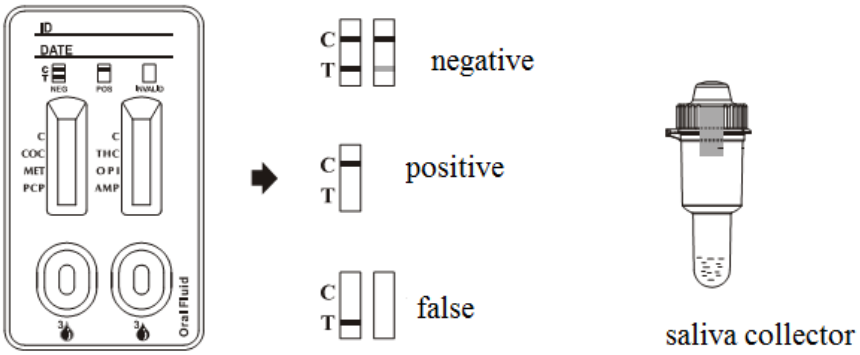


Fig. 4.1 An example of immunoassay test kit [59]

A few studies on toxicological screening and monitoring of abused drugs by CE methods have been published e.g. [52, 60 - 65]. These papers deal with the sample preparation according to different matrices (urine, blood, plasma, and saliva), analyte character, and ability of CE method to screen various types of drugs. Unfortunately the majority of them are focused only on one type of drug (e.g. opiates, amphetamines, benzodiazepines).

In this chapter the simple capillary electrophoretic method was proposed as the verification tool for screening of amphetamines, opiates, benzodiazepines, cocaine and their metabolites. On the Fig. 4.2, chemical structures of studied drugs are presented. The developed method was applied on samples of saliva and urine, collected from addicts of Olomouc region from the Czech Republic. Moreover the results were compared with the multicomponent immunochemical test used for toxicological screening and with the HPTLC method.

The studied analytes could be divided on three groups: stimulants (amphetamine, metamphetamine, and cocaine), opiates (morphine, codeine, ethylmorphine, and diacetylmorphine) and hypnothics (7-aminoflunitrazepam, diazepam, flunitrazepam). Whereas benzoyleckognine is metabolite of cocaine (formed from hydrolysis) and is

a specific marker for the presence of that drug in urine [66]. The 6-monoacetyl-morphine is an active metabolite of diacetyl morphine (heroine) but its presence in urine may be caused also by an intake of drugs containing morphine, ethyl morphine, or codeine [67]. The last analyte from the examined compounds is 2-aminochlor-benzophenone, which is a starting material namely for the manufacture of hypnotic drugs [68].

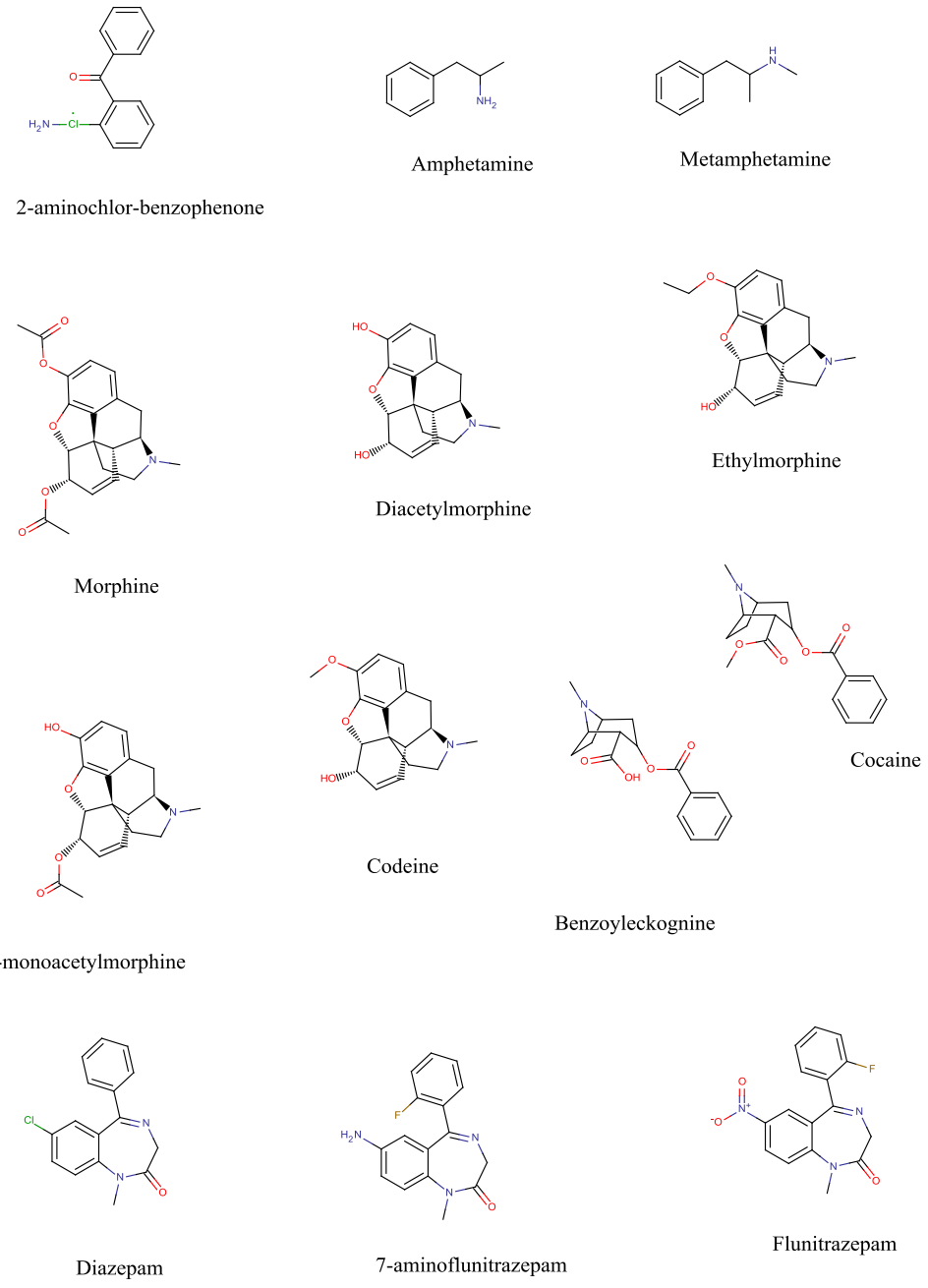


Fig. 4.2 Chemical structures of studied analytes

The method development was based on the production of acceptable peak shape, analyte recognition and analysis time.

4.2 Experimental part

4.2.1 Chemicals

Phosphoric acid, tris(hydroxymethyl)aminomethane (Tris), sodium hydroxide, methanol and ethyl acetate were obtained from Sigma (St. Louis, USA). All of chemicals were analytical grade quality. Drugs and its main metabolites were kindly provided from the Institute of Forensic Medicine and Medical Law, Palacký University, Olomouc, Czech Republic. Biological samples were collected from drug addicts (narcomaniacs) in cooperation with the Drop-in Centre Olomouc. Deionised water (Elga, Bucks, England, 18 $M\Omega \cdot cm^{-1}$) was used for preparation of all solutions.

4.2.2 Instrumentation

The separations were performed on the capillary electrophoresis Spectra PHORESIS 100 with fast-scanning UV/VIS detector SpectraFOCUS (Thermo Separation Products). Uncoated fused silica capillaries (CACO-Silica Tubing and Optical Fibbers, Slovakia) of total and effective length of 75 cm and 45 cm, respectively, with I.D. 75 μm. The capillary was thermostated at 25 °C, the applied voltage was + 30 kV (+ 400 $V \cdot cm^{-1}$). The daily washing procedure include flushing with 0.1 $mol \cdot L^{-1}$ NaOH solution for 10 min, then with water (10 min); 0.1 $mol \cdot L^{-1}$ HCl (10 min); again with water (10 min); and finally with the working

electrolyte for 15 min. The capillary was washed with BGE between individual analysing runs. All measurements were performed five times, if not stated otherwise.

Statistical evaluations were done using the data analysis programme QC Expert 2.5 (TriloByte Statistical Software, Pardubice, Czech Republic). The background electrolyte was prepared by diluting an appropriate amount of phosphoric acid in deionised water and then the solution was titrated with Tris to pH 2.0, finally methanol was added to the background electrolyte. Drugs and their metabolites were dissolved in a ten times diluted background electrolyte. Samples were injected for 0.5 s by vacuum.

4.2.3 Screening assays

As was mentioned above the results obtained by CE were compared with the multicomponent immunochemical QuikScreen^{TM} tests (donated by Exbio, Olomouc, Czech Republic) and HPTLC analysis. The test was utilized for amphetamine (sensitivity 1.0 $\mu g \cdot mL^{-1}$), benzodiazepines (0.3 $\mu g \cdot mL^{-1}$), methamphetamine (1.0 $\mu g \cdot mL^{-1}$), and opiates/morphine (2.0 $\mu g \cdot mL^{-1}$). For HPTLC experiments the Merck plates 60 F-254 (Merck, Darmstadt, Germany) and mobile phase composed by ethylacetate - methanol - ammonium 34 : 4 : 2 (v/v/v) were used. Firstly the sample was filtrated; then alkalized with 50 % (w/v) NaOH to pH 9 - 10. The next step was 3 grades extraction with diethylether; follow by drying with sodium sulfate and acidification with HCl to a pH 3 - 4. Finally the extract was evaporated under gentle stream of nitrogen.

4.2.4 Sample preparation

The real samples of urine and saliva were collected from addicts and prepared according to the extraction procedures presented below.

Extraction of opiates: The sample was adjusted to pH 9 - 10 with concentrated NH_4OH and then shaken for 3 min with a mixture of chloroform and isopropyl alcohol (4:1, v/v). Then the organic phase was dried with anhydrous sodium sulphate. The next step was addition of 0.1 $mol \cdot L^{-1}$ HCl in isopropyl alcohol and evaporation under a stream of the nitrogen at 25 °C.

Extraction of amphetamines and benzodiazepines*:* The sample was adjusted with 1 $mol \cdot L^{-1}$ NaOH to pH 9 - 10 and then shaken for 3 min with ethyl acetate. After extraction the remaining organic layer was shaken with 0.1 $mol \cdot L^{-1}$ HCl for 2 min and the water layer was separated and alkalized with 20 % (w/v) Na_2CO_3. Then water phase was reextracted with ethyl acetate and the organic extract was dried with anhydrous sodium sulphate. In the next step the 0.1 $mol \cdot L^{-1}$ HCl in isopropyl alcohol was added to the organic phase and then the sample was evaporated in a gentle stream of nitrogen at 25 °C.

4.3 Results and discussion

4.3.2 Separation conditions

All of the studied drugs are positively charged in the acidic background electrolytes. Therefore a low pH BGE was taken under consideration. Strong acidic conditions ensure analytes protonization and suppress the EOF. The first experiment was conducted with 50 mmol·L^{-1} phosphate/Tris pH 2.0 electrolyte, what led to the excellent separation of methamphetamine, amphetamine, cocaine, benzoyleckognine and both benzodiazepines from their metabolites. However the separation of opiates and cocaine was not satisfying.

To influence the separation a few BGE additives were tested. Native cyclodextrins (α -, β -, γ - cyclodextrin) were examined as modifiers for the selectivity. The addition of α - cyclodextrin gave the best baseline separation of five morphine analogues. The best concentration of α - cyclodextrin was settled on 15 mmol·L^{-1} in 50 mmol·L^{-1} phosphate/Tris pH 2.0. Unfortunately, separation of 7-aminoflunitrazepam and cocaine under these conditions was lost.

Second group of additives is made of solvents: methanol and acetonitrile. Methanol as opposed to acetonitrile, at concentration of 30 % (v/v) enabled the separation of all studied drugs (Fig. 4.2). As is depicted on the Fig. 4.3 the three group of analytes could be distinguish, what is in agreement with the initial division of studied drugs: stimulants (peaks 1, 2, 3), opiates (peaks 4, 5, 6, 8) together with active metabolite of diacetylmorphine (peak 7) and hypnotics (peaks 9, 10, 12) together with 2-aminochlor-benzophenone (peak 13). The proposed electrophoretic system is first of all preferable for the hypnotics and their precursor as well as for cocaine and its metabolite (peaks 3, 11). The compounds could be then quick and easily separated with good resolution. Their identification was based on migration times

and characteristic UV spectra, what is presented for real sample analyses. Moreover the results show good intraday repeatability of migration time, which not exceeded 1.3 %.

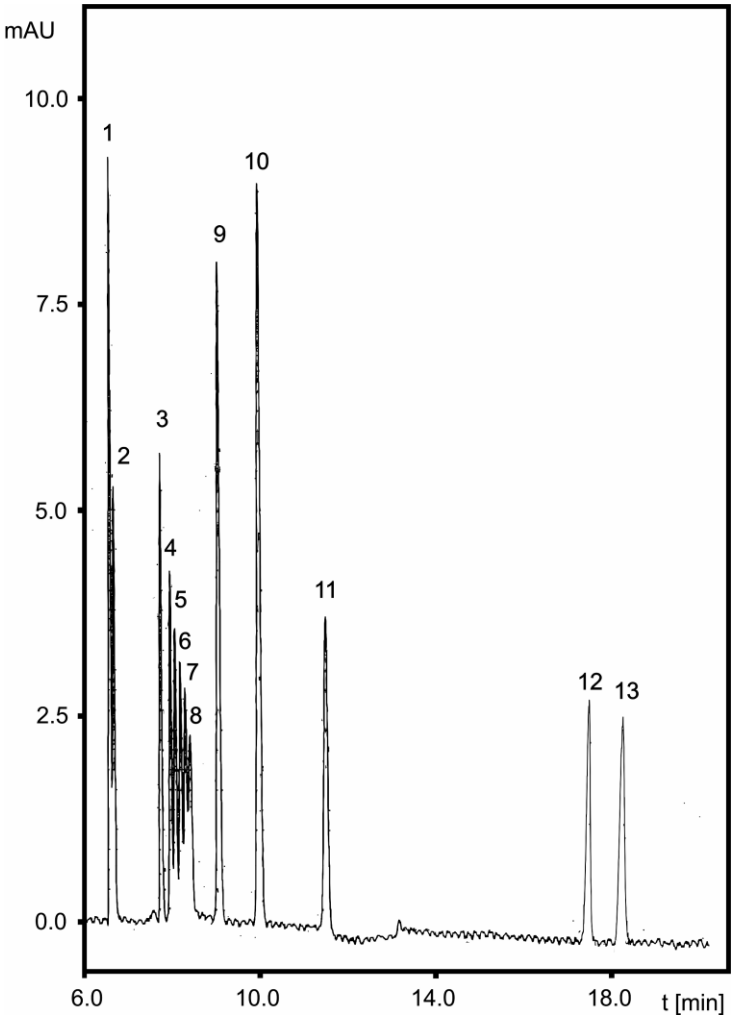


Fig. 4.3 Electropherogram of standard mixture of groups of abused drugs

1 - amphetamine (AMP), 2 - methamphetamine (MET), 3 - cocaine, 4 - codeine, 5 - morphine (MOR), 6 - ethylmorphine (ETM), 7 - 6-monoacetyl-morphine (MAM), 8 - Diacetylmorphine (DAM), 9 - 7-aminoflunitrazepam (7-AF), 10 - diazepam, 11 - benzoyleckognine, 12 - flunitrazepam, 13 - 2-aminochlor-benzophenone

The partial validation of the proposed screening method included repeatability, limits of detection and calibration parameters are presented in Table 4.1. The correlation coefficients varied from 0.978 to 0.998. The precision of peak areas (RSD) did not exceed 3.5 % for intraday assays and 18.6 % for interday assays.

Table 4.1 Partial method validation

Compound	Repeatability of migration times		Tested calibration range ($\mu g \cdot mL^{-1}$)	LOD ($\mu g \cdot mL^{-1}$)
	Intraday, RSD (%)	Interday, RSD (%)		
AMP (1)	0.2	7.3	2.0 - 100.0	1.5
MET (2)	0.3	8.1	2.0 - 100.0	1.6
cocaine (3)	0.5	8.3	5.0 - 100.0	2.1
codeine (4)	0.8	11.7	5.0 - 50.0	2.4
MOR (5)	1.2	11.8	5.0 - 50.0	2.5
ETM (6)	1.2	12.3	5.0 - 50.0	2.5
MAM (7)	1.3	11.5	5.0 - 50.0	2.8
DAM (8)	1.3	13.2	5.0 - 50.0	2.8
7-AF (9)	0.8	11.7	5.0 - 100.0	1.6
diazepam (10)	0.7	12.5	5.0 - 100.0	1.4
BLN (11)	0.9	10.8	5.0 - 100.0	1.6
FNAP (12)	0.9	11.4	5.0 - 100.0	1.8
ACBP (13)	1.0	12.9	5.0 - 100.0	1.9

Intraday precision for 3 days; average RSD values for intraday precision are shown; linear calibrations were made with 6 points (each with 5 repetitions).

AMP - amphetamine; MET - methamphetamine; MOR - morphine; ETM - ethylmorphine; MAM - 6-monoacetyl-morphine; DAM - diacetylmorphine; 7-AF - 7-aminoflunitrazepam; BLN - benzoyleckognine; FNAP - flunitrazepam; ACBP - 2-aminochlor-benzophenone.

4.3.3 Real sample analysis

The analyses of real samples contained saliva and urine matrices and were carried out in 50 mmol·L^{-1} phosphate/Tris pH 2.0 with 30 % (v/v) of methanol. The saliva and urine extracts were checked on presence of amphetamines and morphines. The Fig. 4.4 presents the electropherogram of saliva extract from volunteer and urine extract of addict person. It seems that saliva is suitable sample for monitoring drug abuse due to its relatively simple matrix comparing to urine extracts and easy for sampling.

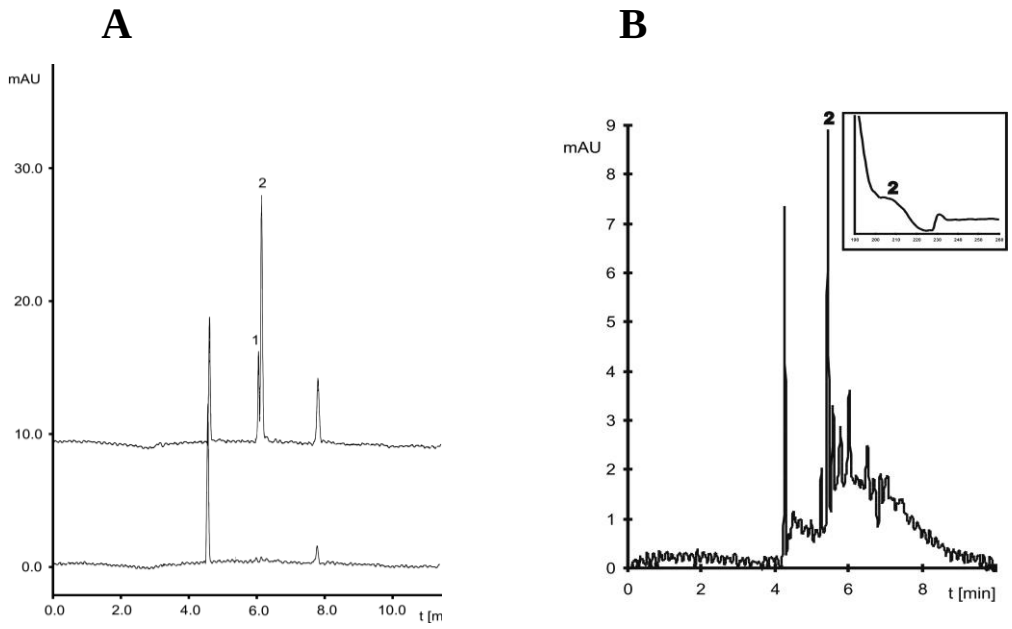


Fig. 4.4 The A electropherogram: saliva extract from volunteer after methamphetamine dosage. 1 - amphetamine, 2 - methamphetamine. Top electropherogram: sample after 24 hours after dosage, lower electropherogram corresponds to sample immediately after dosage.

The B electropherogram: urine extract from drug addict with methamphetamine (6 hours after dosage). 2 - methamphetamine (MET).

In urines samples the presence of morphine and its metabolites were confirmed. One of electropherogram (Fig. 4.5) presented results obtained from volunteer, when the other from drug addicted.

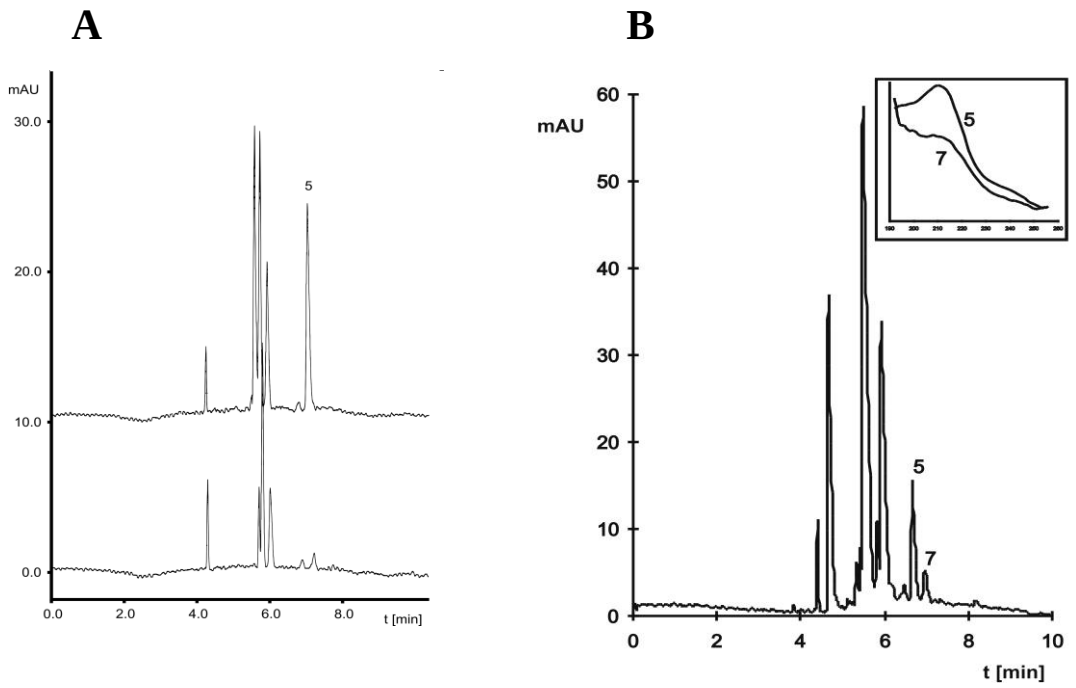


Fig. 4.5 The A electropherograms: urine extract from volunteer with morphine, (upper part: 6 hours after dosage, lower corresponds to sample immediately after dosage).

The B electropherogram: urine extract from drug addict with morphine (48 hours after dosage). 5 - morphine (MOR), 7 - 6-monoacetylmorphine.

The CE method was compared with the HPTLC method and the multicomponent immunochemical screening test QuikScreen^{TM} on a set of samples from teenage drug abusers of the Olomouc region, Czech Republic. The results are listed in Table 4.2. Results obtained by CE and HPTLC demonstrate 99.8 % agreement and those from CE and immunochemical analysis exhibited 95.0 % correspondence.

Table 4.2 Results from immunochemical tests and CE and HPTLC methods

Sample No.	Immunochemical tests*				HPTLC**	CE**
	AMP	MET	OPI	BZD		
1	pos/neg	pos	neg	pos	MET, 7-AF	MET, 7-AF
2	neg	neg	pos	neg	ETM, MOR	ETM, MOR
3	pos	pos	neg	neg	MET, AMP	MET, AMP
4	neg	neg	pos	pos	MOR, 7-AF	MOR, 7-AF
5	neg	neg	neg	pos	7-AF	7-AF
6	pos	pos	pos	neg	MET, AMP, MOR	MET, AMP, MOR
7	pos/neg	pos	neg	neg	MET	MET
8	neg	neg	pos	neg	MOR	MOR
9	neg	neg	neg	pos	7-AF	7-AF
10	pos	pos	neg	neg	MET, AMP	MET, AMP
11	neg	neg	pos	pos	ETM, MOR, 7-AF	ETM, MOR, 7-AF
12	pos	pos	neg	neg	MET, AMP	MET, AMP

*AMP - amphetamines; MET - methamphetamines; OPI - opiates; BZD - benzodiazepines; pos - the test was positive; neg - the test was negative

**MET - methamphetamine; AMP - amphetamine; MOR - morphine; ETM - ethylmorphine; 7-AF - 7-aminoflunitrazepam

4.4 Conclusion

The proposed electrophoretic approach for screening opiates, stimulant and hypnotics is relatively quick (most of the studied analytes, apart from flunitrazepam and 2-aminochlor-benzophenone, were detected within 12 min.) and simple analytical method. It allows recognition of the mostly used abused drugs (amphetamines, opiates, benzodiazepines and cocaine) with good results. The advantages include simplicity with relatively high selectivity for separation and identification of abused drugs and low cost. That is why the CE has a potential to become verification tool for immunochemical screenings and HPTLC screening especially that results are with a good agreement more than 95 %.

5 ANTIANDROGENS SEPARATION

J. Znaleziona, V. Maier, J. Petr, J. Chrastina, J. Ševčík: Separation and determination of antiandrogen drugs flutamide and nilutamide in human serum by high salt sample stacking - micellar electrokinetic chromatography. Chromatographia – under revision.

5.1 Introduction

Nilutamide and flutamide belong to the group of nonsteroidal antiandrogens utilized in a treatment of prostate cancer. They limited the development of disease by blocking the supply of testosterone [69]. They could be also used as supporters in other hormone therapy like hirsutism in women [70, 71]. Furthermore flutamide was reported as a drug for topical treatment of androgenetic alopecia [72].

Both drugs show similar therapeutic effects and chemical structures (Fig. 5.1), their separation could be essential analytical need for pharmaceutical and biomedical purposes (monitoring of drugs levels in body fluids).

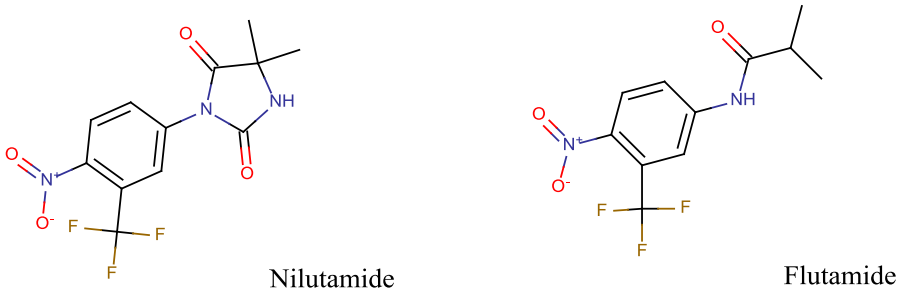


Fig. 5.1 Drugs structures

Nilutamide

5,5-dimethyl-3-[4-nitro-3-(trifluoromethyl)phenyl]-2,4-imidazolidinedione, is a micro-crystalline, white powder. It is freely soluble in ethyl acetate, acetone, chloroform, ethyl alcohol, dichloromethane, and methanol. It is slightly soluble in water [< 0.1 % w/v at 25 °C]. It melts between 153 °C and 156 °C [73].

Flutamide

2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl] propanamide. It is pale, yellow, crystalline powder, practically insoluble in water, freely soluble in acetone and in alcohol. It melts at about 112 °C [73].

A few analytical techniques were employed for determination of studied drugs both in biofluids and pharmaceutical formulations. Most of the papers are focused on flutamide, on its pharmacokinetics, separation, determination procedure, whereas for nilutamide reports deal mainly with drug pharmacology [74] or its medical potential [75].

Tzanavaras *et al.* [76] proposed the direct measurement of the absorbance of the flutamide at 310 nm under flow conditions, using the HPLC as a reference method. The broad polarographic study was presented by Snycerski and was focused on the direct current polarography (DCP), alternating current polarography (ACP), normal pulse polarography (NPP) and differential pulse polarography (DPP) [77]. Other electroanalysis method was proposed by Hammam *et al.* [78], who reported three adsorptive cathodic stripping voltammetric procedures for determination of flutamide in bulk, tablets, and human plasma. Flutamide was also determined by spectrophotometry [79 - 87] which is simple and cost-effective but time consuming method. The chromatographic techniques such as gas

chromatography (GC) [88], or high performance liquid chromatography (HPLC) [89 - 93] were reported, too.

The pharmacokinetics study showed that unchanged nilutamide represents the major active compound [94]. In patients, it has a long half-life of 56 hours (range from 23 to 87 hours). The drug is quickly and completely absorbed and present in high and persistent concentrations in plasma. The mean steady-state plasma concentration of nilutamide in patients, after repeated dose treatment, is 6 - 7 $mg \cdot L^{-1}$ for 300 mg daily dose and 3 - 4 $mg \cdot L^{-1}$ for 150 mg daily dose [74]. Flutamide interaction with androgen receptor in vivo is shorter then for nilutamide [95]. Thus, the lower concentrations of that drug in patients' plasma were found, what means 112.7 $\mu g \cdot L^{-1}$ for steady state doses of 250 mg per day [96].

5.2 Experimental part

5.2.1 Instrumentation

Experiments were performed on a CE instrument (HP ^{3D}CE, Agilent Technologies, Waldbronn, Germany) equipped with diode array detector. Detection was conducted at 200 nm, 275 nm and 300 nm. Uncoated fused-silica capillaries (MicroSolv Technology Corporation, NJ, USA) of 50 μm I.D., 40.0 cm effective length, 48.5 cm total length were used. The capillary was rinsed 20 min with 0.1 $mol \cdot L^{-1}$ sodium hydroxide, 15 min with deionized water and then 5 min with running electrolyte at the beginning of each working day.

The washing procedure should be carefully selected because of observed shift of the migration time of analytes. The capillary was rinsed between individual runs with background electrolyte (BGE) for 5 min. Moreover the vials with running buffer were changed every

10 runs. Injection was performed by pressure 50 mbar for 5 s. The applied voltage was + 20 kV for all experiments. During the measurements the capillary was thermostated at 25 °C. All of the measurements were performed five times unless stated otherwise.

5.2.2 Electrolytes and sample preparation

Running electrolytes were prepared by dissolving of appropriate amount of boric acid or phosphoric acid in deionized water (18 MΩ cm, Elga Bucks, UK) and titrating to desired pH by 50 % (w/v) sodium hydroxide solution. The adequate amount of SDS was added at the end, and the solution was sonicated for 15 min at ambient temperature. The stock standard solutions of flutamide and nilutamide were prepared at 10 $mg \cdot mL^{-1}$ concentration in methanol and then mixed and diluted to the appropriate lower concentration by deionized water. All solutions were stored in the refrigerator at -20 °C.

Human blood was obtained from healthy volunteers. To obtain blood sera for the next experiments the centrifugation at 5000 × g for 15 min was applied. A blood serum, spiked with standards, was mixed with extraction solvent, acetonitrile in the content 1:1. After 10 min centrifugation at 3000 × g, the supernatant was transferred to a plastic conical tube and dried in a gentle stream of nitrogen. The dried serum samples were reconstituted with 200 μL of 150 $mmol \cdot L^{-1}$ NaCl in deionized water and directly injected to the capillary. Using the same procedure the blank serum samples were prepared, but instead of addition of standard the appropriate volume of deionized water was added.

5.3 Results and discussion

5.3.1 Capillary zone electrophoresis - brief study of acidobasic properties

The dissociation equilibriums (pK_a values) of studied antiandrogens are not reported in the literature. However Alvarez-Lueje *et al.* determined the pK_a for flutamide but in the mixture of ethanol : Britton-Robinson buffer (20 : 80, v/v). According to his study the pK_a is 4.58 [80]. Therefore the preliminary experiments covered the study of migration behavior of analytes in wide pH range from 2.5 to 13.0. The working electrolytes were based on sodium phosphate, sodium borate and sodium hydroxide. Nevertheless, in the analyzed range of pH, the antiandrogens comigrated and their separation by capillary zone electrophoresis was not possible. Only the slight migration of nilutamide as very weak anion was observed at pH 13.0, when flutamide is still migrate at EOF velocity. To confirm the obtained results about the acid-base properties the second spectrophotometric study was performed under the above mentioned pH range. The results did not show any significant changes for dissociation equilibrium, the same verifying the correction of the findings and leading to choice of MEKC as a separation mode.

5.3.2 Micellar electrokinetic separation

The most frequently studied buffers factors for MEKC mode are: the concentration of the surfactant, the buffer, its pH and the addition of organic modifier. The effect of SDS concentration, on migration time and resolution between peaks, was investigated in the range 10 - 150 $mmol \cdot L^{-1}$. The results are shown in the Fig. 5.2. During the measurements the buffer concentration, the separation voltage and the cartridge temperature stand constant, what means 10 $mmol \cdot L^{-1}$ sodium borate buffer (pH 9.0), + 20 kV and 25 ºC, respectively.

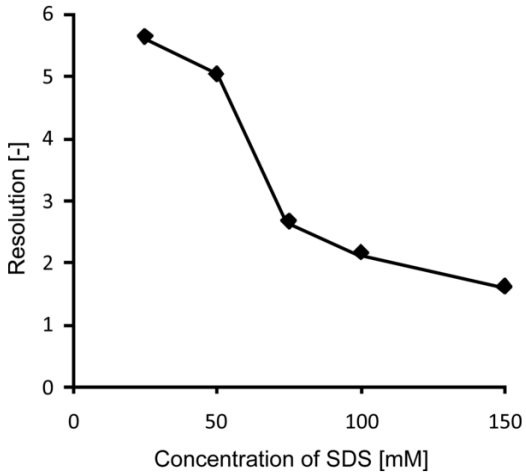


Fig. 5.2 The influence of SDS concentration on the resolution

With increasing the SDS micelles concentration in the running electrolyte, the resolution decreased and retention times increased. The efficiency values exhibit maximum for both studied drugs at concentration of SDS at 50 mmol·L^{-1}. In order to separate analytes in short time and good resolution and high efficiency the 50 mmol·L^{-1} concentration of SDS was selected for next experiments. Then, the influence of sodium borate concentration was studied in the range from 10 mmol·L^{-1} to 100 mmol·L^{-1} in the running electrolyte. The highest peak efficiency and sufficient resolution (> 5.7) was achieved with 50 mmol·L^{-1} sodium borate. Finally, the study of influence of pH value of the running electrolyte was checked in the range from 8.0 to 10.5 using 50 mmol·L^{-1} sodium borate with 50 mmol·L^{-1} SDS. The highest efficiency of peaks, acceptable resolution (> 6.0), and the best repeatability of analyses was achieved at the pH 9.0 (Fig. 5.3)

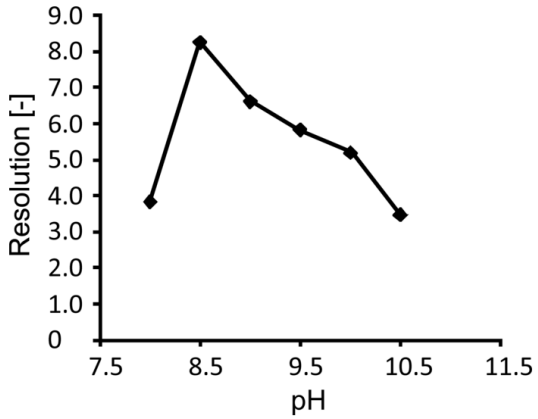


Fig. 5.3 The influence of pH on resolution

The final conditions for separation of nilutamide and flutamide were 50 mmol·L^{-1} sodium borate pH 9.0 with 50 mmol·L^{-1} SDS at 20 kV (Fig. 5.4).

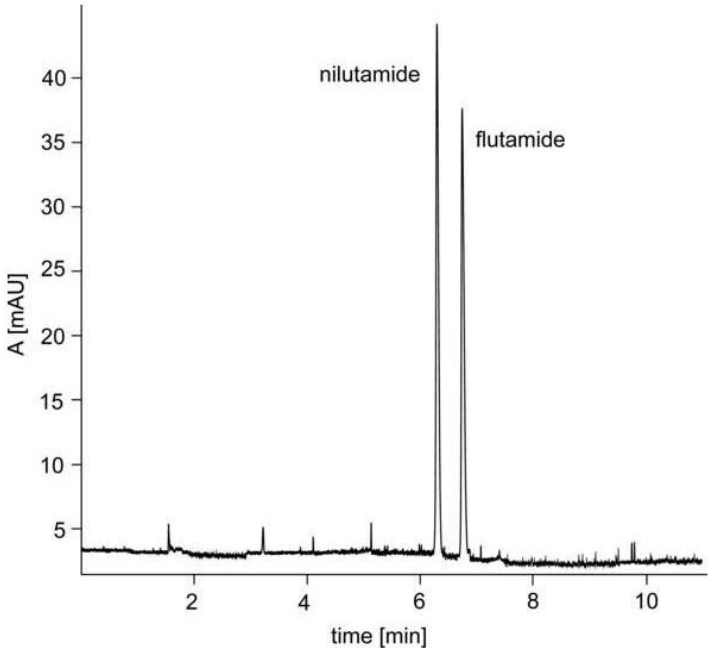


Fig. 5.4 The separation of both drugs at final conditions

5.3.3 The determination of nilutamide and flutamide in human serum

At the final conditions the limit of detection (LOD) and quantification (LOQ) were evaluated basing on 3 S/N ratio and 10 S/N ratio, respectively. LODs were 0.57 $mg \cdot L^{-1}$ for flutamide and 1.03 $mg \cdot L^{-1}$ for nilutamide. These values show that the determination of flutamide on therapeutic level in patient serum is impossible (tens to hundreds of $\mu g \cdot L^{-1}$). From this point of view, the separation procedure should be extended of the preconcentration step, which allows quantification of both drugs in serum. According to the use of MEKC mode for analytes separation the considered preconcentration part was high salt sample stacking. In the following chapter the developing of sample enrichment will be described.

5.3.4 High salt sample stacking

The preconcentration step is based on the injection of analyte in the electrolyte with high -salt concentration. Thus firstly the sodium chloride concentration was changing within the 100 - 200 $mmol \cdot L^{-1}$ range. The sample was injected as a long plug 50 mbar/100 s. To avoid the transfer of analytes to the inlet vial, the short plug of buffer was applied (50 mbar/2 s) after the analytes injection. The change of the analytes peak area is demonstrated on the Fig. 5.5.

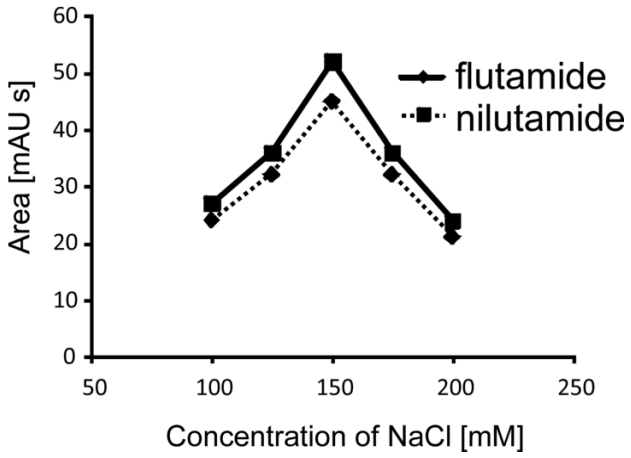


Fig. 5.5 The influence of NaCl concentration in sample on the peak area

As it is seen the best results were obtained for the 150 mmol·L^{-1} concentration of NaCl. The second optimized parameter was the sample plug length, which varied from 60 s to 200 s at the 50 mbar injection pressure. The peaks areas of analytes increased with increasing the injection time up to 150 s what is presented on the Fig. 5.6.

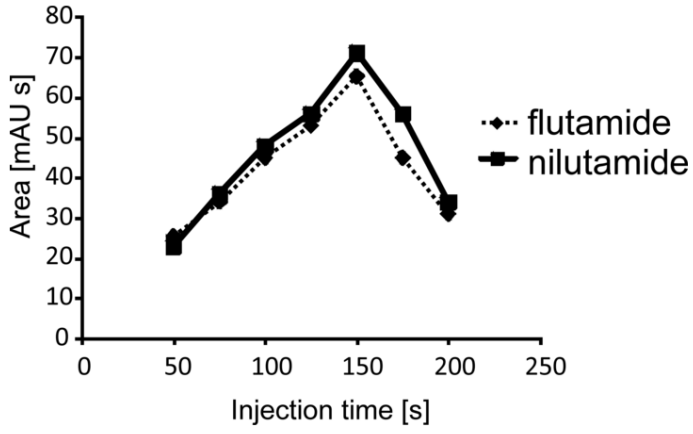


Fig. 5.6 The time of sample injection plug and its influence on the peak area

The proposed preconcentration step included 150 mmol·L^{-1} concentration of NaCl and injection plug at 50mbars/150s. The enrichment factor (calculating as a ratio

between LODs with and without the preconcentration step) was 27.1 for flutamide and 39.6 for nilutamide. Calibrations were linear between 0.05 $mg \cdot L^{-1}$ and 10 $mg \cdot L^{-1}$ (six points; n = 10). LODs (based on 3 S/N ratio) were 21 $g \cdot L^{-1}$ for flutamide and 26 $g \cdot L^{-1}$ for nilutamide. The electropherograms of a blank blood serum and a spiked serum after protein precipitation, employing the preconcentration step is presented on the Fig. 5.7.

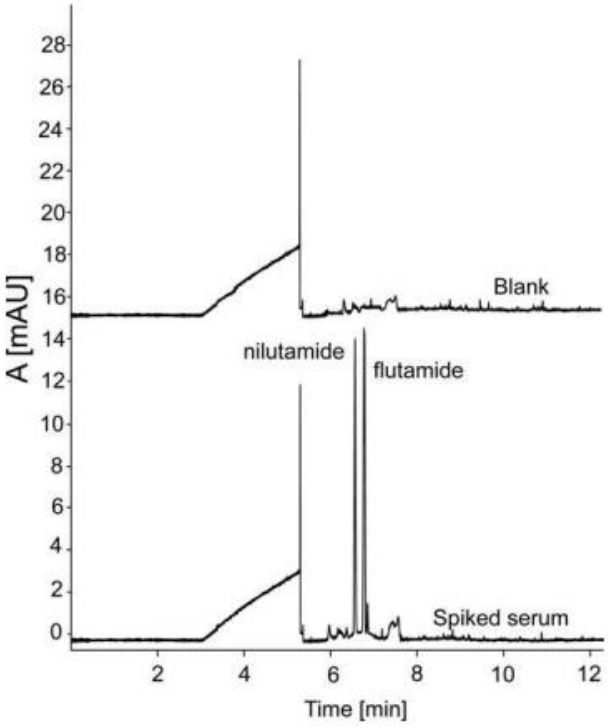


Fig. 5.7 The results of preconcentration step

Recoveries were calculated from the addition of each antiadrogens standard at 100 $\mu g \cdot L^{-1}$ concentration level to the blood serum and were higher than 90 % for both studied drugs.

5.4 Conclusion

The MEKC mode was developed as an alternative method, to other reported in literature, for determination of two widely used antiandrogens. The method allowed efficient separation of structurally similar analytes. Moreover, incorporating the sample stacking to the method enabled the determination of nilutamide and flutamide concentrations in patients' plasma samples.

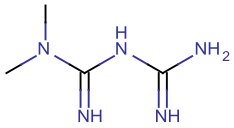
This analytical procedure due to its simplicity and rapidity could be also successfully used for quality control of pharmaceutical formulation. Furthermore, continuing the cooperation with the Laboratory of Experimental Medicine in Olomouc method would be applied in a routine control of pharmaceutical substances.

6 ANTIDIABETICS SEPARATION

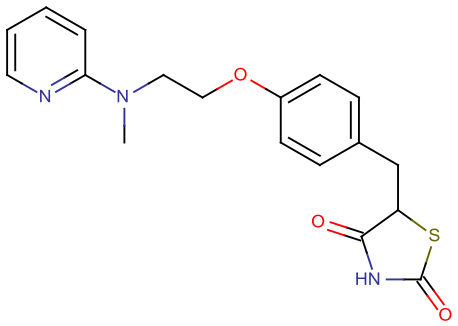
J. Znaleziona, V. Maier, V. Ranc, J. Ševčík: Determination of rosiglitazone and metformin in human serum by capillary electrophoresis with electrospray mass spectrometry. J. Sep. Sci. - accepted.

6.1 Introduction

According to the publication of International Diabetes Federation the number of people affected by diabetes mellitus is increasing year by year. Approximately the 6.4 % of the world's adult population has been affected by this disease till 2009 [97]. Rosiglitazone belongs to the class of medications called thiazolidinediones, it might be used alone or as a combine therapy with biguanides like metformin or glitazones like sulfonylurea [98, 99]. The structures of studied drugs metformin and rosiglitazone used for combined medication are shown in Fig. 6.1.



Metformin



Rosiglitazone

Fig. 6.1 Drugs chemical structure

Metformin

N,N-dimethylimidodicarbonimidic diamide is white to off-white crystalline compound. It is freely soluble in water and is practically insoluble in acetone, ether, and chloroform. The pK_a of metformin is 12.4. The pH of a 1 % aqueous solution of metformin hydrochloride is 6.68 [100].

Rosiglitazone

(RS)-5-[4-(2-[methyl(pyridin-2-yl)amino]ethoxy)benzyl]thiazolidine-2,4-dione. Rosiglitazone maleate is a white to off-white solid with a melting point range of 122 to 123 °C. The pK_a values of rosiglitazone maleate are 6.8 and 6.1. It is readily soluble in ethanol and a buffered aqueous solution with pH of 2.3; solubility decreases with increasing pH in the physiological range [100].

Drugs' concentration levels were monitored individually as well as in biological samples and pharmaceutical formulations. Analytical methods used for rosiglitazone determination in blood samples included liquid chromatography with tandem mass spectrometry (LC-MS/MS) [101 - 105], as well as with UV detection [106 - 108] or with fluorescence detection [109 - 114]. The last work of Al Azzam et al. [115] compares CE with HPLC, however is based on the UV detection and prolonged by the hollow fiber liquid-phase microextraction. Metformin quantification was evaluated by LC-MS/MS [116 - 124] with spectrophotometric [125 - 140] or fluorescence detection [141, 142] or by capillary electrophoresis with UV detection [143, 144].

Up to date there are four publications concerning the determination of both drugs in serum, all based on the liquid chromatography (LC) with UV [145] or MS/MS detection [146 - 148]. Yardimici *et al.* [149] used phenyl column and gradient elution LC; limits of

detection (LOD) determined by this method were 50 $ng \cdot mL^{-1}$ for rosiglitazone and 100 $ng \cdot mL^{-1}$ for metformin with analysis time around 10 min. Blood samples were treated with acetonitrile as a precipitation agent and supernatant was directly injected to the system. Zhang *et al.* [150, 151] proposed the LC with MS/MS to simultaneous drug determination, also applying one step sample preparation method with acetonitrile. Lower detection limits at the level of 1 $ng \cdot mL^{-1}$ for both compounds were achieved by this method with analysis time around 11 min. Similar LODs were obtained by the method developed by Wang *et al.* [152], however, the analysis time was shortened to 5 min and the method was tested on monkey plasma.

Capillary electrophoresis with electrospray ionization mass spectrometry (CE-ESI-MS) is a separation technique which offers unique advantages such as small samples and background electrolytes consumption, easy method development and possibilities of online preconcentration, what makes it a cost effective alternative to HPLC analyses [153]. Moreover, the use of mass spectrometry allows identification of the compounds of our interest in complicated mixtures. The aim of this work was to develop the separation method with mass spectrometric detection of metformin and rosiglitazone in suitable run times with acceptable precision and apply it for determination of both analytes in human serum.

6.2 Experimental part

6.2.1 Instrumentation

The CE-MS measurements were carried out using the CE instrument HP ^{3D}CE, (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and connected to the Agilent G6130 single quadrupole mass-selective detector. Sheath liquid for

the electrospray ionization interface was supplied by an Agilent G1310 LC isocratic pump. Uncoated fused-silica capillary (MicroSolv Technology Corporation, NJ, USA) of 50 μm I.D., with 21.5 cm of the effective length to DAD detector was used. The total capillary length and also an effective length to the MS detector was 81 cm. Each first conditioning of the capillary included rinses as follows: 20 min with 0.1 $mol \cdot L^{-1}$ sodium hydroxide, 15 min with deionized water and 15 min with background electrolyte, all with the capillary end outside of the MS inlet.

6.2.2 Separation conditions

Injection was performed by a pressure 50 mbar for 5 s. The drying gas in ESI is used to accelerate buffer desolvation and to increase sensitivity [149], its flow rate was tested in the range from 1 to 8 $\mu L \cdot min^{-1}$, and the flow rate 2 $\mu L \cdot min^{-1}$ was proved to be suitable. The drying gas temperature was measured in the range 100 - 250 ºC, giving the high and stable MS signal at 160 ºC. The last studied parameter was the potential on the spraying capillary in the range 3.50 - 4.75 kV. The best results were obtained by using 4.25 kV for both analytes.

The direct injection mass spectrometric experiments were performed on the high resolution QqTOF instrument (QqTOF Premiere, Waters, UK), equipped with a linear pump (Harvard apparatus, Holliston, MA, USA). Experimental parameters were selected according to the absolute intensity of target ions. Parameters of ionization were tuned as described: temperatures of desolvation gas and the source temperature were tuned in the range 80 - 120 °C, 100 °C was selected for further use. Applied API voltage was tuned in the range from 1 to 3 kV; 2.4 kV was selected. The flow of desolvation gas (nitrogen) was tuned in the range from 50 to 500 $L \cdot h^{-1}$ (value of 250 $L \cdot h^{-1}$ was selected).

6.2.3 Sample and electrolytes preparation

The background electrolyte was prepared by dilution of appropriate amount of formic or acetic acid in deionized water and then the pH value was measured. The stock solutions of metformin and rosiglitazone (0.01 $mg \cdot mL^{-1}$ of each one) were prepared by a dissolve of corresponding amount of respective compound in a mixture of methanol and water (1:2) and the following standard solutions were made by dilutions.

The blood samples were collected from patients suffering on diabetes mellitus type II (non insulin-dependent diabetes mellitus), in whose treatment the combine medication of rosiglitazone with metformin was used. Samples were kept in the freezer (-20 °C) until the analysis time. The amount of drug combination was 1,000 mg of metformin and 2 mg of rosiglitazone and was taken as a daily dose. Blood was centrifuged at 4,700 × g for 5 min and then the serum was taken. 200 μL of precipitation agent acetonitrile was added to the 100 μL of serum. The sample was vortexed for 20 s and then centrifuged 10 min at 15,900 × g. The volume of 100 μL of supernatant was separated and acetonitrile was evaporated with a gentle stream of nitrogen at room temperature. The residue was reconstituted in 100 μL of the mixture of methanol and water (1:2) and then analyzed.

Identification was done by the standard addition method. The statistical program QC Expert 2.5 (TriloByte Statistical Software, Pardubice, Czech Republic) was used to evaluate the obtained data.

6.3 Results and discussion

6.3.1 Capillary electrophoresis with electrospray mass spectrometry

The influence of various acidic background electrolytes (BGEs) on separation of rosiglitazone and metformin was studied, with a special care for rosiglitazone as a minor component in the medication. According to the pK_a values the both compounds are positively charged under the acidic conditions and can be analyzed by CE. The influence of background electrolyte composition on resolution, electric current during analyses, intensity of MS signals and repeatability were monitored. The acetic and formic acid was used as background electrolyte in the concentration range from 10 $mmol \cdot L^{-1}$ to 100 $mmol \cdot L^{-1}$ was compared. The best results were obtained with 50 $mmol \cdot L^{-1}$ formic acid at pH 2.8 as BGE.

The MS detection was performed in the single ion monitoring (SIM) mode with $m/z = 358 \pm 0.5$ ($[M+H]^+$) for rosiglitazone and $m/z = 130 \pm 0.5$ ($[M+H]^+$) for metformin. The influence of sheath liquid composition and its flow rate, drying gas temperature as well as its flow rate and the voltage applied on the spraying capillary were studied and results are presented on Fig. 6.2.

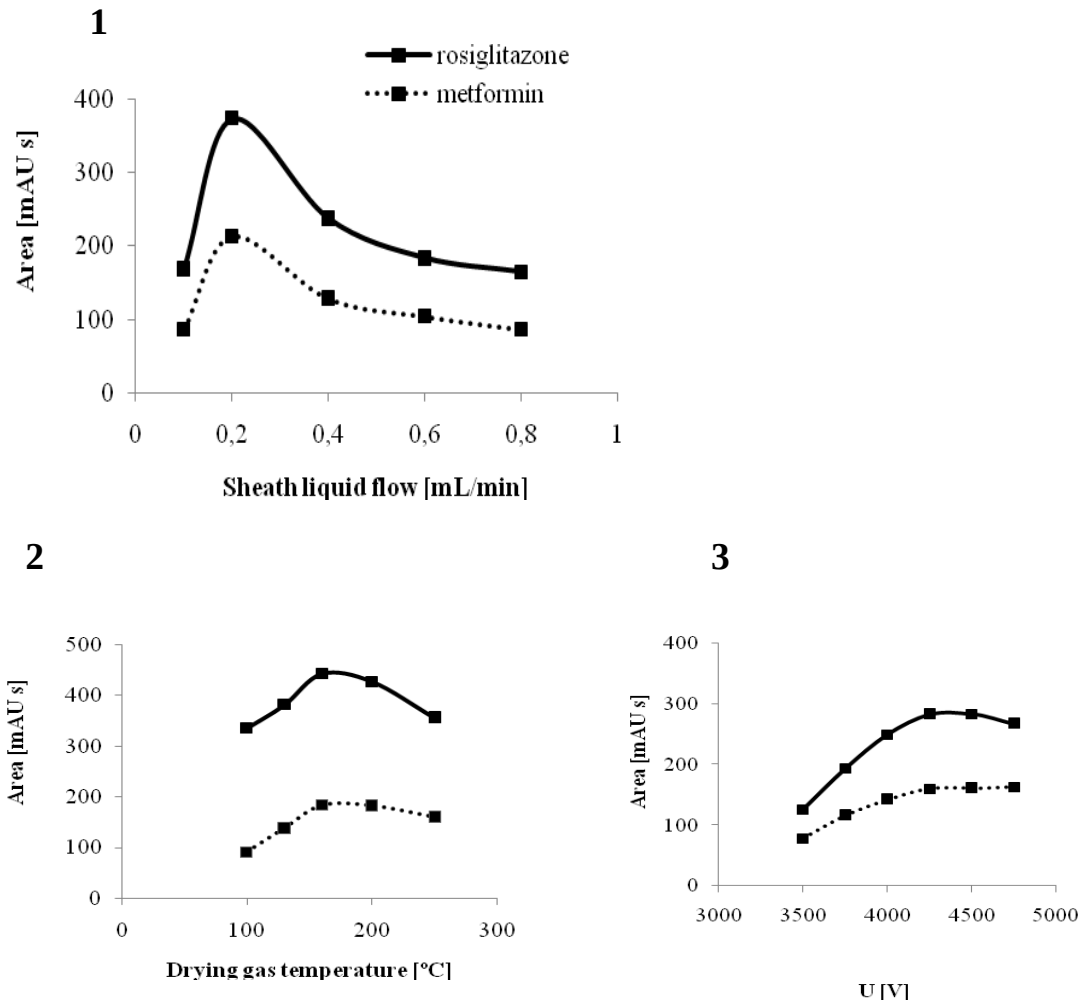


Fig. 6.2 Influence of sheath liquid flow (1), drying gas temperature (2) and voltage applied on the spraying capillary (3) on the peak area

Methanol and isopropanol with mixture of water and formic acid were selected as organic solvents for the optimization of sheath liquid composition. Two different ratios of organic and water phase (50:50, 80:20) were tested for the sensitivity and stability of the MS signal. The formic acid was chosen as an ionization agent to minimize the negative effect between sheath liquid and BGE [111]. Three different contents of formic acid in sheath liquid were checked: 0.25 %, 0.5 % and 0.75 %. The addition of formic acid to the sheath liquid improved the signal intensity. The signals' increasing of about 2 magnitudes was observed for

both analytes. The higher content of methanol than 50 % in the sheath liquid or using isopropanol instead of methanol resulting in poor and unstable MS signals for both studied drugs. In the Table 6.1 the all analysis conditions are presented.

Table 6.1 Final analysis conditions

Final analysis conditions	
Sheath liquid composition	MeOH : H_20 50:50 + 0.5% (v/v) formic acid
Sheath-liquid flow rate	2 μL min^{-1}
Drying gas flow rate	10 L min^{-1}
Drying gas temperature	160 °C
Nebulizer gas pressure	10 psi
Potential on spraying capillary	4250 V
ESI mode	Positive
BGE	50 mmol·L^{-1} HCOOH

The Fig. 6.3 presents the separation under the final conditions. As it is seen the UV-DAD detection allowed drug determination on levels of hundreds of ng·mL^{-1}, thus disabled their determination in patient serum samples. Thus, it had demanded the additional enrichment procedure such as online preconcentration or labor demanding sample preparation, what in this case was omitted due to use of MS.

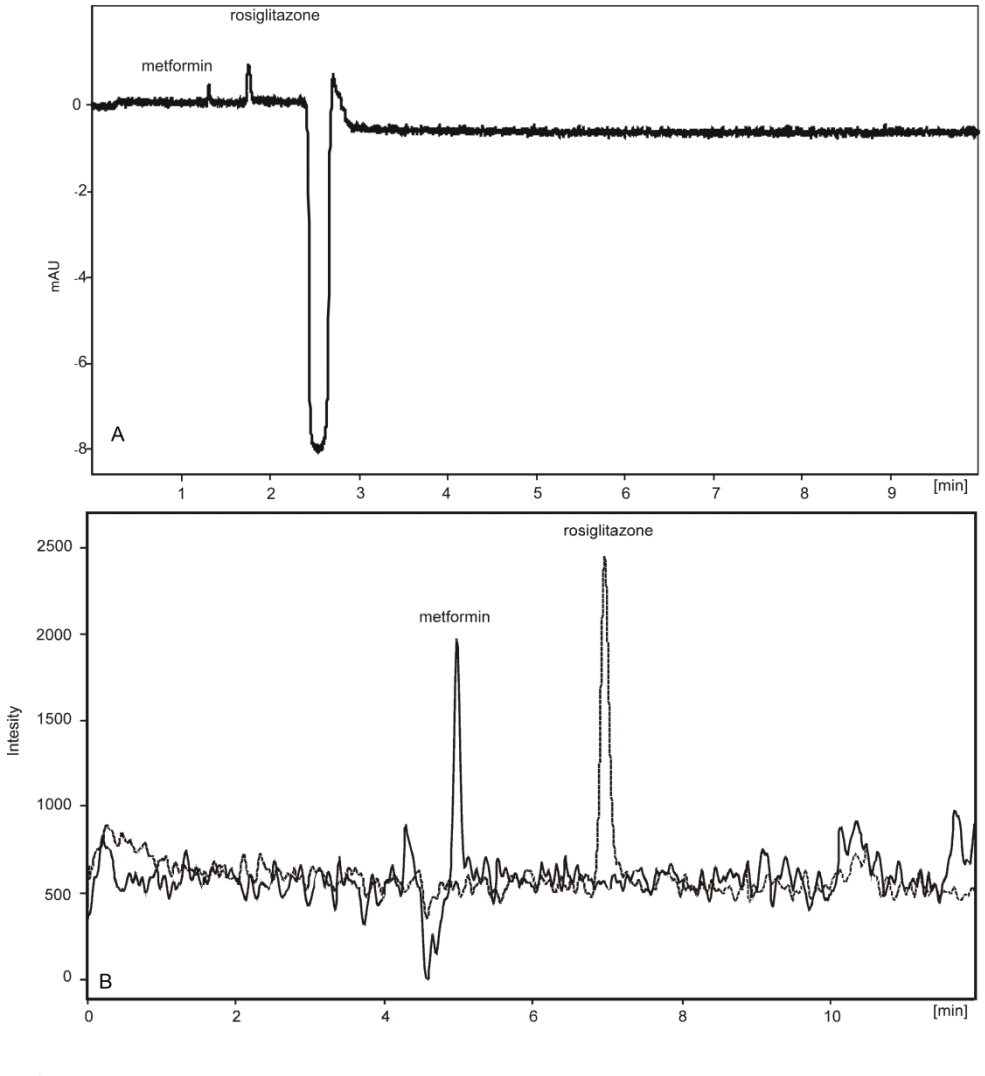


Fig. 6.3 Representative electropherogram of metfromin and rosiglitazone

A) Results obtained with DAD detector, c (met.) = 200 ng·mL^{-1}, c (ros.) = 500 ng·mL^{-1};
B) results obtained with MS detector c (met.) = 8 ng·mL^{-1}, c (ros.) = 20 ng·mL^{-1}

6.3.2 QqTOF experiments

High resolution mass spectrometer (QqTOF) was used as an evaluation tool to prove the presence of target analytes in the human serum. Three identification parameters were used: accurate mass that was provided by the definite instrument with accuracy bellow 3 ppm, where $[M+H]^+$ ions were monitored. Secondly, fragmentation spectra were evaluated in the

way of finding fragments that can potentially represent logical losses from the parent ions. And lastly also isotopic patterns were evaluated. Based on these three aspects, both analytes (rosiglitazone and metformin) were successfully identified. Another important parameter was impurities that can be present in the complex matrix of human serum. Fortunately, no impurities that can interfere during the determination of target analytes were found. QqTOF instrument was also used for an evaluation of presence of unwanted adducts of target analytes with sodium and potassium. The presence of the clusters $[M+Na]^+$ and $[M+K]^+$ can lower sensitivity of proposed method but here no peaks at the corresponding m/z ratios were found and the same results were observed in the CE-ESI-MS experiments, too.

6.3.3 Method characteristic

Primary statistical characterization of the method was made on standards. The linearity was tested in the range 20 - 800 $ng \cdot mL^{-1}$ for rosiglitazone and 8 - 400 $ng \cdot mL^{-1}$ for metformin and was linear over the whole concentrations range for both target analytes. The calculation of limit of detection (LOD) was based on the signal to noise ratio (S/N = 3). The proposed CE-ESI-MS method does not need the introduction of any additional analytical steps of preconcentration or special sample treatment. Precision values were determined from calibrations from the peak area as well as for migration time and was expressed as the percentage relative standard deviations (% RSD). It was studied by repeated injections of serum samples with rosiglitazone concentration at 50 $ng \cdot mL^{-1}$, 100 $ng \cdot mL^{-1}$, 200 $ng \cdot mL^{-1}$ and 20 $ng \cdot mL^{-1}$, 40 $ng \cdot mL^{-1}$, 80 $ng \cdot mL^{-1}$ for metformin on the same day and on three following days. Overall, the results of intraday experiments showed RSD % $\leq$ 1.2 % for metformin and 2.2 % for rosiglitazone peak areas. Detailed data are presented in Table 6.2. According to the

guidelines for pharmaceutical analysis, these values are acceptable and the method could be used for the determination of such compounds in serum samples.

Table 6.2 Statistical characteristic of the method

		ROSIGLITAZONE				**METFORMIN**			
Conc. range [ng·mL^{-1}]		20-800				8-400			
Correlation coefficient		0.998				0.999			
LOD* [ng·mL^{-1}]		4.42				2.14			
LOQ*[ng·mL^{-1}]		14.58				7.06			
SERUM SAMPLES		**Interday (RSD %)**		**Intraday (RSD %)**		**Interday (RSD %)**		**Intraday (RSD %)**	
Ros. conc [ng·mL^{-1}]	**Met. conc. [ng·mL^{-1}]**	***A***	***t_M***	***A***	***t_M***	***A***	***t_M***	***A***	***t_M***
50	20	10.5	1.2	1.2	3.1	21.8	2.9	2.1	1.0
100	40	11.3	6.4	0.3	2.7	10.8	4.3	0.8	1.1
200	80	8.0	5.6	0.5	3.4	15.8	4.0	0.7	1.1

*LOD calculated as 3 S/N ratio, and LOQ as 10 S/N ratio, t_M – migration time, *A* – peak area

6.3.4 Real sample analysis

Blood serum is a complex mixture containing various interferences, e.g. proteins and a corresponding sample processing has to be carried out before CE-ESI-MS measurements. The first step has to be the elimination of proteins from the sample and disruption of protein-drug binding. Two organic precipitation agents: methanol and acetonitrile, in three different ratios of precipitant to serum (1:1, 1:2, 1:3) were studied. The best results, with focus on the rosiglitazone recovery as a minor component, were for acetonitrile in a ratio 1:2 as summarized in Table 6.3.

Table 6.3 Comparison of drugs recoveries for different precipitation agents

	Recovery [%]					
	Serum : Acetonitrile			**Serum : Methanol**		
	1:1	**1:2**	**1:3**	**1:1**	**1:2**	**1:3**
Rosiglitazone	82.8	93.0	64.4	69.5	66.8	71.3
Metformin	88.7	85.5	86. 8	84.2	86.9	97.2

Acetonitrile has a higher dielectric constant and lower viscosity than methanol, which plays significant role in the process of protein elimination [154]. Measurements of serum samples met a few difficulties concerning the reproducibility, shape of peaks and time of analysis due to the residue of proteins presented in the serum, which adsorbed on the capillary wall. The prolongation and improvements introduced to the flushing step minimized those negative effects.

The developed sample pretreatment was then used for the analysis of rosiglitazone and metformin in patients' serum. Rosiglitazone concentration found in patient's serum was 45 ± 5 ng·mL^{-1} and for metformin 168 ± 2 ng·mL^{-1}. These values are in good agreement with other published results, e.g. [101, 155]. The representative SIM electropherograms of the human serum from patient treated with metformin and rosiglitazone and corresponding electrophoregrams of blank serum are shown in Fig. 6.4. There were two peaks in SIM electropherogram with metformin, one representing studied drug and the second one was unidentified. The proper metformin identification was confirmed by the method of standard spiking.

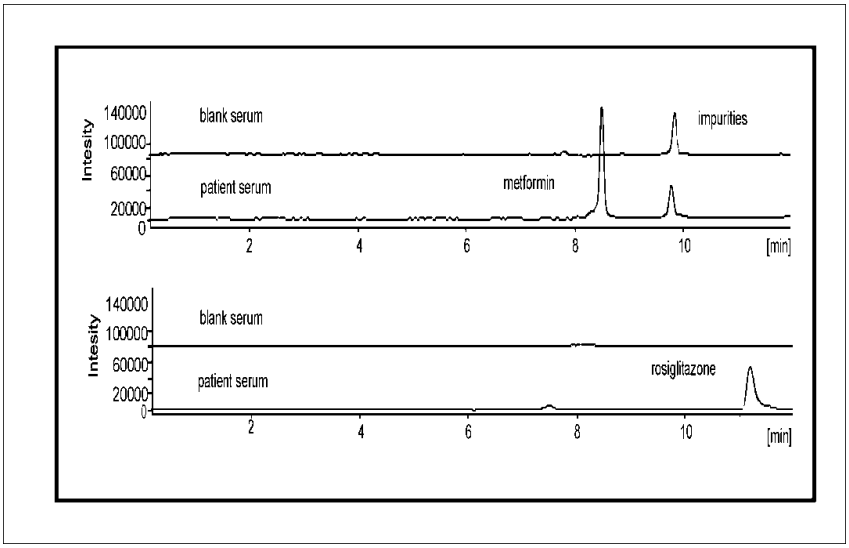


Fig. 6.4 Electropherogram of blank and patient serum sample

6.4 Conclusion

The aim of this work was to develop the separation and determination method of anti-diabetic drugs using capillary electrophoresis with mass spectrometric detection. As it was proved on real blood samples, the developed method could be successfully used for quantitative and qualitative determination of metformin and rosiglitazone, and could be useful alternative to HPLC analyses. Advantages of the method include small sample and background electrolyte (BGE) consumption. Besides the proposed sample preparation step is not work and time consuming. Additional quality is clear analyte identification according to the MS spectra and low detection limits. Moreover, the study was supplemented with the MS and MS/MS spectra obtained from QqTOF to find possible interferences and to confirm or contradict the presence of unwanted analyte-metal complexes in serum. Applying this method, metformin was quantified in human serum at 168 ± 2 $ng \cdot mL^{-1}$ concentration levels and rosiglitazone at 45 ± 5 $ng \cdot mL^{-1}$ concentration levels. The proposed method is suitable for simple and relatively quick therapeutic monitoring of both studied drugs.

7 CONCLUDING REMARKS

The aim of this work was to show that capillary electrophoresis could be successfully applied for clinical purposes, especially when is hyphenated with mass spectrometry. Proposed methods do not required complicated and time consuming sample preparation. Main sample clean–up procedure utilized the precipitation. Although in the third chapter the L-L extraction was employed due to wide spectrum of drugs being analysed. The capillary electrophoresis screening approach for all studied abused drugs' was accomplished within 18 min. Analysis of antiandrogens took 10 min; whereas for antidiabetics was 12 min. Thus analysis time is another positive aspect of these methods. Manipulating the composition of the electrophoretic system, the separation of wide range of analytes, in one run, is possible. Moreover all experiments are carried out in silica capillaries, which inner surface could be easily modified at laboratory conditions. In opposite the HPLC uses usually expensive columns, in some cases (e.g. separation of isomers) highly analyte specified. As was also proved the capillary electrophoresis enabled the preconcentration of analytes during the separation step. What had minimal effect on duration of analysis and used the advantages of the micellar system and complex nature of real samples.

I do not dare to say that this technique has a power to replace HPLC, but is for sure great complementary technique, which in some cases could take advantage over HPLC. Especially that CE is relatively low costs, thus sample and background electrolyte consumption and has less hazardous impact on environment, when consider limited use of solvents.

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9 ABBREVIATIONS LIST

7-AF - 7-aminoflunitrazepam

A – peak area

ACBP - 2-aminochlor-benzophenone

ACP - alternating current polarography

AMP - amphetamine

BGE - background electrolyte

BLN - benzoyleckognine

CE - capillary electrophoresis

cIEF - capillary isoelectric focusing

CMC - critical micelle concentration

CTAB - cetyltrimethyl ammonium bromide

DAM - diacetylmorphine

DCP - direct current polarography

DPP - differential pulse polarography

EOF - electroosmotic flow

ESI - electrospray ionization

ETM - ethylmorphine

FNAP - flunitrazepam

GC - gas chromatography

HPLC - high performance liquid chromatography

HPTLC - high performance thin layer chromatography

LIF - laser induced fluorescence detection

L-L - liquid-liquid

LOD - limit of detection

LOQ - limit of quantification

MAM - 6-monoacetyl-morphine

MECK - micellar electrokinetic chromatography

MEECK - microemulsion electrokinetic chromatography

MET - methamphetamine

MOR - morphine

MS - mass spectrometry

MS/MS - tandem mass spectrometry

NACE - nonaqueous capillary electrophoresis

NPP - normal pulse polarography

RSD - relative standard deviation

SDS - sodium dodecyl sulfate

SIM - single ion monitoring

SPE - solid phase extraction

SPME - solid phase microextraction

QqTOF – quadrupole time of flight

UV – DAD - ultraviolet diode array detection

ε - dielectric constant

η - dynamic viscosity

v_{EOF} - electroosmotic velocity

ζ - zeta potential

k - retention factor

K - distribution coefficient

t_0 - migration time of the EOF marker

t_{mc} - migration time of the micelle marker

t_M - migration time of an analyte

V_{aq} - volume of the aqueous phase

V_{mc} - volume of the micellar phase

α - degree of dissociation

μ_{app} - apparent mobility

μ_{eff} - effective mobility

μ_{EOF} – electroosmotic mobility

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