Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Chemie
Studijní obor: Analytická chemie

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Study of interaction between protamine and heparin and its applicability in capillary electrophoresis

Studie interakce protaminu s heparinem a její využitelnosti v kapilární elektroforéze

Diplomová práce

Vedoucí diplomové práce: RNDr. Tomáš Křížek, Ph.D.

Praha 2017

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Abstrakt

Heparin je kyselá směs sulfatovaných polysacharidů (glykosaminglykanů) s velkou hustotou záporného náboje nacházející se přirozeně v lidském organismu. Díky své schopnosti vázat se na antithrombin III a tím urychlovat inhibici thrombinu má silné antikoagulační účinky, čehož se hojně využívá v klinické praxi při operacích, emboliích či infarktu myokardu. Protamin je naopak směs malých bazických peptidů využívaná jako antidotum při předávkování heparinem. To je možné díky elektrostatické interakci kladně nabitého protaminu se záporně nabitým heparinem. Této interakce se též využívá pro stanovení heparinu v plazmě či krvi pomocí afinitních metod. V rámci práce bylo zjištěno, že pokud jsou protamin s heparinem smíchány v jedné vialce, tvoří komplex, jehož výsledný náboj je dán vzájemným poměrem koncentrací protaminu a heparinu. Naopak v případě, že se protamin dávkuje jako vzorek a heparin je přidán do základního elektrolytu jako ligand vázající protein, je možné z úbytku plochy píku protaminu stanovit koncentraci heparinu. Kvůli složitosti interakce protaminu s heparinem byl pro zvýšení opakovatelnosti měření použit tetraarginin jako protaminu strukturně blízký model. Byla optimalizována metoda, která využívá 20 mM, resp. 60 mM kyselinu fosforečnou jako základní elektrolyt, roztok tetraargininu o koncentraci 1 mg/ml, jehož úbytek se pozoruje, a UVdetekce při 200 nm. Měření je prováděno v křemenné kapiláře o vnitřním průměru 50 μm a délce 50,0 resp. 41,5 cm, při teplotě 25 °C. Po nadávkování protaminu je na systém vloženo napětí 30 kV po dobu 30 s, poté je systém mobilizován tlakem 5 kPa. Ve 20 mM kyselině fosforečné je dosaženo limitu detekce 1,9 μg/ml, limitu kvantifikace 6,3 μg/ml a horní meze lineárního dynamického rozsahu 29,5 μg/ml při relativní standardní odchylce plochy píku tetraargininu bez přídavku heparinu 0,9 % (n = 10). V 60 mM kyselině fosforečné je dosaženo limitu detekce 0,9 μg/ml, limitu kvantifikace 2,9 μg/ml a horní meze lineárního dynamického rozsahu 16,9 μg/ml při relativní standardní odchylce plochy píku tetraargininu bez přídavku heparinu 7,8 % (n = 10).

Klíčová slova: Heparin, protamin, tetraarginin, kapilární zónová elektroforéza, afinitní elektroforéza

Abstract

Heparin is an acid mixture of glycosaminoglycans with high negative charge density which naturally occurs in human body. Due to its ability to bind antithrombin III and thus accelerate inhibition of thrombin it has anticoagulant effect. This is abundantly used in clinical practice for operations, in case of embolia or heart-attacks. Protamine is a mixture of small basic peptides, which is used in clinical practice as a heparin antidote. The interaction between heparin and protamine is electrostatic and is also used for determination of heparin in human plasma or blood using affinity methods. In my study it was found that if protamine and heparin are mixed in one vial, a complex is formed. Its resulting charge depends on concentration ratio of protamine and heparin. On the other hand, in case the protamine is injected as a sample and heparin is added to background electrolyte as a protein-binding ligand, it is possible to determine heparin from decreasing protamine peak area. Because of the complexity of protamine-heparin interaction, tetraarginine was used as structurally close model of protamine to increase repeatability of measurements. The method for determination of heparin was optimalised. It uses 20 mM or 60 mM ortho-phosphoric acid as background electrolyte, 1 mg/mL solution of tetraarginine as heparin-binding protein and UV detection at 200 nm. The measurements were performed in an uncoated silica capillary of 50 μm I. D. and 50,0 cm of length (41,5 cm to detector) using a temperature of 25 °C. After protamine is injected, the voltage of 30 kV is applied for 30 s, then the system is mobilised using a pressure of 5 kPa. In 20 mM ortho-phosphoric acid the limit of detection is 1.9 μg/mL, the limit of quantification is 6.3 μg/mL and the upper limit of linear dynamic range is 29.5 μg/mL. Relative standard deviation of tetraarginine peak area with no added heparin is 0.9 % (n = 10). In 60 mM ortho-phosphoric acid the limit of detection is 0.9 μg/mL, the limit of quantification is 2.9 μg/mL and the upper limit of linear dynamic range is 16.9 μg/mL. Relative standard deviation of tetraarginine peak area with no added heparin is 7.8 % (n = 10).

Keywords: Heparin, protamine, tetraarginine, capillary zone electrophoresis, affinity electrophoresis

Poděkování

Ráda bych poděkovala svému školiteli RNDr. Tomáši Křížkovi, PhD., za neocenitelnou odbornou pomoc při tvorbě této práce. Dále chci poděkovat rodině, kamarádům a především svému příteli za všechnu podporu, pomoc a motivaci, kterou mi během studia a psaní této práce poskytli.

Chapter contents

List of abbreviations

ACE – affinity capillary electrophoresis

ACT – activated coagulation test

API – active pharmaceutical ingredient

APTT – activated partial thromboplastin time

AT – antithrombin

BGE – background electrolyte

CPB – cardiopulmonary bypass

CS – chondroitin sulfate

CTAB – cetyltrimethylammonium bromide

CZE – capillary zone electrophoresis

DNA – deoxyribonucleic acid

DS – dermatan sulfate

FDA – Food and Drug Administration

FGFs – fibroblast growth factors

GAG – glycosaminoglycan

HS – heparan sulfate

ISE – ion selective electrode

LMWH – low-molecular-weight heparin

LDR – linear dynamic range

LOD – limit of detection

LOQ – limit of quantification

MEKC – micellar capillary electrophoresis

PVC - polyvinylchloride

RSD – relative standard deviation

THC – target heparin concentration

UFH – unfractionated heparin

USP – United States Pharmacopoeia

UV-Vis – ultra-violet – visible

List of symbols

l_d – lenght of capillary from the inlet to detector

l_t – total lenght of capillary

μ - electrophoretic mobility

Q – value tested in Dixon's Q test

$Q_{tab.}$ – comparative value in Dixon's Q test

t_1 – time needed for heparin to go through the detector

t_2 – time needed for thiourea to go through the detector

t_U – time for which the voltage was applied

U – voltage

1. The aim of the study

Heparin is currently a very important drug due to its broad application in surgery, cardiology and hematology. Therefore it is necessary to have fast, precise, accurate and inexpensive method to determine heparin in human plasma or blood. The aim of my diploma thesis is to study interaction between heparin and protamine in the conditions of capillary electrophoresis and then exploit the findings for development of a suitable method, which would be applicable to determination of heparin in clinical practice.

2. Introduction

2.1 Heparin

Heparin is an acid mixture of glycosaminoglycans, which has the highest negative charge density of all known biologicaly active molecules [1]. Heparin molecule is long-chained and linear with uronic acid-(1,4)-D-glucosamine disaccharide subunits. This long chain is called unfractionated heparin (UFH) – Fig 2.1. Its molecular weight ranges from 500 to 30 000 Da [1]. However, due to its polydispersity and various disaccharide sequences it is impossible to define exact structure [3]. It is only possible to get fractions of similar molecular weights. The fractions containing just short oligosaccharide subunits are called low-molecular-weight heparin (LMWH). Heparin naturally occurs in human body within granules of basophil granulocytes and mast cells. Usually, it is recovered from pigs‘ intenstinal mucosa and cattles‘ lungs. The concrete structure of the molecule depends just on its source. Because the glycosidic bond of heparin has limited rotation, heparin does not form proper tertiary structure known for example from proteins, nevertheless, extended right-handed helical structure is present. In this structure, tetrasaccharide sequences repeat along the chain [3].

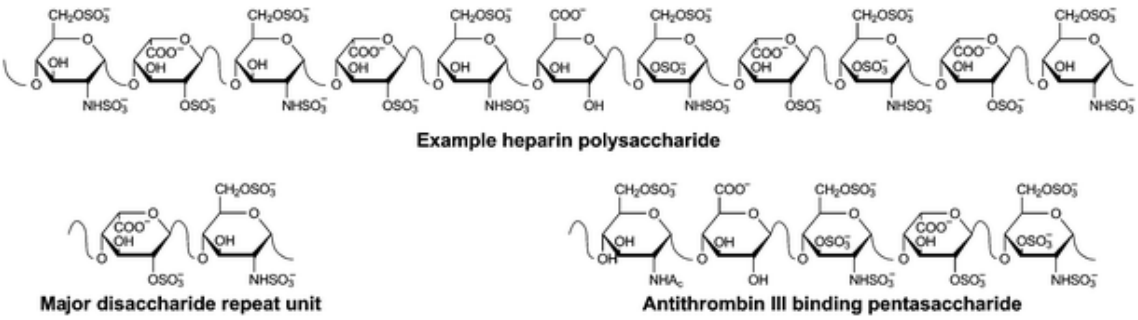

Fig. 2.1 The structure of unfractionated heparin – example of polysaccharide chain – major disacharide repeat unit – active heparin side for antithrombin III binding [2].

Thanks to its high negative charge, heparin has great ability to bind with positively charged guanidinium, ammonium or imidazolium groups of proteins. Until now, a lot of heparin-binding proteins with the same heparin-binding sequence have been described [4]. The best explained heparin-protein interaction is the one with

antithrombin III (AT). Antithrombin inhibits thrombin by a very slow reaction, which naturally regulates the coagulation of blood. The reaction is however up to 1000 times faster, when heparin is bound both to antithrombine and active enzyme and thus works as an anticoagulant [1]. This function is very important in clinical use during operations, in case of embolia or heart-attacks. In surgery, using of both types of heparin is possible. For the better prediction of pharmacodynamics the LMWH of molecular weight from 4000 to 5000 Da is used, however the both types have their preferred usage. LMWH has also longer half-life, about four to five hours, whereas UFH has half-life of only one to two hours which results in necessity of almost continual dosing [1].

Typical dose for cardiopulmonary bypass operation (CPB) is approximately 500 U/kg for infants younger than five years and 300 U/kg for the older children and adults which leads to target heparin concentration (THC) in blood between 3.0 – 4.0 U/mL, which is about 15.5 – 20.7 μg/mL depending on heparin type [5]. However, the treatment of post-operation complications requires lower blood concentration of heparin, just about 0.2 – 0.7 U/mL, which results in necessity to develop a method with limit of detection around 1.0 μg/mL [6].

Heparin can interact with some naturally occuring proteins and thus has other than only anticoagulant pharmaceutical activity. It works as a carrier for several heparin-binding cytokines, for example fibroblast growth factors (FGFs), which are important in healing or embryonic development due to their role in processes of proliferation [7]. However, high doses of heparin lead to risk of bleeding, so some control has to be employed. One possibility is preparation of low-molecular-weight heparin/protamine nanoparticles resulting from the mixture of LMWH of 5000 Da molecular weight and protamine of 3000 Da molecular weight in the ratio of 7:3. These nanoparticles have no anticoagulation activity, however they still can work as FGF and another cytokines carriers [7].

The importance of heparin use in medical practice leads to necessity of fast and precise determination of heparin in human blood or human plasma. This is, however, considerably complicated by the complex structure of heparin, no matter if it is UFH or

LMWH, and different level of sulfonation depending on manufacturing processes and type of heparin. Heparin itself is electrochemicaly inactive, so it is impossible to detect it directly using some electrochemical methods. Molecule of heparin also does not absorb in the commonly used range of UV-VIS spectra (Fig. 2.2), which causes problems with simple direct UV detection and leads to necessity of finding more sophisticated detection methods. Nevertheless several different methods have been developed.

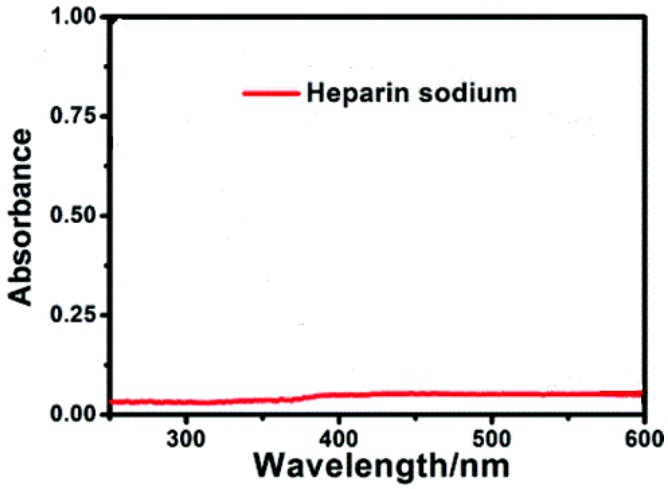

Fig. 2.2 UV spectrum of heparin sodium. Adapted and adjusted from [8].

2.2 Existing heparin determining methods

Due to heparin's importance in surgery it is currently one of the most observed substances in biological analysis. In case of too small doses of heparin thrombotic complications can occur, on the other hand, in case of too big doses a patient can die on bleeding. Heparin is however complex and not exactly defined molecule, so it's direct determination in blood or blood plasma is still very complicated.

Depending on type of heparin, there are few currently practicaly used methods for determination of unfractionated heparin or low-molecular-weight heparin and a lot of other methods published in literature.

2.2.1 Determination of heparin in blood and its components

2.2.1.1.1 Activated partial thromboplastin time and Active coagulation test

For determination of UFH in blood plasma, activated partial thromboplastin time (APTT) is usually measured [9, 10]. To the tested decalcified plasma phospholipide cephalin is added and a reaction is started by adding calcium chloride. Time to creation of a fibrin clot is measured. Several problems can however occur. Especially that UFH reacts differently *in vivo* and *in vitro*. Proteins which occur during acute thrombosis or acute increased fibrinogen level can interfere with the measurement and distort acquired results. This method is also useable just for small concentrations of heparin in plasma, so it is inapplicable for example during dialysis and other extracorporeal circulations, where high concentrations of heparin are used. In this case, Hattersley in 1966 implemented an activated coagulation test (ACT) [11]. This test is similar to APTT, but uses the whole blood, not just blood plasma and is usable also for high concentrations of heparin.

The determination of LWMH is based on its ability to create binary complex with antithrombin and then to bind Factor Xa. This Factor Xa is added in abundance and the rest is measured by method of chromogenic substrates. The advantage is that in this method no interferences with fibrinogen as in APTT or ACT are present [9]. These methods are usually known as “gold standard”.

2.2.1.1.2 APTT based methods

Hussain developed new method based on APTT [10]. In this method a quartz crystal microbalance with dissipation platform covered with molecularly imprinted polymer is coated with a thin layer of heparin-containing sample mixed with calcium chloride. Then it works as APTT, the time needed for creation of a fibrin clot is measured. This way of measuring reduces coagulation time to about 45-50 % of original value, is 3-times more precise than original APTT and needs just about 2 μL of plasma.

2.2.1.1.3 Electrochemical methods

Due to heparin's electrochemical inactivity it is impossible to use any direct electrochemical methods, however electrochemical reaction of other compounds in presence of heparin can be observed. V. Gadzekpo et al. used cyclic voltammetry based on ion-channel sensor for determination of heparin in artificial and horse serum [12]. Self-assembled monolayer of thioctic acid on gold electrode is covered with protamine as a receptor. Oxidation of $[Mo(CN)_8]^{4-}$ or reduction of $[Fe(CN)_6]^{3-}$ in presence of heparin is monitored. Negatively charged heparin in high concentration binds with protamine on the electrode. Electrode is now negatively charged and repulses present complex, which leads to decrease of redox current. Linear dynamic range of method is from 0.5 to 1.5 μg/mL of heparin, but the determination is more precise when fresh electrode is used for every measurement.

Lei et al. used the same method as a flow injection analysis [13]. This arrangement is linear between 0.1 and 2.0 U/mL (0.5 – 10.1 ng/mL) of heparin with LOD 0.06 U/mL (0.3 ng/mL). They used their method successfully for determination of heparin in whole sheep blood.

In nineties, Meyerhoff et al. published series of articles which deal with development of heparin-sensitive electrochemical sensor [14]. They developed ion selective electrode (ISE), which would enable determination of the equivalence point for titration of heparin with protamine. This ISE is made from polyvinylchloride (PVC) doped with tridodecymethylammonium chloride (TDMAC) [15] alone or in combination with dioctylsebacate [16]. TDMAC is structurally close to polybrene, which is cationic polymer known as antagonist of heparin. Heparin-sensitive electrode was connected with pseudo-reference argentchloride electrode and the potential was measured while the sample of undiluted blood was titrated with protamine. The method was very fast especially when compared with APTT or ACT. Authors used it for online determination of heparin in blood during operations both for blood with dose of heparin and with dose of heparin and protamine as an antidote. They showed its high accuracy by comparing the sensor with the results of measurements of the same samples on commonly used

Hepcon HMS Titrator. Another advantage of the membrane is its high selectivity, which makes it possible to determine heparin in whole blood with the normal physiological concentration of sodium chloride. The sensor covers common therapeutical range of heparin concentrations from 1 to 10 U/mL [14].

After they used another membrane doped with dinonylnaphtalensulphonate, which upgraded the linear dynamical range of measurable heparin concentrations from 0.5 to 20 U/mL [17]. They used their upgraded membrane also as a rotating electrode. Using the rotating electrode they were able to develop quality control method of 3 LMWHs drugs approved by FDA. The best results were obtained for 3000 rpm velocity of the electrode and membrane thickness of 50 μm using TRIS buffer, pH = 7.4, which is physiologicaly neutral. It may be possible to use the electrode also for determination of heparin in whole blood [18].

2.2.1.1.4 Spectrochemical methods

Wang et al. developed method for determination of heparin in serum for Raman spectroscopy using nanoparticles [19]. 4-mercaptopyridine functionalized silver nanoparticles interact by electrostatic interaction with protamine, which leads to significant signal in Raman spectra. The interaction of protamine with heparin is however stronger than interaction of protamine with nanoparticles. When heparin is added to the sample, free protamine interacts with heparin, which results in dissipation of protamine-nanoparticles complex and thus to decrease of Raman enhancement effect. The method is linear in range from 0.5 to 150 ng/mL in aqueous solution and from 1 to 400 ng/mL in bovine serum.

Fluorescent sensors of Long et al. are based on similar effect as Wand's method for Raman spectroscopy [20, 21]. They however used gold nanoparticles. The „turn on-off" fluorescent sensor is based on silicon quantum dots, which fluoresce. This fluorescence is suppressed by addition of gold nanoparticles. When protamine is added, it starts to compete with silicon dots for the binding on nanoparticles and fluorescence appears. When heparin is added to this solution, it interacts with protamine, which suppresses the

fluorescence again. The advantage of this relatively complicated method is that it is possible to determine both protamine and heparin. Long et al. acquired LOD of 6.7 ng/mL and linear dynamic range from 0 to 1.2 μg/mL for protamine and LOD of 0.67 ng/mL and linear dynamic range from 0.002 to 1.4 μg/mL for heparin in serum. On the same principle, but using NaYF4:Yb, Er upconversion nanoparticles instead of silicon quantum dots works Long's another sensor. It has LOD of 6.7 ng/mL and linear dynamic range from 0.02 to 1.2 μg/mL for protamine and LOD of 0.7 ng/mL and linear dynamic range from 0.002 to 2.0 μg/mL for heparin.

2.2.2 Determination of heparin in other liquids

2.2.2.1.1 Separation methods

Patel et al. used reverse-phased ion-pair liquid chromatography for separation of some commercial LMWHs [22]. Separation takes place on C18 column using ACN:H_2O 32:68, 15 mM tetrabutylammonium hydroxide and 50 mM ammonium acetate at pH = 7.0 as a mobile phase. The method was also coupled with evaporative light scattering detection. The chemical structure of different LMWHs and its changes while they are stressed was studied. This method is usable for separation, identification, characterization and testing pharmaceutical stability of different types of LMWHs, it was not however used for determination of heparin in blood.

Sun et al. used combination of capillary electrophoresis with mass spectrometry detection [23]. As BGE they used volatile ammonium-bicarbonate electrolyte and as sheath fluid methanol-formic acid. Positive-ion electrospray ionization and normal voltage capillary electrophoresis led to LOQ of 2.0 – 5.9 ng/mL and LOD of 0.6 – 1.8 ng/mL for different disaccharides. The method is suitable for heparin disaccharides, oligosaccharides and LMWHs.

2.2.2.1.2 Electrochemical methods

Guo and Amemiya used stripping voltammetry with PVC membrane plasticized with 2-nitrophenyl octyl ether on a gold electrode modified with poly(3-octylthiophen) [24]. They used salty solution and were able to get LOD of 0.005 U/mL (0.03 ng/mL).

Chen et al. used polycationic-sensitive membrane electrode with dinonylnapthalene sulfonate as an ion-exchanger [25]. There is equilibrium between free protamine and electrode. If heparin is added to the solution, it binds with free protamine and equilibrium is disrupted. More protamine has to be released from the membrane being exchanged with free sodium ions and potential of membrane decreases. Membrane has linear reaction between 0.025 and 1.25 μg/mL of heparin and LOD 0.01 μg/mL of heparin.

2.2.2.1.3 Spectrochemical methods

Fu et al. developed method based on color quenching capacity of graphene oxide [26]. Gold nanoparticles stabilized by hexadecyltrimethylammonium bromide electrostaticaly interact with graphene oxide, which leads to change of color of nanoparticles. Protamine is added as electrostatic interaction disruptor, as described above. When heparin is added, it interacts with protamine and interaction between nanoparticles and graphene oxide is again possible, with change of nanoparticles color. Method has LOD of 5 ng/mL with linear dynamic range from 0.02 to 0.28 μg/mL for heparin. For determination of protamine it was not tested.

2.2.3 Determination of heparin impurities

A great part of published methods which deal with heparin is used rather for determination of other glycosaminoglycans as dermatan sulfate or chondroitin sulfate which are present as heparin impurities in pharmaceutical products.

Wielgos et al. developed CZE method for separation of oversulfated chondroitin sulfate (CS), dermatan sulfate (DS) and heparin sulfate (HS) [27]. They used 600 mM sodium phosphate buffer, pH = 3.5, and indirect UV detection using detection wavelength 200 nm and reference wavelength 450 nm. The separation was performed in reverse polarity mode. High resolution improvement was tested and confirmed using 600 mM lithium phosphate buffer, pH = 2.8.

Process based on enzymatic degradation of glycosaminoglycanes was developed by Bendazzoli et al [28]. This method is possible to use for determination of dermatan sulfate and chondroitin sulfate. Glycosaminoglycanes are degraded to disaccharides subunits using chondroitinase ABC and chondroitinase AC. The first one is active with both DS and CS, the second one is usable only for CS. Unsaturated subunits are then separated using 110 mM phosphate buffer, pH = 3.5, and reverse polarity mode. Detection wavelength is 200 nm, they used direct detection. The method was tested on real samples with LOD of 0.01 % (w/w).

Zhang et al. developed method for determination of sulfate anions present in heparin [29]. They used 20 mM nitrate electrolyte, pH = 5.5, because of close migration time of nitrate to the migration time of sulfate, which leads to narrow peak shape. 0.2 mM CTAB was added to the buffer. Indirect detection using detection wavelength of 200 nm and reverse polarity mode were used. The method was tested for measurements of real samples with LOD of 18 μM and LOQ of 53 μM.

Zhao et al. developed method for determination of heparin and its impurities in plasma [30]. After some improvements it could be used for pharmacokinetics studies of heparin. They used 80 mM diethylenetriamine phosphate buffer, pH = 5.0, to determine hyaluronan acid, CS, DS and heparin in one sample. They tested their method in range from 50 to 600 μg/mL for hyaluronan acid and from 500 to 6000 μg/mL for CS.

Another CZE method was used by Loegel et al. for qualitative identification of heparin and its impurities in pharmaceutical industry [31]. Method is based on different affinity of heparin and another glycosaminoglycanes (GAGs) to polyamine electrolyte. The best

run conditions are 200 mM ethylenediamine buffer with addition of 45.5 mM phosphate. Fused silica capillary and negative voltage are used for separation with conductivity detection to show the mechanism of polyamine-GAG ion-pairing.

2.3 Protamine

Protamine (Fig. 2.3) is a mixture of small basic peptides obtained from fish milt. The most used one is salmon protamine called salmine. It contains four major nearly identical components. Their composition is variable but they always consist of seven aminoacids: glycine, valine, alanine, isoleucine, proline, serine and arginine. The absence of free primary amino groups led to an opinion, that the chains terminate with proline [32]. Chains consists from up to 60-80 % of arginine. Arginine is arranged in longer segments divided with neutral aminoacids. Typical molecular weight of protamine is from 7000 to 10 000 Da [32], but also shorter chains of about 4000 Da can occur [33]. It has heterogeneous secondary structrure, which contains both α-helixes and β-turns, as well as parts not stabilized by hydrogen bonding [34]. The ratio of these structure motifs differs in different types of protamine. According to infrared spectroscopy salmine contains 20 % of α-helixes, 40 % of β-turns and 40 % of non-stabilized structure. Some models say that arginine segments should be straight but protamine contains more arginine residues than non-stabilized parts of chains, so at least some arginine residues have to participate in some secondary structure [34].

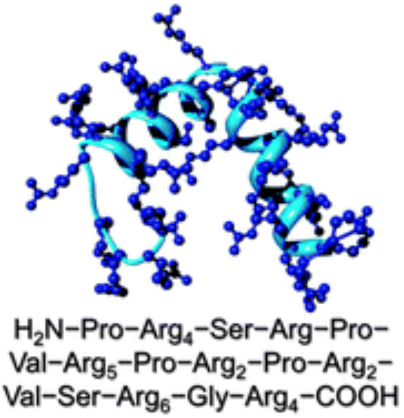

Fig. 2.3 An example of protamine chain structure [2].

Protamine is often used in food industry as a preservative [35] and in pharmacy [36]. Insulin-zinc-protamine complex is used due to its long-lasting effect [37]. Another important feature of protamine is its ability to bind heparin and thus function as a heparin antidote.

2.4 Interaction between heparin and protamine

Ability of protamine to work as heparin antidote is caused by protamine-heparin interaction, which is electrostatic. Negative carboxyl and sulfate groups of heparin bind to positive guanidino groups of arginine residues in protamine in one-to-one ratio (Fig. 2.4). When all positive binding sites are occupied, another binding occurs. This is caused by different affinity of carboxyl and sulfate for guanidino groups. Thus guanidino groups prefer binding with sulfate groups in the complex, which leads to expulsion of some carboxyl groups. Moreover, the resultant complex has greater volume than the one that would be predicted in case of simple pairing of individual components so some degree of cross-linking binding is present [33]. Because of this spatial binding, the time needed for complete formation of the complex can be relatively long. In literature, different times needed to bind the protamine to heparin are reported ranging from few seconds to 15 minutes [38-43]. For complete saturation of protamine molecule of Mr = 4000 with heparin from 20 to 21 molecules of heparin are needed, however, in case of heparin abundance up to 40 molecules are bound to one protamine molecule. So some type of interaction between free heparin and heparin-protamine complex also has to occur. Due to protamine composition, about 70 percent of arginine, about 20 guanidino groups appear in one protamine molecule, so all or almost all positive guanidino sites bind the heparin negative sites [33].

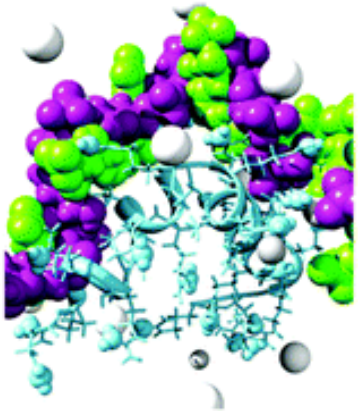

Fig. 2.4 The heparin-protamine complex [2].

It was empiricaly found out that for neutralization of 100 U of heparin from 1 to 1.3 mg of protamine, depending on the specific situation, is needed (pharmac.). This means that for example the initial dose 300 U/kg of heparin is beeing neutralized with 3.9 mg/kg protamine in CPB surgery [5].

2.5 Affinity electrophoresis

2.5.1 History

Affinity electrophoresis is an analytical separation method developed by Kocourek and Hořejší at Charles University in Prague in 1973 [45]. This method combines principles of affinity chromatography and electrophoresis. It combines advantages of high resolution of electrophoresis with the possibility to exploit noncovalent molecular interactions, which are widespread in nature [46].

2.5.2 Application

Currently two different arrangements are possible. The first one is original affinity gel electrophoresis, using stationary gel phase with covalently bound affinity ligands [45], which is now used for example for analyses of DNA on polyacrylamide gel [47]. Modern affinity capillary electrophoresis is arranged similarly to Hummel and Dreyer gel filtration [48]. Measurements take place in fused silica capillary. One of the

interacting coumpounds is dissolved in separation buffer and its co-interactant is injected as a sample and the influence on the mobility of resulting complex is observed [49].

Using Hummel-Dreyer method [48] it is possible to determine binding constants of ligands to proteins, their stoichiometry and kinetics [50-53]. It is widely used even in spite of critism focused on limitations of this method related to the way of mathematical processing of results.

Binding ligands to proteins is an important part of pharmaceutical analysis because it provides information about activity of receptors as well as about concentration of active pharmaceutical ingredient (API) or its metabolites in blood plasma. ACE gives a way to characterize and analyze proteins and peptides using their affinity to the specific antigens. Antibody interaction is usable for analysis of DNA fragments with minimum size of 16 bases. In enzymology ACE can be used to study interactions between enzymes and their substrates and inhibitors. Proteins also can be used for enantiomeric separations. Commonly exploited interaction is the one between glycopeptide vancomycin and D-alanine-containing peptides, which provides this possibility [54].

However proteins are not the only compounds that could be used in this field, another variant how to separate enantiomers is using polysaccharides. It is also possible to use cavities of crown-ethers and cyclodextrines or add detergents to background electrolyte [46, 55]. However the last mentioned technique is often not assigned as affinity electrophoresis, but better as micellar capillary electrophoresis (MEKC).

Low limits of detection are obtained using a fluorescent marker, typically fluorescein, using laser-induced fluorescence detector for detection. Interactions between protein G marked with fluorescein isothiocyanate and its antibodies from human serum enables determination of protein G in range of pM. Similarly sensitive is labeling of Protein A with fluorescein diisocyanate [46].

Due to high number of heparin-binding proteins the use of heparin interactions with proteins was already succesfully employed [56, 57]. Also shape changes and migration time shifts of observed peaks depending on concentration of heparin in background electrolyte are already known, but were used for comparison of two forms of lactoferrin from different sources [57].

3. Experimental

3.1 Chemicals and instruments

Heparin, sodium salt from porcine intestinal mucosa, Grade I-A, 193 USP units/mg (Sigma-Aldrich, St. Louis, USA); protamine sulfate, Grade X (Sigma-Aldrich, St. Louis, USA); Tetraarginine, trifluoroacetate salt (Bachem, Bubendorf, Switzerland); ortho-phosphoric acid 85%, p. a. (Lachner, Neratovice, Czech Republic); sodium hydroxide, p. a. (Penta, Praha, Czech Republic)

For all experiments, electrophoretic system 1600 CE (Agilent Technologies, Waldbronn, Germany) was used. Measurements were performed in uncoated silica capillary, 50 μm of inner diameter and 50.0 cm of length, respectively 41.5 cm to the detector. For detection 8543 UV-Vis detector was used, absorbance at 200 nm for protamine, tetraarginine and complexes with heparin and 254 nm for thiourea was measured.

3.2 Method

All measurements, unless otherwise stated, were done by following approach: injection was hydrodynamic with 5 kPa for 3 s, than the injected zone was mobilized by a pressure of 5 kPa for 210 s. After this, a voltage of 30 kV was applied for 30 s and then all zones of analytes were washed out of the capillary by a pressure of 5 kPa. You can see the whole procedure on Fig. 3.1.

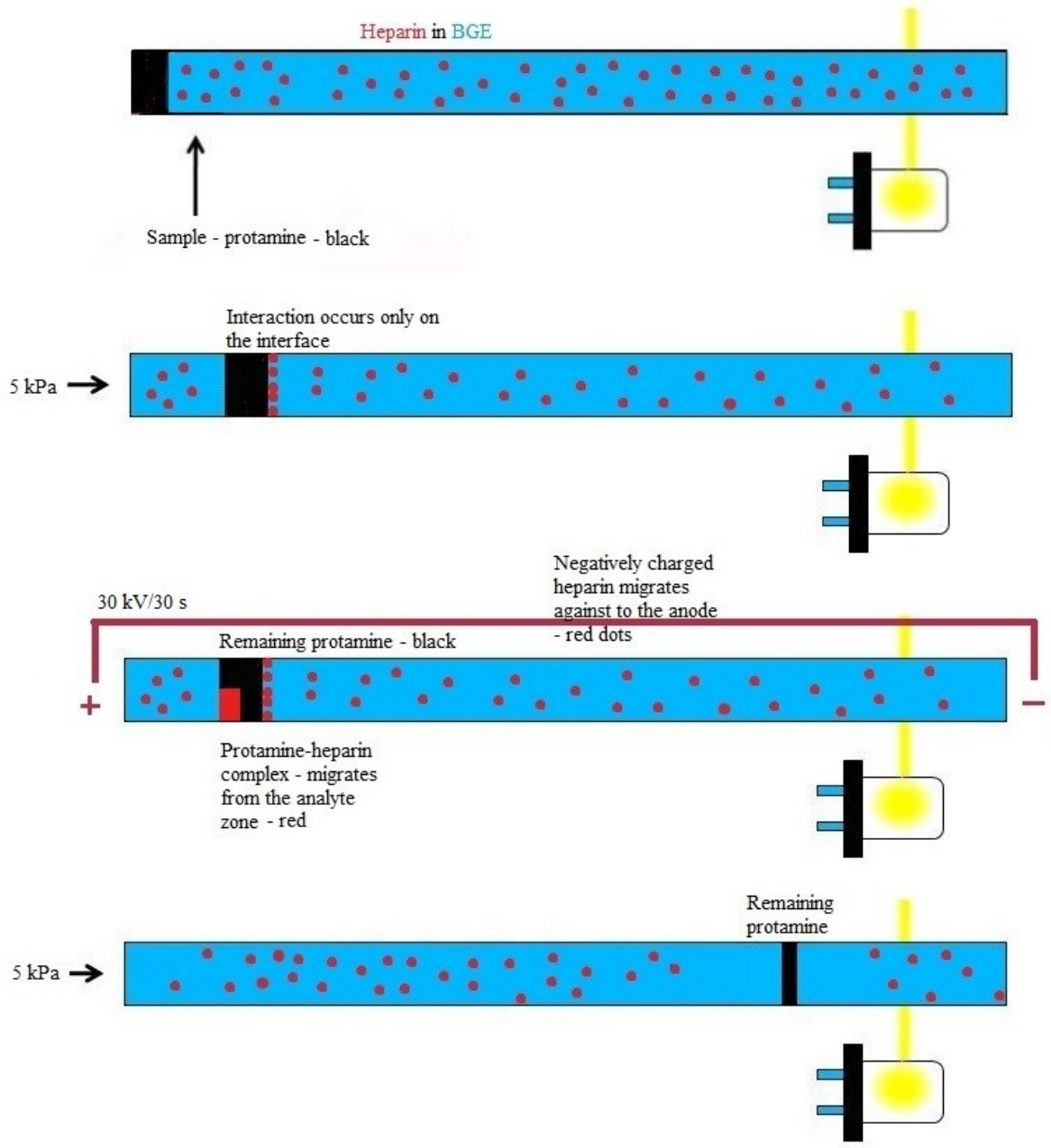

Fig. 3.1 Graphical representation of the measurement procedure: a) heparin is added to the BGE, protamine is injected as a sample; b) a pressure of 5 kPa/210 s is applied. Interaction occurs only on the interface; c) a voltage of 30 kV/30 s is applied. Negatively charged heparin migrates against positively charged protamine, immediate interaction can occur. Nascent complex is carried away from the protamine due to different charge; d) remaining protamine is washed out of the capillary through detector using a pressure of 5 kPa.

Before all measurements capillary was flushed with background electrolyte (BGE) for 3 min. Every new capillary was flushed with 1M sodium hydroxide and deionized water, both for 10 min using a pressure of 92 kPa. Vials with electrolyte were changed at least every 3 measurements. The cassette was thermostated at 25 °C.

For all interaction-describing measurements 20 mM ortho-phosphoric acid was used. It was prepared by dilution of concetrated ortho-phosphoric acid. During some measurements also 30 mM, 40 mM, 60 mM and 80 mM ortho-phosphoric acid was used, these solutions were prepared equally. Electrolyte was stored in a refrigerator at 4 °C.

Stock solutions of heparin, protamine and tetraarginine were prepared by dissolving required amount of given substance in 20 mM or 60 mM ortho-phosphoric acid, depending on the following use, and were stored in the freezer.

3.3 Calculations

Mobility μ was calculated according to the equation [58]:

$$\mu = \frac{(t_2 - t_1) l_d}{t_2 t_U} \frac{l_t}{U} \quad (1)$$

where t_1 is time needed for heparin to go through the detector, t_2 is the same time of thiourea used as an electroosmotic flow marker, l_t is total length of capillary , l_d is length of capillary from the inlet to the detector, U is applied voltage and t_U is time for which voltage was applied.

All results were processed in MS Excel. Outliers were rejected using Dixon's Q test [59].

$$Q = \frac{\text{gap}}{\text{range}} \quad (2)$$

where gap is the absolute difference between the tested value and the one which is closest to it and range is the absolute difference between the biggest and the smallest value of tested file. If Q > than $Q_{tab.}$, than Q is rejected from the tested file. $Q_{tab.}$ is corresponding to the sample size and confidence level.

4. Description of heparin-protein interactions

4.1 Protamine-heparin characterization

Due to the fact that protamine is a cation in acidic environment, it migrates to the cathode. On the other hand, heparin migrates as an anion back to the anode. When both substances are mixed a complex starts to form. As said before, the time needed for complex to form can be relatively long, therefore, the way in which heparin and protamine are mixed is important. If the both substances are pipetted into one vial, complex can form immediately and for the whole time of analysis. However, if the heparin is added into BGE and protamine alone is injected, interaction can only occur on the border of a sample and BGE zones, which has different effect on resulting observation.

4.1.1 Heparin in a sample

At first, the experiment with 1 mg/mL of protamine and addition of heparin mixed together in one sample vial was done. When there is no other heparin in BGE, resulting charge of the complex depends on ratio in which the substances are mixed. If the protamine is in abundance, all positive guanidino groups are not bound to heparin anionic sites. The whole complex has still a positive charge and migrates as a cation. On the other hand, in the abundance of heparin all positively charged binding sites of protamine are saturated, the whole complex is negatively charged and migrates as an anion. The last possible case that can occur is the one in which the complex is close to neutral and migrates approximately in one zone with EOF marker. In Fig. 4.1 you can see changes of mobility of heparin-protamine complex for different concentrations of heparin in sample. Used heparin has activity of 193 USP U/mg. Based on the literature it was theoreticaly calculated that 1 mg of protamine neutralizes 0.52 – 0.67 mg of heparin [5, 6]. In Figure 4.1 you can see that complex is neutral in concentration of heparin about 0.5 mg/mL, which is consistent with previously published research. In Fig. 4.2, the influence of heparin concentration on complex peak shape is demonstrated.

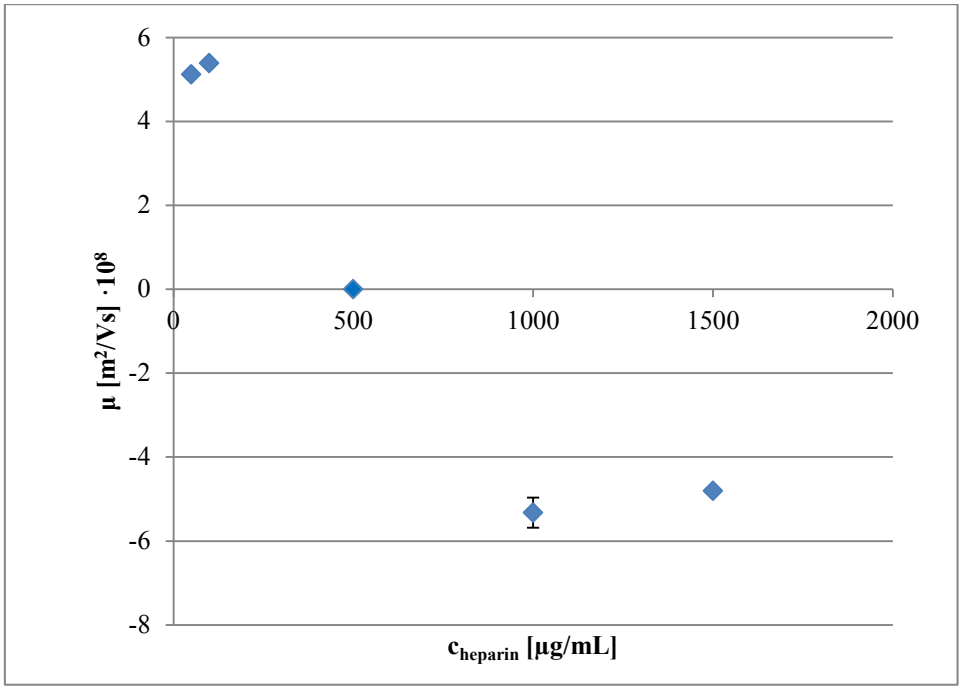

Fig. 4.1. Mobilities μ of heparin-protamine complex in 20 mM phosphoric acid and for concentrations of heparin in range from 50 to 1500 μg/mL injected as a sample in mixture with 1 mg/mL protamine.

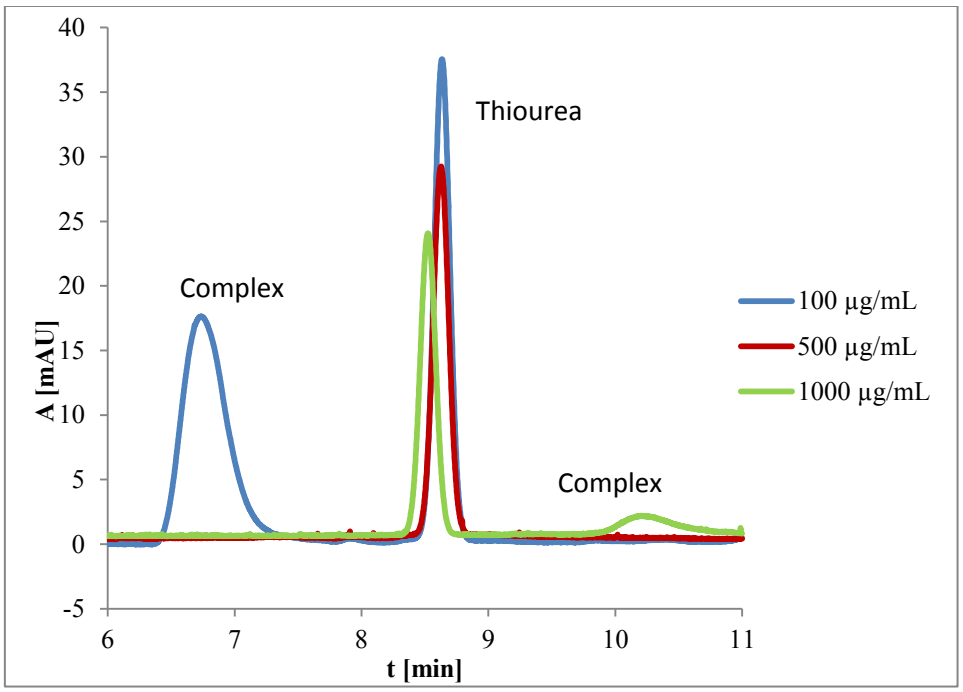

Fig. 4.2. The influence of heparin concentration on protamine peak shape and its mobility in case that heparin-protamine mixture is injected as a sample. 20 mM

ortho-phosphoric acid was used as BGE with no addition of heparin. Concentration of protamine is 1 mg/mL.

Time needed for complete interaction of heparin with protamine is dependent on reaction conditions. According to literature, complete binding can occur in order of tens of seconds to more that 10 minutes. If the reaction under the current conditions would be slow, it could influence resulting peaks‘ areas in consecutive measurements of the same sample.

4.1.2 Protamine-heparin reaction rate

The dependence of complex peak area on the time past from mixing was measured for a mixture of protamine and heparin with heparin in abundance. The concentration of heparin was 1.5 mg/mL and the concentration of protamine was 0.5 mg/mL. Each of three separately prepared mixtures was measured four times within one hour from mixing, immediately after mixing (time 0) and than every 20 minutes till 60 minutes. The area of peak of protamine-heparin complex was related to the area of thiourea as an internal standard in order to minimize the influence of bad run-to-run repeatability. As you can see in (Fig. 4.3), differences in time dependences between the three mixtures are very small. The table 4.1 shows the values of relative standard deviations of relative peak areas for the three mixtures measured within one hour. The RSD of all three samples is about 12 %. This means that the interaction between protamine and heparin in used experiment conditions is very fast. The whole complex forms in order of seconds and does not grow in time, so there is no need to take the reaction time into account in following experiments.

Table 4.1 Relative standard deviation of protamine-heparin complex peak areas for three mixtures of protamine and heparin measured in time t = 0 min and then every 20 minutes till 60 minutes. Concentration of protamine is 0.5 mg/mL, concentration of heparin is 1.5 mg/mL. Peak area of complex is related to peak area of 0.1 mg/mL thiourea.

Mixture	RSD [%]
1	12.2
2	13.4
3	14.1

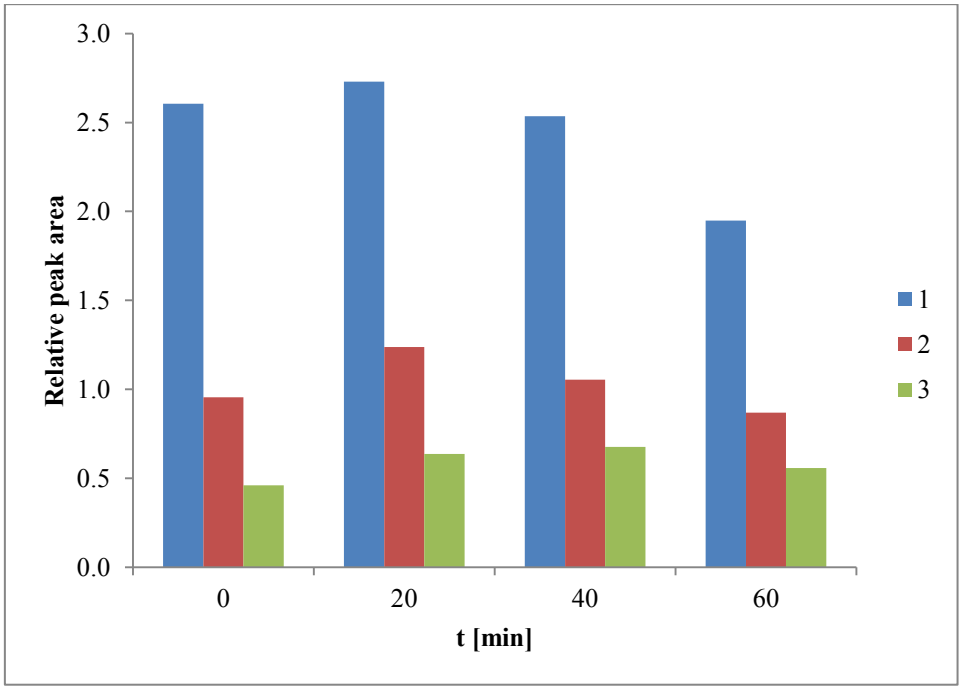

Fig. 4.3 Comparison of relative peak areas of protamine-heparin complex for three mixtures of protamine and heparin measured in time t = 0 min and than every 20 minutes till 60 minutes. Concentration of protamine is 0.5 mg/mL, concentration of heparin is 1.5 mg/mL. Area of complex is related to area of 0.1 mg/mL of thiourea.

4.1.3 Heparin in BGE

Another situation occurs, when protamine is injected as a sample and heparin is added to the BGE. Mobility of observed peak is nearly the same for growing concentration of heparin in BGE, however, what changes is peak area and shape. With growing concentration of heparin the area of protamine peak declines and the peak is becoming wider and lowering until it disappears in the baseline noise. The peak of nascent complex is probably carried away due to the fast kinetics of the interaction and thus is not observable in the system (Fig. 4.4 and 4.5).

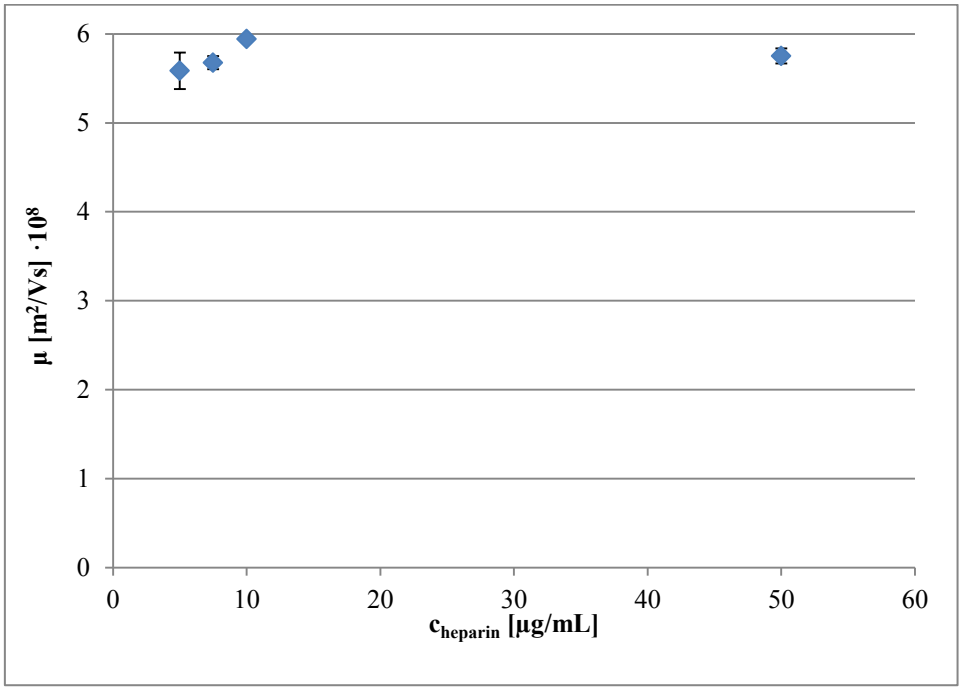

Fig. 4.4. Electrophoretic mobilities *μ* of heparin-protamine complex heparin added to BGE in concentrations ranging from 0 to 50 μg/mL. Concentration of protamine injected as a sample is 1 mg/mL.

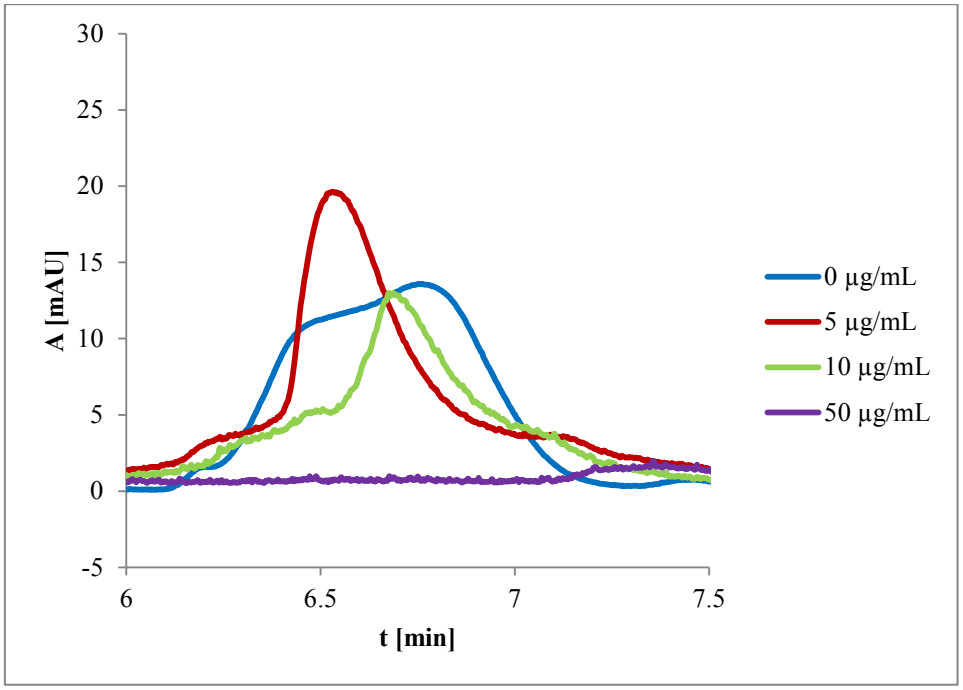

Fig. 4.5. The influence of heparin concentration on protamine peak shape and its mobility in case that heparin is added to the BGE. Concentration of protamine is 1 mg/mL.

4.1.4 The influence of voltage application period

Because in system with applied voltage protamine from the sample and heparin from the BGE migrate against each other, their zones mix and thus can better interact together instead of a contact only at the interface. It was experimentally confirmed that the peak area and shape also depend on the time for which the voltage was applied. Several measurements were done, in which the time of applied voltage was changed. In Fig. 4.6 it can be seen, that the longer exposure time causes broadening of the protamine peak as well as a small shift of its migration time. This could be caused by partial decrease of positive charge of protamine partly saturated with heparin in case that they are allowed to interact for longer time as well as by different migration time in case of extending voltage application period.

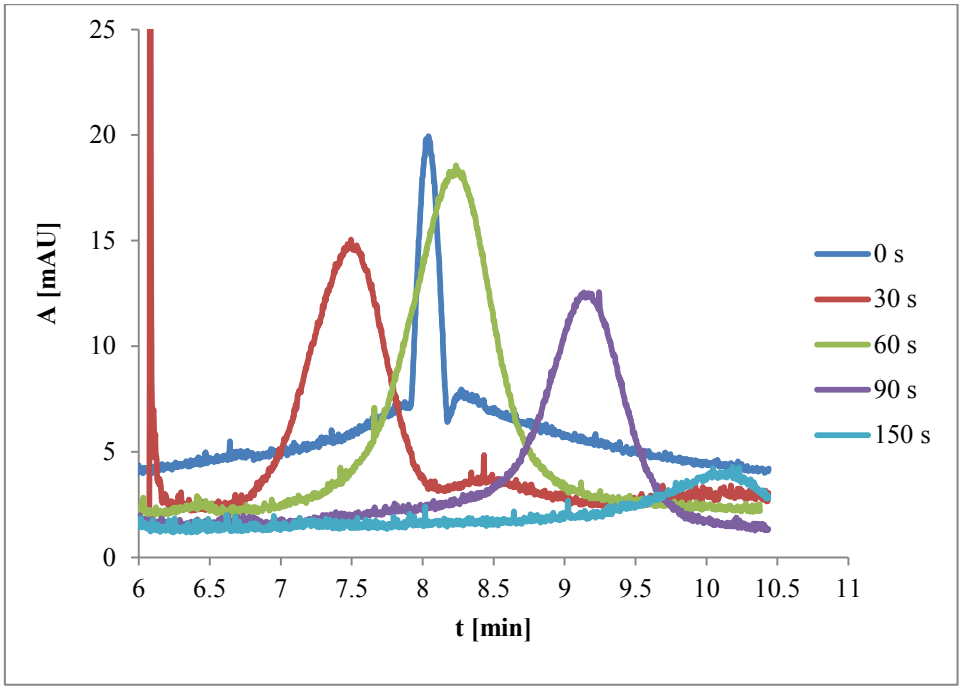

Fig. 4.6. The influence of voltage application period, on protamine peak shape, when heparin is added to BGE. Concentration of protamine is 2.0 mg/mL, concentration of heparin is 0.1 mg/mL

4.2 Tetraarginine-heparin characterization

Already during the first measurements poor run-to-run repeatability of protamine peak area was mentioned. Measurements using protamine have relative standard deviation (RSD) of 10 injections 36.3 %. Such low run-to-run repeatability is consistent with the fact that composition of both protamine and heparin is variable and the interaction between them can thus occur in a slightly different way every run and leads to differences in peak areas between individual runs that are too big for practical usage. Due to this problem with reproducibility, protamine was replaced with tetraarginine with exactly defined structure. Protamine consists from about 70 % of arginine residues in longer sequences, which are moreover the main binding centre for heparin, therefore, tetraarginine is structurally very close model, which should eliminate problems caused by variable protamine structure.

Run-to-run repeteability of 10 injections using the same arrangement, only with tetraarginine instead of protamine is much better with RSD only 13.2 %. It was decided, that tetraarginine will be used for following measurements. The same experiments with complex mobility were done to confirm that previously determined properties are still correct in the case of tetraarginine.

4.2.1 Heparin in sample

Experiments with heparin both in the sample and in BGE were done. For the one with heparin added to one vial with tetraarginine the same trend was observed. Complex of tetraarginin and heparin changes its mobility according to degree of saturation of the peptide with heparin. The concentration in which the whole complex is neutral is the same for both, protamine and tetraarginine. As you can see in Fig. 4.7, resulting mobility of tetraarginin-heparin complex for very low or very high concentration of heparin is similar to the one with protamine. However, for 500 μg/mL concentration of heparin protamine-heparin complex is neutral, while the tetraarginine-heparin complex is still positively charged. This is caused by the fact that for the same heparin concentration all positively charged binding centres of protamine are saturated with heparin and neutral amino acid residues left. On the other hand tetraarginine consists only of positively charged arginine residues, so it needs more heparin for saturating them.

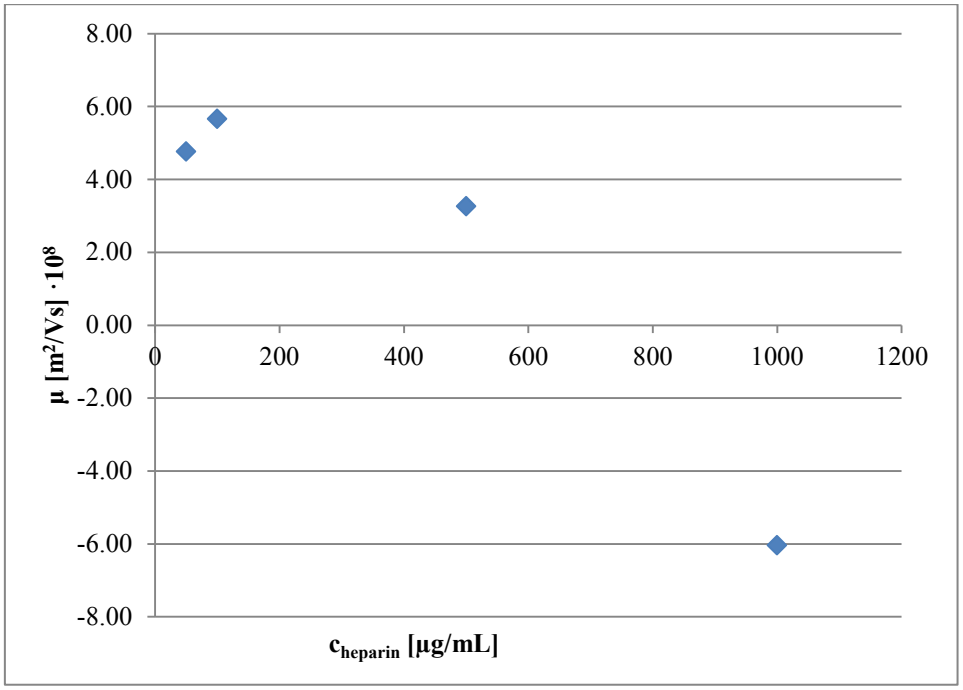

Fig. 4.7. Electrophoretic mobilities *μ* of heparin-tetraarginine complex for concentrations of heparin in the range from 50 to 1500 μg/mL heparin injected in mixture with tetraarginine as a sample. Concentration of tetraarginine is 1 mg/mL. Complex is neutral in higher concentration than 0.5 mg/mL of heparin.

4.2.2 Heparin in BGE

In case of heparin added to BGE almost no differences between mobilities are observed (Fig. 4.8), which means that remaining tetraarginine is present, as in the case of protamine-heparin interaction. But opposed to that interaction, peak of remaining tetraarginine maintains its shape, which enables its integration in wide range of heparin concentrations.

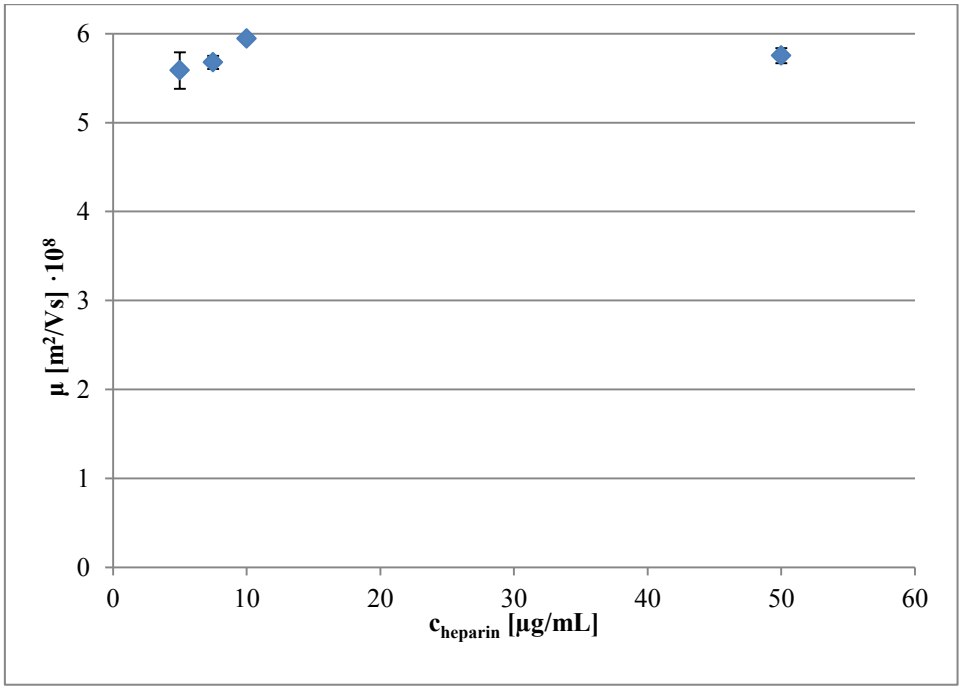

Fig. 4.8. Electrophoretic mobilities *μ* of heparin-tetraarginine complex for concentrations of heparin in the range from 0 to 50 μg/mL added to the BGE. Concentration of tetraarginine is 1 mg/mL.

If the decrease of tetraarginine peak area would be linear in sufficient range of concentrations, the interaction could be used for determination of heparin. The dependence of peak area on heparin concentration and several different periods of applied voltage were measured and it showed that area decrease is proportional to the heparin concentration increase (Fig. 4.9). With longer time of applied voltage the relative peak area decreases more than with shorter one. This is caused by longer time for interaction, which takes place only when tetraarginine and heparin migrate against each other. However, between 30 and 45 seconds there is no significant difference. Consistently with the influence of the time of applied voltage on the shape of protamine peak, 30 s period was chosen for following measurements.

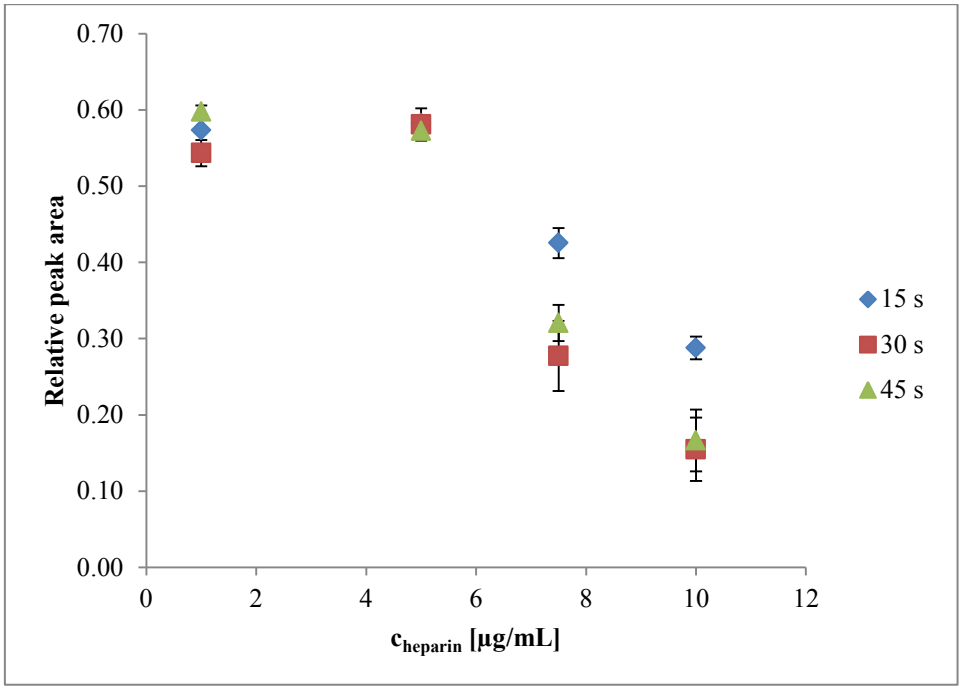

Fig. 4.9. The influence of voltage application period on remaining tetraarginine peak relative area. Concentration of tetraarginine is 1 mg/mL. The peak area is related to the area of 0.1 mg/mL thiourea.

Interaction-describing experiments were done in 20 mM ortho-phosphoric acid, however, principles of electromigration processes and our previous experience indicated that the more concentrated the ortho-phosphoric acid is, the higher and sharper peak is observed beacause lower pH supresses the dissociation of silanol groups and thus interaction with positively charged peptides. On the other hand, too concentrated electrolyte is more conductive, which leads to overheating of the system and thus to experimental difficulties.

4.3 Optimization of new method

4.3.1 Optimization of BGE

4.3.1.1.1 Volt-ampere characteristics

At first, volt-ampere characteristics of the system was measured to exclude the risk of overheating. The dependence of the current on the applied voltage for different concentrations of ortho-phosphoric acid was measured. Due to Ohm's law, current is

directly proportional to voltage using the conductivity as a constant of the proportionality. If this dependence is linear, no problem should occur. Conductivity nevertheless depends on the temperature. If the dependence is nonlinear, it means that conducticity changes rapidly and system overheating is present. Overheating causes convective flows which lead to undesirable mixing of liquid and thus for example broadening of the peaks, which are then less reproducible and limits of quantification and detection increase. Six different concentrations of orthophosphoric acid from 10 to 80 mM were tested. As you can see in Fig 4.10, for concentrations from 10 to 30 mM, the dependence is linear, for concentrations higher than 30 mM, the deviation in case of higher voltage occurs.

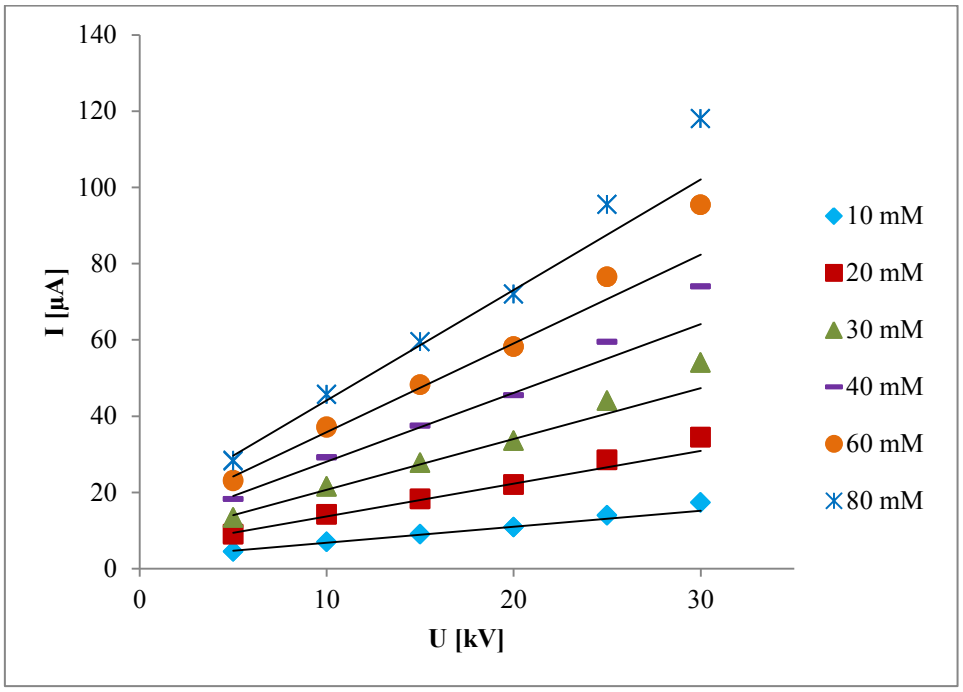

Fig. 4.10. The volt-ampere characteristics for ortho-phosphoric acid in the range of concentrations from 10 to 80 mM.

4.3.1.1.2 Ortho-phosphoric acid concentration

Because the volt-ampere characteristics using voltage of did not show any striking problem with higher concentrations of ortho-phosphoric acid, all previous concentrations were included into optimization. The sample of 1 mg/mL tetraarginine

was measured using different levels of ortho-phosphoric acid in usual electrophoretic arrangement with voltage of 30 kV applied for the whole measurement. For all these levels, the height of the peak and its efficiency [plates/capillary] were calculated. Results are shown in table 4.2. As a suitable combination of these two parameters, 60 mM ortho-phosphoric acid was chosen. At this level, the observed current is about 100 μA, which is recommended electrophoretical upper limit, the peak efficiency is the best and the peak is highest of all tested levels.

Table 4.2. The influence of concentration of ortho-phosphoric acid on tetraarginine peak efficiency [plates/capillary] and height [mAU]. Concentration of tetraarginine is 1 mg/mL. The voltage was applied for the whole measurement.

Concentration [mM]	Efficiency [theoretical plates]	Height [mAU]
20	496	40.0
30	1314	63.5
40	2110	57.3
60	2558	79.0
80	1444	57.1

4.3.2 Optimization of injection

4.3.2.1.1 Hydrodynamic vs. electrokinetic injection

For all previous experiments hydrodynamic injection by 5 kPa for 3 s was used. Electrokinetic injection for varied injection periods as well as change of injection period of hydrodynamic injection were tested. For the evaluation of the acquired results the USP Tailing factor was used. This factor is measured in 5 % of peak height. It is defined as the ratio of distance from the front slope to the back slope of the peak and the double distance between the front slope and the centre line of the peak. If the values vary between 1.0 and 1.5, there is no problem with symmetry, althoug it is not possible to generalize this rule for all cases of CZE separations [60]. The longer the injection period is, the higher the peak is, which is desirable by itself, however, also the USP Tailing factor of peak grows, which can cause inaccuracies during integration. In table 4.3, you can see comparison of USP Tailing factors and the heights of the peaks acquired by the

hydrodynamic injection with 5 kPa or electrokinetic injection with 5 kV. As you can see, USP Tailing factor does not differ significantly between hydrodynamic and electrokinetic injection, however, using hydrodynamic injection leads to peaks more than five times higher that for electrokinetic injection. Hydrodynamic injection using 5 kPa for 3 s was then used. In Fig. 4.11 you can see comparison of peak acquired by hydrodynamic and electrokinetic injection. The difference is caused by heparin-protamine interaction, which occurs already during injection, when electrokinetic injection is used, and changes initial conditions of the separation.

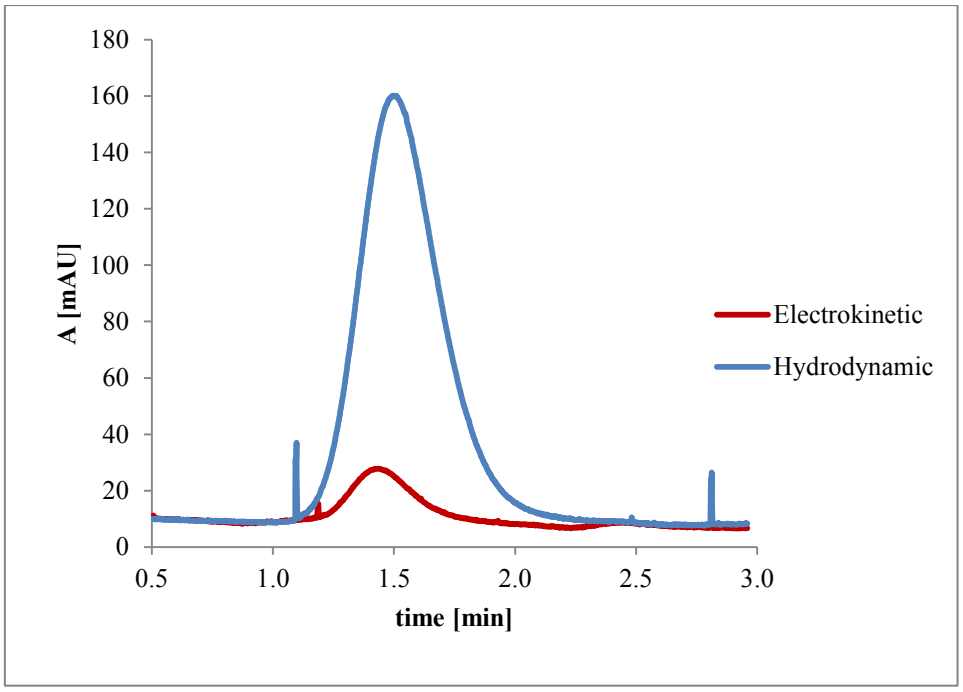

Fig. 4.11. Difference between tetraarginine peak shape using hydrodynamic injection 5 kPa/3 s and electrokinetic injection 5 kV/3 s.

Table 4.3. Tetraarginine peak parameters for hydrodynamic injection 5 kPa and electrokinetic injection 5 kV. Concentration of tetraarginine is 1 mg/mL.

	5 kPa		5 kV	
Time [s]	USP Tailing	Height [mAU]	USP Tailing	Height [mAU]
3	1.12	153	1.17	21
6	1.48	236	1.25	49
9	1.69	285	1.36	60

4.3.2.1.2 The influence of tetraarginine concentration

Important factor, which makes electrokinetic injection inappropriate, is that it prefers cations during injection. Previous experiments were done with 1 mg/mL of tetraarginine. The influence of higher and lower concentration was tested. Results are shown in table 4.4.

Table 4.4. Tetraarginine peak parameters for electrokinetic injection by 5 kV and 10 kV. Concentration of tetraarginine is 0.5 or 2.5 mg/mL

Time [s]	USP Tailing	Height [mAU]	USP Tailing	Height [mAU]
0.5 mg/mL TA	5 kV		10 kV	
3	1.12	1.8	1.09	6.3
6	1.06	8.6	1.06	17.2
9	1.07	12.8	1.09	23.2
2.5 mg/mL TA				
3	1.15	19.4	1.22	46.9
6	1.25	64.8	1.48	106.3
9	1.37	81.1	1.59	124.9

As you can see above, the influence of low tetraarginine concentration on the USP Tailing factor is also not significant while using different periods of electrokinetic injection, only height of peak changes due to greater amount injected during longer injection period. The influence of high tetraarginine concentration on the USP Tailing factor is significant, the longer the period is, the higher the USP Tailing. According to the acquired data, hydrodynamic injection by 5 kPa for 3 s and 1 mg/mL concentration of tetraarginine were maintained for further measurements. This type of injection led to the best values of USP Tailing factor, although the differences between electrokinetic and hydrodynamic injection were insignificant, and the heighest peak, which is important for higher sensitivity of the developed method.

4.4 Calibration

After optimization of ortho-phosphoric acid concentration and mode of injection, a calibration curve was obtained (Fig. 4.12). It was measured using 60 mM ortho-phosphoric acid and hydrodynamic injection 5 kPa/3 s. 1 mg/mL concentration of tetraarginine was used and samples were measured using a voltage of 15 kV applied for 30 s. Before the voltage was applied, the tetraarginine zone was pushed into the capillary using a pressure of 5 kPa for 210 s. For the limits of detection (LOD) and quantification (LOQ) and the upper limit of linear dynamic range for all following calibration curves were calculated using equation of the calibration curve obtained from MS Excel (table 4.5). As you can see in fig. 4.12, calibration is, however, not linear enough and still has low run-to-run repeatability. The calibration also has small linear dynamic range.

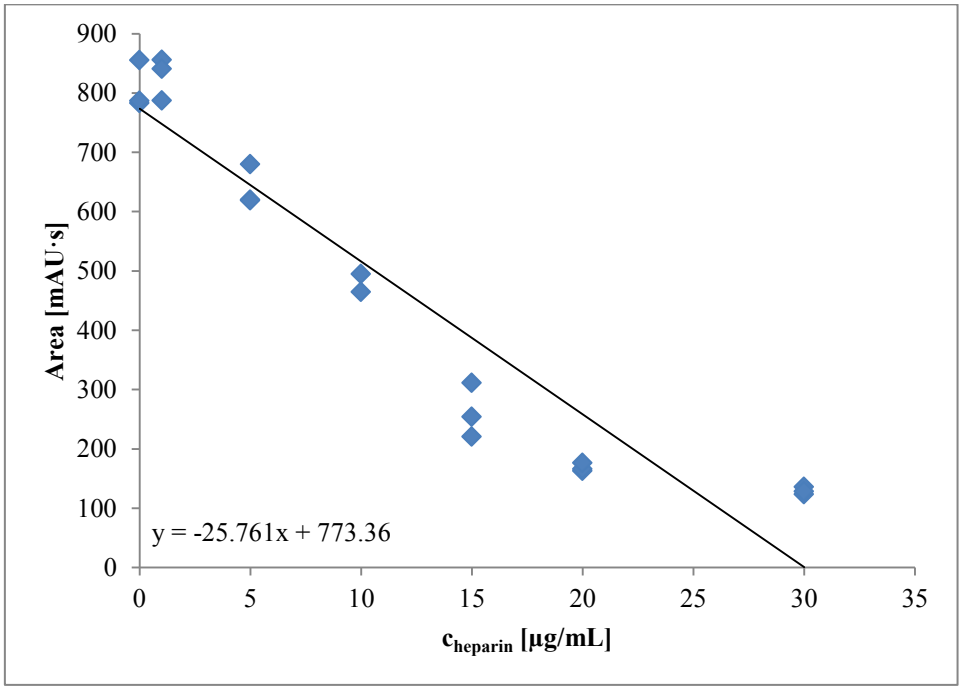

Fig 4.12. Calibration curve obtained using 1.0 mg/mL tetraarginine in sample vial and 60 mM orthophosphoric acid. Initial pressure 5 kPa/210 s was used.

Table 4.5. Parameteres of the developed method with 1.0 mg/mL of tetraarginine in sample vial and 60 mM ortho-phosphoric acid. Initial pressure 5 kPa/210 s was used.

Parameter	Value
R^2	0.8990
LOD [µg/mL]	3.9
LOQ [µg/mL]	13.0
LDR [µg/mL]	3.9 – 26.0

To achieve wider linear dynamic range, a higher concentration of tetraarginine was used. The higher the tetraarginine concentration is, the higher the linear dynamic range should be, because tetraarginine can bind more heparin. The 2.5 mg/mL concentration of tetraarginine was used to obtain a new calibration curve under the same experimental conditions. You can see parameters of the method in table 4.6. Linear dynamic range is wider, however, the run-to-run repeatability is the same as for 1 mg/mL of tetraarginine and deviations from linear decrease of tetraarginine peak area occur for concentrations of heparin from 5 to 20 µg/mL (Fig. 4.13). This is the most important range according to doses used in medical practice [5, 6].

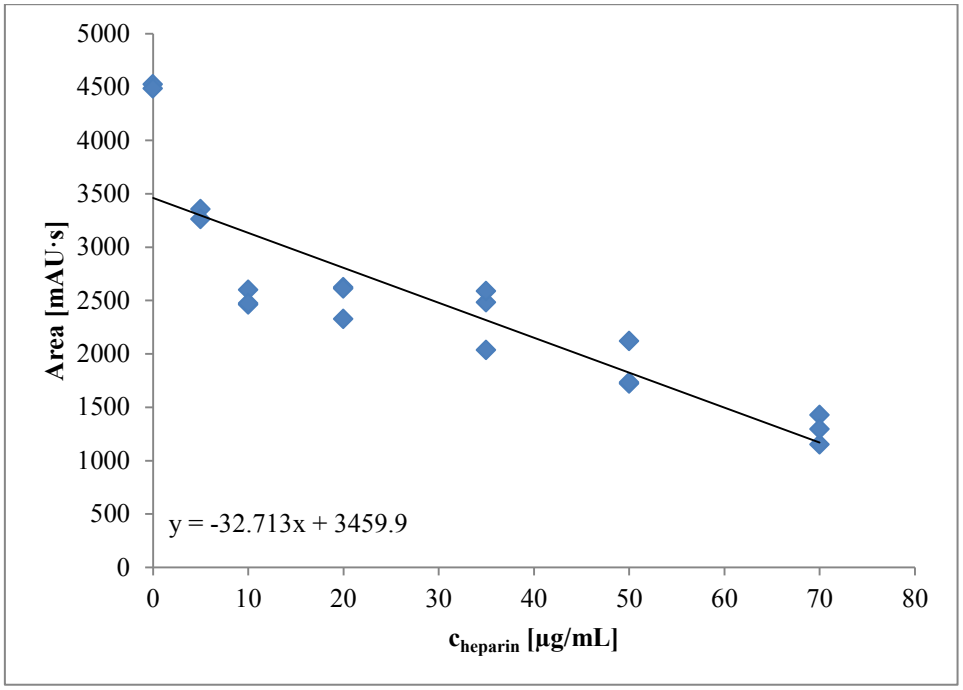

Fig 4.13. Calibration curve obtained using 2.5 mg/mL tetraarginine and 60 mM ortho-phosphoric acid. Initial pressure 5 kPa/210 s was used.

Table 4.6. Parameters of developed method with 2.5 mg/mL of tetraarginine in sample and 60 mM ortho-phosphoric acid. Initial pressure 5 kPa/210 s was used.

Parameter	Value
R^2	0.7384
LOD [µg/mL]	1.8
LOQ [µg/mL]	6.0
LDR [µg/mL]	6.0 - 104.0

4.4.1 Arginine

As a next step the replacement of tetraarginine with arginine was tested. Arginine is basic structural unit and should interact even more reproducibly with heparin than tetraarginine and it is also less expensive. Nevertheless, measurements with arginine proved to be impossible, fluctuations in current appeared and often no peak was observable. This could be caused by the fact that the tertiary structure of the resulting complex is very important for interaction between protamine (tetraarginine) and

heparin, but there is none in interaction with so simple molecule as arginine. It was decided to continue measurements with tetraarginine, but it was necessary to resolve problems with insufficient run-to-run repeatability.

4.4.2 Factors influencing RSD

An experiment was suggested to find out what causes the high RSD of peak area. The whole method was tested, always with one step of it skipped. It was found out that the interaction between tetraarginine and heparin itself has no influence on overall RSD. The experiment, on the other hand, showed that the RSD are strongest influenced by pressure used in the beginning of measurement before the voltage is applied. This step is not necessary in calibration measurements, because contrary to measurements of the mobilities, the peak of the observed remaining tetraarginine always migrates as a cation. Skipping of the initial pressure leads to improvement of RSD of 10 following injections below 10 % (Table 4.7).

Table 4.7. The influence of single parts of the whole method on the relative standard deviation of relative peak area.

Procedure	RSD (10 injections)
Whole method using protamine	36.3 %
Whole method using tetraarginine	13.2 %
Whole method, no heparin to BGE added	14.2 %
No voltage, no heparin to BGE added	10.1 %
No initial pressure, no heparin to BGE added	7.8 %
No initial pressure, heparin to BGE added	7.8 %
No initial pressure, no heparin added, 20 mM H_3PO_4	0.9 %

For the next calibration this step was finally left out. Due to fact that higher concentration of tetraarginine leads to deviations from linearity in the most important range of the calibration, 1.0 mg/mL of tetraarginine was used again. Much better calibration curve was now obtained. It is linear in range from 0.0 to 17.8 μg/mL of heparin (Fig 4.14) with determination factor R^2 = 0.9678. You can see method

parameters in table 4.8 and compare them with table 4.5 of method parameters using initial pressure for 210 s.

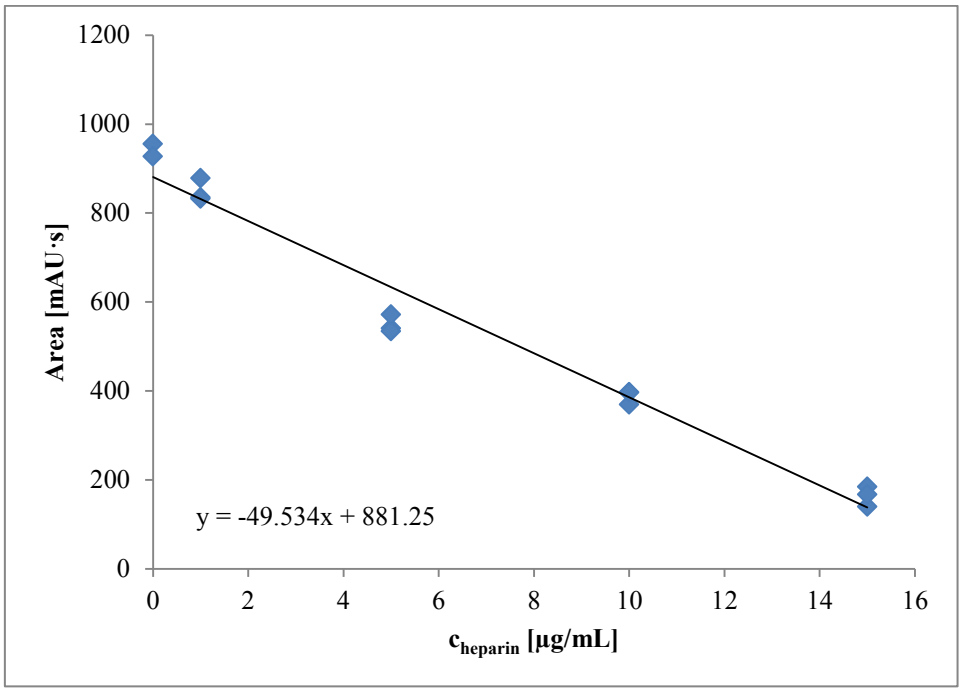

Fig 4.14. Calibration curve obtained using 1.0 mg/mL tetraarginine and 60 mM ortho-phosphoric acid. Initial pressure was skipped and voltage was applied immediately after the injection.

Table 4.8. Parameters of the developed method with 1.0 mg/mL tetraarginine in sample and 60 mM ortho-phosphoric acid. Initial pressure was skipped and voltage was applied immediately after the injection.

Parameter	Value
R^2	0.9678
LOD [µg/mL]	0.9
LOQ [µg/mL]	2.9
LDR [µg/mL]	2.9 - 16.9

This calibration curve is usable for determination of heparin in a sample in the most common concentrations used in surgery. Limit of detection is low enough to distinguish minimal heparin concentration needed for post-operation medication, which is about

1 µg/mL heparin and LDR is wide enough to determine heparin during operations. However the practical implementation still has to be solved.

Another experiment was performed in order to obtain higher run-to-run repeatability. In the previous optimization, the best parameters were obtained from measurement with 60 mM ortho-phosphoric acid because of the highest peak and the best peak efficiency. Also the massive overheating of the capillary was experimentally excluded. Neverthless, the current during measurement reached 100 µA, which can cause inaccuracies because of heat. A run-to-run repeatability from 10 injections was measured using 20 mM ortho-phosphoric acid. As you can see in table 4.7 the RSD is less than 1.0 %, which is the best result of all. Then the calibration curve was measured (Fig. 4.15). It has good parameters with low limit of detection (table 4.9), the best run-to-run repeatability, and the linear dynamic range covers the most important range of heparin concentrations.

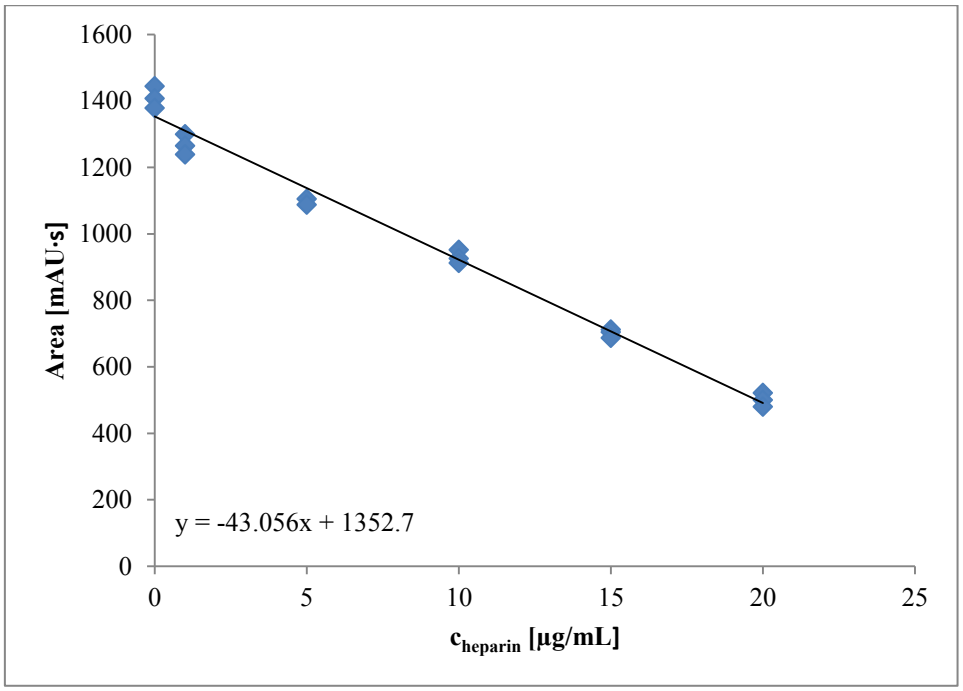

Fig 4.15. Calibration curve obtained using 1.0 mg/mL tetraarginine and 20 mM ortho-phosphoric acid. Initial pressure was skipped and voltage was applied immediately after the injection.

Table 4.9. Parameters of developed method with 1.0 mg/mL of tetraarginine in sample and 20 mM ortho-phosphoric acid. Initial pressure was skipped and voltage was applied immediately after the injection.

Parameter	Value
R^2	0.9857
LOD [µg/mL]	1.9
LOQ [µg/mL]	6.3
LDR [µg/mL]	6.3 - 29.5

5. Conclusion

The interaction between protamine and heparin in the environment of capillary zone electrophoresis was studied and this study was used to develop a new method for determination of heparin.

In an acidic medium protamine is a cation and heparin is an anion. It was found, that their electrophoretic behavior depends on the ratio of their concentrations as well as on the way the both compounds are mixed. The difference in electrophoretic behavior was observed when protamine and heparin were mixed in a vial and injected together and when protamine was injected as a sample to BGE containing heparin.

When protamine and heparin were mixed before injection in one vial, in an excess of protamine a positively charged complex was created. It's mobility was lower than mobility of protamine. In an excess of heparin, the resulting complex was negatively charged. In the weight ratio of approximately 1:0.5 protamine:heparin, which is consistent with literature, the whole complex was neutral and migrated in the zone of EOF marker. It was found, that the reaction is very fast (in range of seconds) under the used conditions, so the reaction kinetics does not influence observed peak migration times and peak areas.

If heparin was used as a BGE additive the migration time of observed peak did not change with increasing heparin concentration, however the peak area decreased. This led to a hypothesis that the decreasing peak represented only remaining protamine that did not participate in the interaction with heparin. No complex peak was observable probably due to fast broadening of this peak. The reduction of observed protamine peak area was linear and it was directly proportional to the concentration of heparin in BGE. It also depended on the period of time, for which the voltage was applied on the system. If the voltage was applied for the whole time of the measurement, the peak shape deteriorated and the peak broadened. Better results were obtained with shorter voltage period followed by mobilisation of protamine using a pressure.

The method for determination of heparin which employs this fact was suggested. Protamine was injected into the capillary filled with ortho-phosphoric acid using heparin as an additive. Then it was pushed into the capillary using pressure and after this the voltage was applied. Concentration of ortho-phosphoric acid, the way and time of injecting and the voltage applied period were optimized. The best results were obtained using 60 mM ortho-phosphoric acid, hydrodynamic injection of 5 kPa for 3 s and voltage of 30 kV applied for 30 s.

Based on poor run-to-run repeatability it was assumed, that protamine-heparine interaction was too complex due to varying protamine composition. Substitution of protamine with tetraarginine was suggested as tetraarginine has a similar structure but in the same time is well defined. Tetraarginine showed similar behavior as protamine in the case when it was mixed with heparin before injection as well as when it was used as a BGE additive. Its structure, however, led to better run-to-run repeatability. Due to still poor run-to-run repetability an experiment was done to show, which part of the method has the biggest contribution to inaccuracies. It revealed, that RSD under 10 % is possible to obtain in case the initial pressure was skipped. The interaction between tetraarginine and heparin itself had no influence on RSD in contrast to interaction of heparin with protamine. This experiment also showed that though 60 mM ortho-phosphoric acid provides sharper and higher peaks, measurements done in 20 mM ortho-phosphoric acid led to less inacurracies and RSD lower than 1.0 %.

Two similar calibration curves were obtained using 20 mM and 60 mM ortho-phosphoric acid, 30 s voltage application period and no initial pressure. Both curves had low limits of detection. The one obtained using 60 mM ortho-phosphoric acid was better to determine lower concentrations of heparin with LOD of 0.9 μg/mL, LOQ of 2.9 μg/mL and LDR upper limit of 16.9 μg/mL. This is important in post-operation medication, where THC is about 1.0 μg/mL of heparin. On the other hand, the calibration curve obtained using 20 mM ortho-phosphoric acid with LOD of 1.9 μg/mL, LOQ of 6.3 μg/mL and LDR upper limit of 29.5 μg/mL covers the range of heparin concentrations from 15.0 to 25.0 μg/mL, which is used during operations.

All of these experiments were done in deionised water which led to necessity of testing more complex matrix before it would be possible to try to use these methods in medical practice. The next step is to find out any interferences with proteins present in blood plasma and eventually to suggest a process which would suppress all interfering interactions.

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