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Biomolecular interaction studies of xanthurenic acid using capillary electrophoresis

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Abstract Xanthurenic acid, which is a metabolite product emanating from the degradation of tryptophan by the enzyme indoleamine-2,3 dioxygenase (IDO), is present in our body and can induce cell death and also denaturation of some proteins by binding to them. The primary goal of this project was to study the interaction between xanthurenic acid and insulin at physiologic pH. The affinity of xanthurenic acid to calcitonin and cyclodextrins was also determined. To study the mentioned associations, capillary electrophoresis was used where focus was on the partial filling technique. The xanthurenic acid and insulin interacted weakly whereas no interaction was detected between the acid and calcitonin. The affinity of xanthurenic acid to γ -cyclodextrin and 2-hydroxypropyl β -cyclodextrin was determined to 60 M^{-1} and 90 M^{-1} , respectively. Furthermore, it was shown that the 2-hydroxypropyl groups facilitated the binding of xanthurenic acid to 2-hydroxypropyl β -cyclodextrin.		
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Martin Holmgren

Sammanfattning

Xanturensyra är en metabolit som bildas vid nedbrytningen av aminosyran tryptofan. Syran interagerar med bland annat proteiner vilket kan leda till denaturering och/eller inaktivering av dessa. När xanturensyra binder till insulin leder det till en inaktivering av insulin då inbindningen till insulinreceptorerna på cellerna hindras. Målet med projektet var att undersöka hur starkt xanturensyra binder till insulin. Interaktionen mellan syran och kalcitonin samt cyklodextriner studerades också. Genom att undersöka insulins samt de senare två molekylnas interaktion med xanturensyra var det möjligt att bestämma till vilken slags struktur som syran binder till starkast. Tekniken som användes för att studera interaktionerna var kapillärelektrofores. Kapillärelektrofores är en separationsteknik där små provvolymmer snabbt och effektivt kan analyseras. Separationen bygger på skillnader i laddning-massa förhållande mellan olika molekyler. När provet injiceras i kapillären och en spänning appliceras kommer olika molekyler att vandra olika snabbt igenom kapillären. Genom att titta på hur den elektroforetiska hastigheten ändras när två molekyler binder till varandra kan man bestämma hur stark interaktionen är. Resultatet av detta projekt är att xanturensyra interagerar starkare med cyklodextriner jämfört med insulin och kalcitonin. Insulin visade en tendens att binda till syran, men styrkan i interaktionen kunde inte bestämmas vid undersökta förhållande.

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1 Introduction

Xanthurenic acid, which is a metabolite product emanating from the degradation of tryptophan by the enzyme indoleamine-2,3 dioxygenase (IDO), is present in our body and can induce cell death and also denaturation of some proteins by binding to them [1]. The concentration of xanthurenic acid in blood and urine is normally 0.7 and 5-10 μM , respectively. People who suffer from vitamin B6 deficiency, for instance tuberculosis, show a several fold increase in the concentration of the acid. The structure of xanthurenic acid is shown in Figure 1 below.

The primary goal of this project was to study the interaction between xanthurenic acid and insulin by using capillary electrophoresis. Even though focus was on insulin, some efforts were also devoted to studying the binding of xanthurenic acid to calcitonin and cyclodextrins. The latter two were chosen to elucidate which of them interacts stronger with xanthurenic acid. In contrast to insulin that is negatively charged, calcitonin is positively charged at physiological pH and cyclodextrins are electrically neutral molecules. By comparing the interaction between these three compounds and xanthurenic acid, it was possible to conclude what type of structure xanthurenic acid binds most strongly to.

It has been previously shown that xanthurenic acid interacts with insulin and prevents insulin from binding to the insulin receptor. The interaction between xanthurenic acid and insulin is unfavourable in the presence of Zn^{2+} [2]. Addition of Zn^{2+} causes the xanthurenic acid-insulin complex to break down, which activates the insulin molecule and it can bind to the insulin receptors once again. The acid probably binds to the imidazole group of the histidine residue in the β chain of insulin, but how strongly it binds is not known.

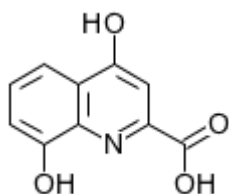


Figure 1. The chemical structure of xanthurenic acid.

The insulin used in this project was bovine insulin, which differs from human insulin in only three amino acid residues and is biologically functional in humans. Insulin consists of two peptide chains that are called α and β , respectively, and they are bound to each other by two disulfide bonds [3]. The α -chain is an acidic peptide whereas the β -chain is a basic peptide. The sequence of the β -chain was determined in this project, see Appendix 2.

2 Theory - Capillary electrophoresis

Electrophoresis as a separation method was first invented in 1937 by Tiselius [4]. In the late 1960s Stellan Hjerten was one of the pioneers who first tried to run electrophoresis in open capillaries. Capillary electrophoresis has since then improved and is today an invaluable tool for analysis of biological compounds. In comparison to classical electrophoresis, the negative influence of temperature gradients on the efficiency is greatly decreased since separations are carried out in narrow capillaries, e.g. 5-100 μm in diameter. Furthermore, the separation time is short, sample volumes in the nanoliter range and high efficiency are some advantages of

capillary electrophoresis over classical electrophoresis [5, 6]. In this project capillary zone electrophoresis (CZE) has been used where the capillary is filled with a buffer, referred to as background electrolyte (BGE). Apart from CZE, there are several other capillary electrophoresis modes available, for instance, capillary isotachopheresis (CIP), micellar electrokinetic chromatography (MEKC) and capillary isoelectric focusing (CIF). The method of choice depends mainly on the physical and chemical properties of the compound studied and the goal of the separation.

As mentioned above, capillary electrophoresis (CE) is a separation technique based upon differences in mobility of different ions under the influence of an electric field [7]. At steady state the electrophoretic mobility, μ , of a molecule can be described by the following equation;

$$\mu = \frac{E_F}{|F_F|} = \frac{q}{6\pi\eta r} \quad \text{Eq. 1}$$

where E_F stands for the electrostatic force, F_F corresponds to the friction force, q is the charge of the molecule, r is the radius of the molecule, and η is the viscosity. Consequently, highly charged small ions have higher mobility than large weakly charged ions.

2.1 Electroosmotic Flow (EOF)

Most commonly used capillaries are made of fused silica where silanol groups (Si-OH) are present on the interior wall of the capillary. If the pH value in the running buffer is higher than three, these groups will be ionized to negatively charged silanoate ions (Si-O⁻). Positively charged cations in the BGE will be attracted to the negatively charged silanoate groups resulting in the formation of two layers of cations on the capillary wall, which creates a potential called the zeta potential. The first layer that is closest to the wall is called the fixed layer because it is bound tightly to the silanoate groups. The outer layer is less tightly bound and is called the mobile layer. Upon application of a voltage, the outer layer is pulled towards the negatively charged cathode giving rise to a flow of buffer solution referred to as electroosmotic flow (EOF) [8].

The total velocity of a certain molecule is the sum of its electroosmotic (V_{eo}) and electrophoretic velocity (V_{ep}). When the electroosmotic flow is greater than the electrophoretic mobility of the anions present in the sample, both cations and anions are carried along with the buffer solution towards the cathode. Those molecules that are neutral, regardless of size, will move with the EOF. Consequently, the separation sequence is as follows; first cations, then neutral molecules and last anions [5, 8].

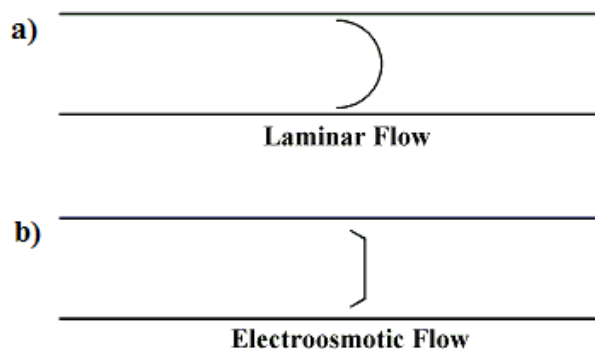


Figure 2. a) The flow profile generated by an ordinary pump. b) The flow profile of electroosmotic flow. In comparison to the laminar flow, the flat flow profile of the EOF does not contribute to zone broadening.

In contrast to the flow profile generated by an external pump that is laminar, the flow profile of EOF is almost flat, which is depicted in Figure 2 [4]. As a result, the band broadening originating from the flow profile is not as prominent in capillary electrophoresis as in for instance HPLC. The strength of the electroosmotic flow is mainly dependent on the level of ionization of the silanolate groups on the interior capillary wall. The charge density of the wall is in turn proportional to the pH of the BGE and the electroosmotic flow increases with pH until all silanol groups on the wall of the capillary are ionized. Neutral compounds, such as DMFA, benzene or mesityl oxide can be used as EOF markers. The following equations describe the velocity and mobility of the EOF;

$$v_{eo} = \frac{E \cdot \epsilon \cdot \zeta}{\eta} \quad \text{Eq. 2}$$

where E is the electrical field, ζ is the zeta potential at a certain distance from the negatively charged wall and ϵ is the dielectric constant.

$$\mu_{eo} = \frac{\epsilon \cdot \zeta}{\eta} \quad \text{Eq. 3}$$

There are occasions when the EOF has to be suppressed or completely eliminated. For instance, adsorption of positively charged analytes onto the capillary wall is undesirable because it gives rise to peak broadening and loss of separation efficiency [9]. To reduce such interactions, the capillary wall must be modified, which in turn changes the magnitude and/or the direction of the EOF. Most commonly the magnitude of the EOF is manipulated by changing the pH or the ionic strength of the BGE. As mentioned, the pH of the BGE affects the ionisation of silanol groups at the capillary surface. An increase in the ionic strength of the BGE reduces the zeta potential due to compression of the double layer, which suppresses the EOF [10]. The disadvantage of enhancing the ionic strength, however, is an increase in the electrical current within the separation column which results in Joule heating and thereby reduced separation efficiency. Sometimes it is possible to compensate this adverse effect by reducing the applied electric field [4].

There are basically two different ways of coating the capillary walls; dynamic and static coatings. The major difference between these two is how the attachment of the coating to the capillary wall surface is done. The coating is bound covalently in static coatings whereas it is

adsorbed onto the surface in dynamic coatings. In comparison, static coatings are stronger than dynamic coatings, but, on the other hand, static coatings are more labour intensive and generally more expensive than dynamic coatings [9].

2.3 Mobility

The mobility of the electroosmotic flow can experimentally be calculated as follows:

$$\mu_{eo} = \frac{L_{tot} \bullet L_{det}}{U \bullet t_0} \quad \text{Eq. 4}$$

where L_{tot} is the total length of the capillary, L_{det} is the length of the capillary from the inlet to the detector, U is the applied voltage and t_0 is the migration time for an uncharged molecule. The observed mobility, which is the vectorial sum of the electroosmotic mobility and the electrophoretic mobility of an ion, can be calculated by the same equation as above but substituting t_0 with t_{obs} , which is the migration time of the analyte.

$$\mu_{obs} = \frac{v_{eo}}{E} = \frac{L \bullet I}{U \bullet t_{obs}} \quad \text{Eq. 5}$$

The effective electrophoretic mobility μ_{eff} is easily calculated by the subtraction of μ_{eo} from μ_{obs} :

$$\mu_{eff} = \mu_{obs} - \mu_{eo} \quad \text{Eq. 6}$$

2.4 Efficiency

The efficiency of capillary electrophoresis separations is generally much higher than the efficiency of other separation techniques. The high efficiency of CE is mainly due to the lack of Eddy diffusion, and the flat flow profile of the electroosmotic flow mentioned above, see Figure 2. The efficiency, or the number of theoretical plates, is given by the following equation assuming that diffusion is the dominant factor for band broadening:

$$N = \frac{E \bullet L \bullet \mu_{obs}}{2 \bullet D} \quad \text{Eq. 7}$$

where D is the diffusion coefficient of the analyte. The equation shows that the efficiency decreases as the diffusion coefficient (D) of the analyte increases. Efficiency is adversely affected by factors such as the temperature gradient in the capillary, interaction between the analyte and capillary surface, pressure difference between the inlet and outlet of the capillary and injection plug length. Ideally, the injection length of the sample should be 1-2% of the total length of the capillary. If the injected plug is longer than the recommended length band broadening is to be expected.

2.5 Resolution

Separation in capillary electrophoresis is regulated by selectivity and efficiency. Since the efficiency is very high, a small difference in the mobility of analytes makes it possible to completely separate the analytes from each other. It means that a small increase in the selectivity leads to a large increase in the resolution. Assuming that diffusion is the only factor that contributes to band broadening the resolution factor (R_s) can be calculated with the following equation [4]:

$$R_s = 0.177 \Delta \mu_{eff} \sqrt{\frac{E \bullet L}{D((\mu_{eff1} + \mu_{eff2})/2 + \mu_{eo})}} \quad \text{Eq. 8}$$

As mentioned before, separation in CE is based on differences in charge and/or mass of the interacting molecules. Even a small charge difference is enough for obtaining an adequate separation because the mobility is linearly proportional to the charge of the molecule. On the other hand, a large difference in mass is required in order to obtain a sufficiently good separation between two molecules of the same charge [11, 12]. In other words, the molecular mass is not linearly related to the mobility. The relationship between charge, mobility and mass of a small peptide is as follows [17];

$$\mu \propto q / m^{2/3} \quad \text{Eq. 10}$$

Where q is the charge and m is the mass of the molecule.

2.6 Determination of association constants by means of capillary electrophoresis

By studying the mobility change of a certain molecule when it interacts with another molecule of different mobility it is possible to determine the binding constant between the two compounds. There are several types of interactions such as protein-protein, protein-carbohydrates, protein-metal, protein-drug, protein-DNA and DNA-DNA interactions that can be studied [11].

Although CZE is a powerful technique for determination of association constants, there are also some limitations; for instance, it is impossible to evaluate the interaction between neutral molecules since they move at the same mobility with the EOF.

There are several related capillary electrophoresis methods that can be used for determining association constants. The choice of method depends mainly on the properties of the molecules studied [13]. In this study, the determination of association constants was based on the conventional CE mode as well as on the partial filling technique (PFT). In the conventional method, one of the interacting molecules was added to the BGE while the other one was injected in the capillary as an analyte.

In the PFT the effective length of the capillary is partially filled with BGE containing ligand at certain concentration, see Figure 3. During the separation the capillary ends are connected with ligand free BGE. The PFT is especially useful when UV-absorbing ligands such as

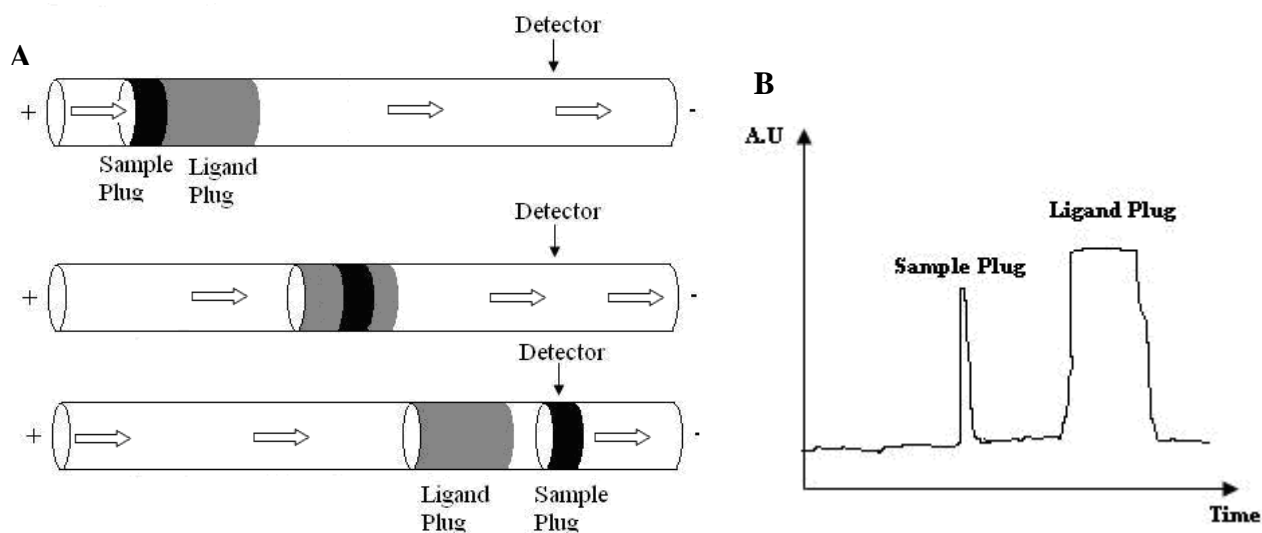


Figure 3. Schematic illustration of the flow through partial filling technique (A). A short ligand plug is applied into the capillary prior to the injection of the sample. During the separation, the analyte will migrate through the ligand plug and will interact with the ligand. The mobility of the analyte is higher than the mobility of the plug and thus it will be detected before the ligand (B).

proteins are used. One of the benefits of using the PFT is that the consumption of the ligand, which sometimes can be very expensive, is reduced. When the analyte and the ligand absorb at different wavelengths or the analyte absorbs strongly it is possible to detect the analyte inside the plug, which is illustrated in Figure 4. The association constant can be determined by varying the plug length or the concentration of the ligand and studying the change in the migration time of the analyte [14, 15].

The length of the plug is adjusted depending on the affinity of the interacting molecules for each other. For instance, the association between two tightly interacting molecules can be estimated by applying a short plug in the capillary. The effective length of the plug will be longer than the applied one (when the analyte and ligand migrate in the same direction but at different velocities) or shorter than the applied plug (when the ligand and analyte migrate in opposite directions).

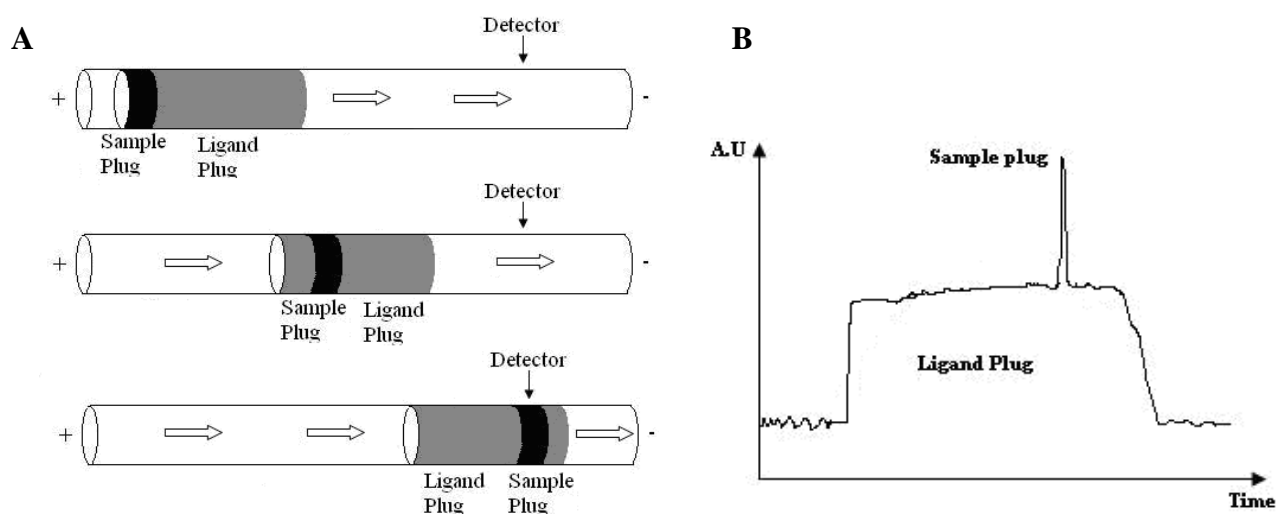


Figure 4. Schematic illustration of the partial filling technique (PFT) (A) when the analyte is detected inside the ligand plug (B).

2.7 Calculation of association constants

2.7.1 Concentration dependent aggregation of insulin monomers

Two insulin monomers (m) can form a dimer (D)



The association constant is calculated by the following formula:

$$K = \frac{[D]}{[m]^2} \quad \text{Eq. 10}$$

The total concentration of insulin is

$$C_0 = [m] + 2[D] \quad \text{Eq. 11}$$

The effective mobility of insulin can be written as follows:

$$\mu_{eff} = \frac{[m]}{C_0} \mu_{(m)} + \frac{C_0 - [m]}{C_0} \mu_{(D)} \quad \text{Eq. 12}$$

where $\mu_{(m)}$ and $\mu_{(D)}$ are the electrophoretic mobilities of monomeric and dimeric insulin. The combination of equations 9, 10, 11, and 12 results in equation 13:

$$\mu_{eff} = (\mu_{(m)} - \mu_{(D)}) \times \frac{\sqrt{1 + 8KC_0} - 1}{4KC_0} + \mu_{(D)} \quad \text{Eq. 13}$$

Equation 13 can be used to determine the self association constant between insulin monomers by using curve fitting. In curve fitting different values for K and $\mu_{(D)}$ are inserted in eq. 13 in order to minimize the difference between the calculated and the observed μ_{eff}

2.7.2 Determination of association constants between xanthurenic acid and neutrally charged cyclodextrins

It was assumed that one xanthurenic acid (X) binds to one cyclodextrin (C) to form a 1:1 complex (XC):



The association constant is defined by the following formula:

$$K = \frac{[XC]}{[X][C]} \quad \text{Eq. 15}$$

The total concentration of xanthurenic acid is

$$C_0 = [X] + [XC] \quad \text{Eq. 16}$$

Since the concentration of cyclodextrin is chosen to be much higher than that of xanthurenic acid, it was assumed that the total concentration of cyclodextrin was not significantly changed upon complexation. Therefore it is reasonable to approximate the total concentration of cyclodextrin with C. The mobility of xanthurenic acid can be written as follows:

$$\mu_{eff} = \frac{[X]}{C_0} \mu_{(X)} + \frac{C_0 - [X]}{C_0} \mu_{(XC)} \quad \text{Eq. 17}$$

where $\mu_{(X)}$ and $\mu_{(XC)}$ are the electrophoretic mobilities of free and complexed xanthurenic acid, respectively. By combining equations 14-17 the following equation is obtained:

$$\mu_{eff} = \left(\frac{1}{K[C] + 1} \right) \mu_{(X)} + \left(1 - \frac{1}{K[C] + 1} \right) \mu_{(XC)} \quad \text{Eq. 18}$$

Equation 18 can be rearranged as follows:

$$\frac{1}{\mu_{eff} - \mu_{(X)}} = \left(\frac{1}{\mu_{(XC)} - \mu_{(X)}} \right) K \frac{1}{C} + \left(\frac{1}{\mu_{(XC)} - \mu_{(X)}} \right) \quad \text{Eq. 19}$$

$\frac{1}{\mu_i - \mu_x}$ is plotted versus $\frac{1}{C}$ and K is obtained by dividing the intercept with the slope of the resulting plot.

3 Experimental

3.1 Instrumentation

3.1.1 Capillary electrophoresis

The experiments were performed on two different instruments; Beckman P/ACTM MDQ system and HP^{3D}CE. The two instruments were both equipped with DAD -detectors and two wavelengths, 223 nm and 340 nm, were simultaneously monitored. Two different types of capillaries were used; neutral coated capillaries (acrylamide coating, the diameter of capillaries was 50 μ m, total length 60 cm and length to detector 50 cm) and untreated fused silica capillaries (diameter 75 μ m, total length 63 cm and length to detector 54.5 cm). The latter was used in the experiments 3.3.1-3.3.2.3 and the former was used in 3.3.2.4-3.5.

The fused silica capillary was washed by applying a pressure of 40 psi for 4 min with first 0.1 M NaOH then deionized water and finally background electrolyte (BGE). The same procedure was done for the neutral coated capillary but the NaOH wash was omitted. Two different buffers were used as BGEs; 25 mM tris(hydroxymethyl)aminomethane, 192 mM

glycine at pH 8.4 and 25 mM phosphate buffer at pH 7. The analytes were dissolved in the same buffer as was used as BGE.

Injection of sample was done by applying 0.5 psi for 5 s and the sample was dissolved in the BGE (see section 3.3). Mesityloxide was used as an EOF marker when fused silica capillaries were used. The analyte was detected at the cathodic side of the capillary when using the fused silica whereas the solutes were detected at the anodic end of the neutral coated capillary (reverse polarity mode) when using the neutral coated capillary. The capillary was thermostated at 20 ± 0.5 °C and applied voltage varied between 30 kV and -15 kV.

3.1.2 Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

The instrument used for analysis of insulin and 2-Hp-CD was MALDI-TOF-MS autoflex from Bruker (Bruker Daltonics, Bremen, Germany). The instrument was equipped with a pulsed nitrogen laser working at 337 nm. One μ l of the sample was mixed with 1 μ l of the matrix, and 1 μ l of this mixture was applied to the MALDI-plate (Bruker Scout 384/400 AnchorShip). A number of dilutions with additional matrix were made. The analyses were performed in positive reflector mode with delayed extraction at an accelerating voltage of 20 kV and a variable voltage reflectron. The ion selector was set at a chosen m/z value and the presence of neighbouring ions was considered by setting a window around the target mass ($\pm 10\%$). The laser intensity was kept in the interval 20-25% and 200 shots were obtained. The data interval was adjusted to fit the actual protein.

3.2 Chemicals

The following chemicals were obtained from Merck: β -cyclodextrin, γ -cyclodextrin, glycine and phosphoric acid. Bovine insulin, bovine insulin chain β , 2-hydroxypropyl β -cyclodextrin, hydroxypropyl methyl cellulose, mesityloxide, xanthurenic acid (4,8-dihydroxyquinoline-2-carboxylic acid) and tris(hydroxymethyl)aminomethane were obtained from Sigma Aldrich.

3.3 Procedures

3.3.1 Concentration dependent aggregation of insulin

Different concentrations of bovine insulin ranging from 10 mM to 300 mM were analyzed by CZE at pH 8.4 to estimate the self association constant between the insulin monomers. Mesityloxide was added to the insulin sample as an EOF marker to a final concentration of 5 mM and the applied voltage was 10 kV. Tris buffer was used as BGE.

3.3.2 Interactions between xanthurenic acid and insulin

3.3.2.1 Mixture of insulin and xanthurenic acid as analyte

Mixtures of insulin and xanthurenic acid were analyzed with CZE using Tris buffer as BGE. The concentration of insulin was held constant at 50 μ M and the concentration of xanthurenic

acid was varied between 0.1mM to 5 mM. In addition, the same concentration series were analyzed in the absence of insulin. Applied voltage was 10 kV.

3.3.2.2 Insulin in the BGE

Different concentrations of insulin were dissolved in the BGE; 0.0 μ M, 20 μ M and 100 μ M. Two samples of xanthurenic acid, 20 μ M and 100 μ M, were injected separately and the mobility of the acid was measured when the concentration of insulin in the BGE was varied. The voltage was held constant at 10 kV. Tris buffer was used as BGE.

3.3.2.3 Xanthurenic acid as separation plug in PFT

Up to 7 % of the total volume of the capillary was filled with 5 mM xanthurenic acid. Insulin at 50 μ M was then injected into the capillary as an analyte. As a negative control, a 7 % xanthurenic plug was analyzed without the injection of insulin. The voltage was set to 10 kV. Tris buffer was used as BGE.

3.3.2.4 Insulin chain β as separation plug in PFT

A plug of 1 mM insulin chain β was injected and thereafter 0.25 mM xanthurenic acid was injected into the capillary as an analyte. The insulin plug length was varied between zero and 50 cm. In these experiments the current was held constant at 15 μ A. Phosphate buffer was used as BGE.

3.4 Calcitonin

The capillary was partially filled (3 min 5 psi) with 15 mM calcitonin (approximately 30 cm plug length) and 0.25 mM xanthurenic acid was injected and analyzed at 30 kV. Phosphate buffer was used as BGE.

3.5 Cyclodextrin

The capillary was filled with either γ -cyclodextrin, β -cyclodextrin or 2-hydroxypropyl β -cyclodextrin and 0.25 mM xanthurenic acid was then injected. Several different concentrations of each cyclodextrin were used;

γ -cyclodextrin 0-70 mM

β -cyclodextrin 0-5 mM

2-hydroxypropyl β -cyclodextrin 0-15 mM (The molecular mass of 2-hydroxypropyl β -cyclodextrin was determined by MALDI, see Appendix 1)

The applied voltage was 20 kV and phosphate buffer was used as BGE.

4. Results and Discussion

4.1 Prestudies

To determine the monitoring wavelengths, absorbance spectra for xanthurenic acid and bovine insulin were taken in the range of 190 to 600 nm. In 25 mM tris(hydroxymethyl)aminomethane, 192 mM glycine at pH 8.4 insulin had an absorbance maximum at 223 nm. At the same wavelength xanthurenic acid also absorbed strongly. In addition, xanthurenic acid has an extra absorbance maximum at 340 nm. Therefore, the two wavelengths 223 nm and 340 nm were chosen for the detection of the solutes. The signal at 340 nm provided the possibility of identifying xanthurenic acid.

4.2 Concentration dependent aggregation of insulin

Before studying the interaction between xanthurenic acid and insulin, interactions between insulin molecules was studied. It is known that there exists a concentration dependent equilibrium between insulin monomers and its dimeric form [16].

The first experiment aimed to investigate whether the formation of dimers may influence the mobility of insulin. The effective mobility of insulin at different concentrations can be seen in Figure 5. The migration time of insulin increased with increasing concentration of insulin from 10 μM , which was the lowest detectable concentration, to 300 μM . The concentration dependent shift in the mobility of insulin was a result of the formation of dimers which migrated slower towards the cathode than the monomers. Upon dimerization both charge and mass of the molecule doubles (see equation 10) which results in higher effective mobility compared to that of monomers (see section 2.7.1).

The association constant between monomers and dimers was calculated in accordance with equation 13. As can be seen in Figure 5, the calculated values of the effective mobilities were adjusted by curve fitting to minimize the difference between the calculated and the experimentally obtained data (for further information see section 2.7.1). By doing so the association constant was determined to be $7900 \text{ M}^{-1} (1/\text{mol})$.

As shown, the mobility of insulin is concentration dependent and the insulin concentration should therefore be held constant when studying its association with xanthurenic acid.

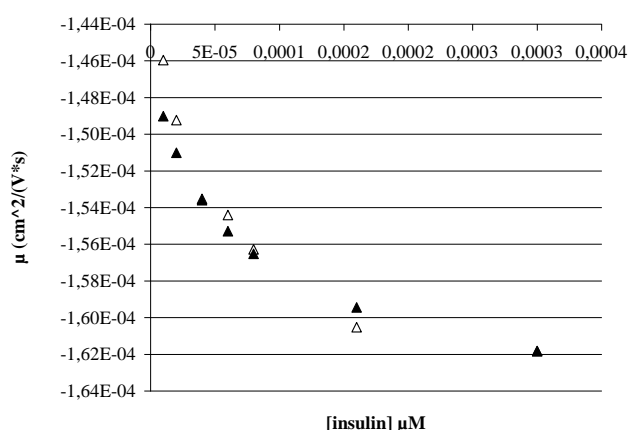


Figure 5. Determination of self association constant of insulin monomers where Δ corresponds to experimentally determined and \blacktriangle corresponds to calculated values of the effective mobility of insulin. For experimental conditions see sections 3.1.1 and 3.3.1.

4.3 Interactions between xanthurenic acid and insulin

4.3.1 Mixture of insulin and xanthurenic acid as analyte

Insulin and xanthurenic acid were mixed (see section 3.3.2.1) and injected into a capillary filled with Tris buffer at 8.4. The insulin concentration in the mixture was held constant at 50 μM (approximately 70% monomers and 30% dimers) while the concentration of xanthurenic acid was varied between 0.1 and 5 mM. It was thought that the interaction between insulin and the acid was so strong that the presence of insulin in the sample could affect the migration time of the acid. However, it was found that the mobility of insulin was not affected by the acid concentration, see Figure 6. The results demonstrated that insulin and xanthurenic acid were not tightly associated. Otherwise, the mobility of insulin would have been affected in the presence of xanthurenic acid. In contrast, the electrophoretic mobility of xanthurenic acid was decreased with increasing concentration of the acid, which may indicate complex formation, see Figure 7.

In order to investigate the mechanism behind this concentration dependent mobility change, the mobility of the acid at different concentrations was investigated. It was found that the electrophoretic mobility of the acid was decreased with its concentration, indicating that the mobility of the acid was not affected by insulin present in the analyte sample, Figure 8. The concentration dependent change in mobility might be due to local EOF or adsorption of the acid to the capillary wall. However, this must be further studied.

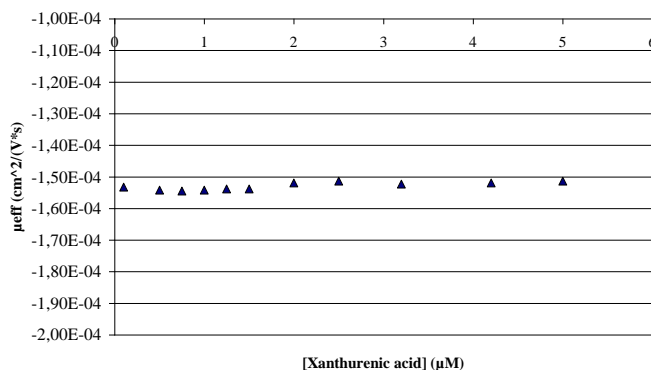


Figure 6. Effective mobility of insulin versus the concentration of xanthurenic acid. For experimental conditions see sections 3.1.1 and 3.3.2.1.

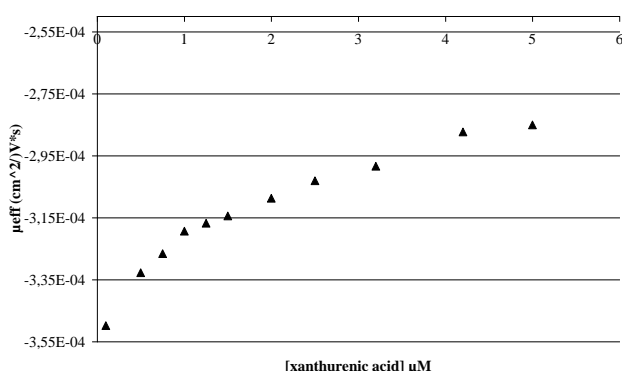


Figure 7. The effective mobility of xanthurenic acid versus the concentration of xanthurenic acid when mixed with insulin. For experimental conditions see sections 3.1.1 and 3.2.2.1.

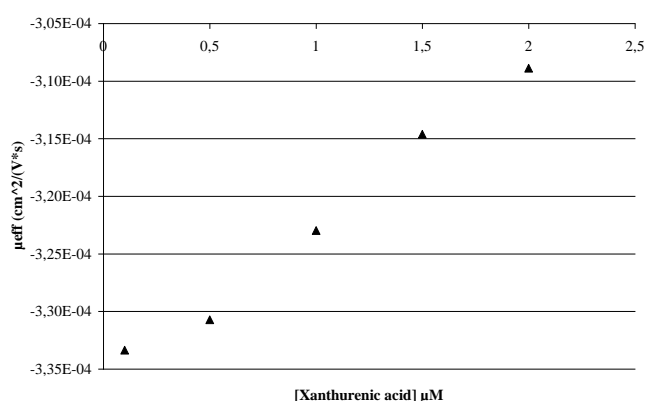


Figure 8. The effective mobility of xanthurenic acid versus concentration of it. As can be seen, the mobility is concentration dependent. For experimental conditions see sections 3.1.1 and 3.3.2.1.

4.3.2 Insulin in the BGE

Two different concentrations of xanthurenic acid were analyzed using BGEs containing different insulin concentrations, i.e., 0, 20 and 100 μM . The results summarized in Figure 9, where the mobility of the acid at 20 μM and 100 μM was plotted versus the concentration of insulin in the BGE, indicated no interaction between insulin and the acid. The mobility of xanthurenic acid was independent of the insulin concentration in the BGE. The results indicate that the association between insulin and xanthurenic acid were not strong enough to significantly affect the migration velocity of the acid.

Ideally when determining association constants, the concentration of the receptor, in this case insulin, in the BGE should be 50-100 times higher than the concentration of the ligand, i.e., xanthurenic acid. However, insulin is poorly soluble ($C_{\text{max}} = 400\mu\text{M}$) and the absorbance of xanthurenic acid is not sufficiently strong to obtain a detectable signal at low concentrations, e.g. 5 μM , to fulfil the above mentioned requirement. Therefore, the concentrations used in this experiment were not optimal to study the association between insulin and xanthurenic acid.

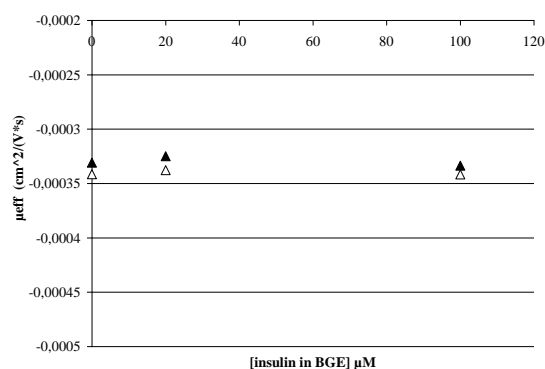


Figure 9. The effective mobility of xanthurenic acid at 20 μM (Δ) and 100 μM (\blacktriangle) versus the concentration of insulin in the BGE. For experimental conditions see sections 3.1.1 and 3.3.2.2.

4.3.3 Xanthurenic acid as separation plug in PFT

The capillary was partially filled with xanthurenic acid at different plug lengths ranging from 0.5 to 5 cm. The experiment was optimized by choosing plug lengths so that insulin moved through the plug and was detected before the acid. Insulin and xanthurenic acid moved in the same direction through the capillary towards the cathode. As a result, the effective plug length that insulin moved through was actually longer than the applied one [15]. Consequently, insulin should have had enough time to interact with xanthurenic acid.

The mobility of insulin was the same regardless of the length of the plug. Surprisingly, a small peak, which had absorbance at both 223 nm and 340 nm, was detected between the insulin and the xanthurenic acid peak, peak C in Figure 10. This peak was first thought to be from a complex between xanthurenic acid and insulin. However, if the small peak originated from the insulin-acid complex the area of the

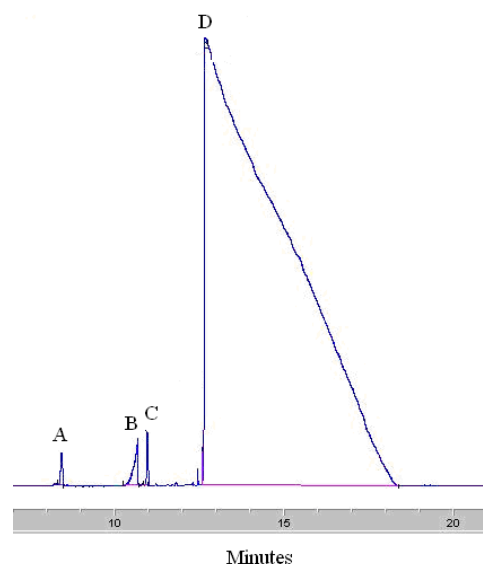


Figure 10. analysis of insulin in a partially filled capillary with xanthurenic acid. Peaks A, B, C and D represent mesityloxide, insulin, impurity from xanthurenic acid and xanthurenic acid, respectively. For experimental conditions see sections 3.1.1 and 3.3.2.3.

peak would change with the concentration of insulin. However, this was not the case, i.e., the peak area was independent of the insulin concentration.

In order to investigate the origin of this peak a 5 cm long plug of the acid was introduced in the capillary and analyzed. The results revealed that this peak originated from an impurity in the xanthurenic acid sample. This was not a surprise since the purity of the xanthurenic acid used was 95 % according to the manufacturer.

The results, which were in agreement with the previous results, indicated that the interaction between the acid and insulin was not possible to measure under the experimental conditions.

4.3.4 Insulin chain β as separation plug in PFT

Insulin chain β is a basic peptide and more soluble than the protein as a whole. Two different experiments were carried out to study xanthurenic acid - insulin chain β interactions.

In the first experiment a sample of xanthurenic acid was analyzed in the presence of different plug lengths of insulin chain β at 1mM. A shift in the migration time of the acid was observed when varying the plug length between zero and 15 cm. However, no change was seen when changing the plug length from 15 cm to 30 cm, as can be seen in Figure 11. The experiment was repeated using several different plug lengths but the result was still ambiguous.

Sometimes a long plug gave a shorter migration time than a short one. The fluctuations in the migration times were too big to draw any conclusion regarding molecular interactions. Still, the shift in migration time indicated interactions between xanthurenic acid and insulin chain β , which is in agreement with previous studies that xanthurenic acid binds to the imidazole group of the histidine residue in β chain [2]. However, this must be verified by further studies.

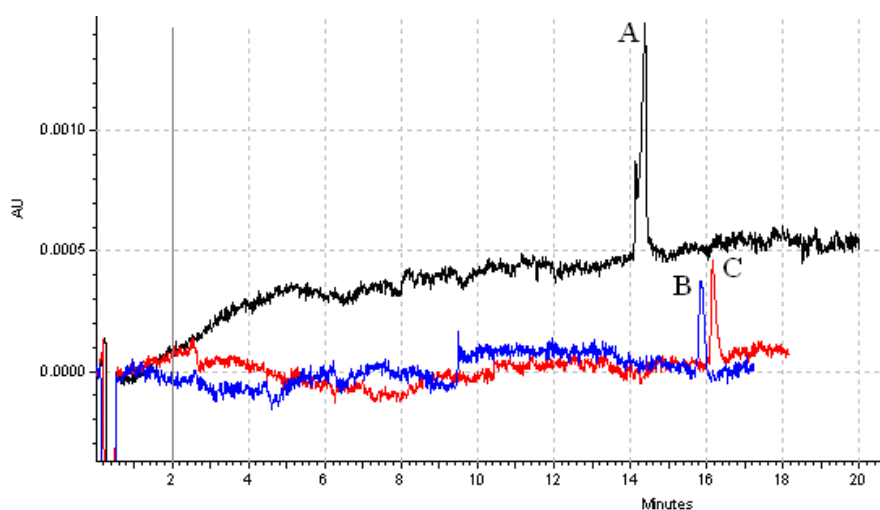


Figure 11. Analysis of xanthurenic acid in the presence of different plug lengths of insulin chain β . The applied plug length of xanthurenic acid was 0 cm, 15 cm and 30 cm in A, B and C, respectively. For experimental conditions see sections 3.1.1 and 3.3.2.4.

4.4 Calcitonin

There is no information on whether xanthurenic can bind to calcitonin. Calcitonin was selected to be studied for two reasons; (a) its high solubility and (b) calcitonin is positively

charged at the experimental pH, which could contribute to electrostatic interaction with the oppositely charged acid. To avoid adsorption of calcitonin to the capillary wall a neutral coated capillary was used.

The capillary was partially filled with 15 mM calcitonin prior to the injection of xanthurenic acid. The migration time of xanthurenic acid was compared to its migration time in the absence of calcitonin. No significant shift was detectable, Figure 12. However, the shape of the peak changed from a broad to a narrow peak. It seemed that the presence of calcitonin in the capillary resulted in a focusing of the xanthurenic acid zone. It might be a result of the increased conductivity in the calcitonin containing BGE that sharpened the acid zone.

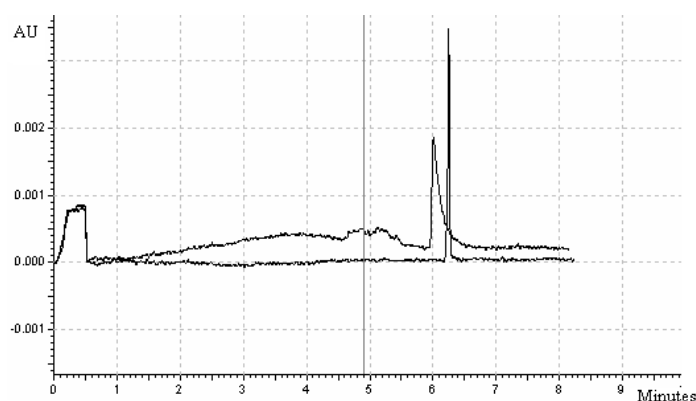


Figure 12. Xanthurenic acid run in the presence and absence of calcitonin plug where the broader peak is without any calcitonin. No detectable interactions were seen. For experimental conditions see sections 3.1.1 and 3.4.

4.5 Cyclodextrin

4.5.1 γ -Cyclodextrin

To study the affinity of xanthurenic acid towards electrically neutral molecules, cyclodextrins, which interact with molecules through hydrophobic interactions and hydrogen bonding, were selected as ligands (for structures of cyclodextrins see Appendix 3).

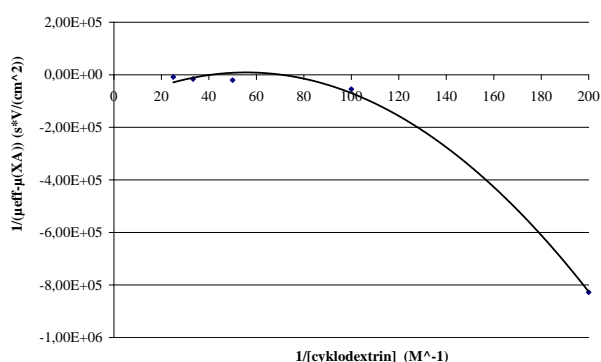


Figure 13. Xanthurenic acid run through different concentrations of γ cyclodextrin in the BGE. Non-linear relationship between the conc. of γ -CD and the effective mobility of the acid when using high concentrations of γ -CD. For experimental conditions see sections 3.1.1 and 3.5.

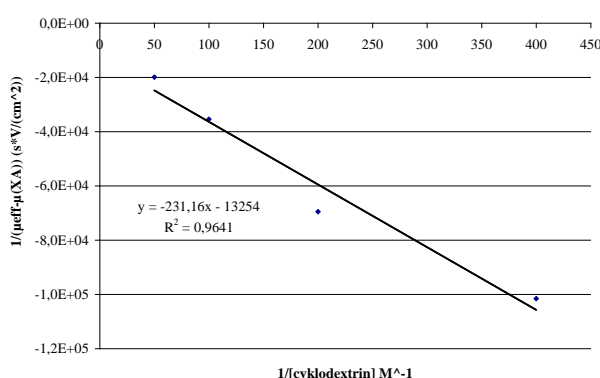


Figure 14. Xanthurenic acid run through different concentrations of γ cyclodextrin in the BGE. For experimental conditions see sections 3.1.1 and 3.5.

The capillary was filled with γ -cyclodextrin (γ -CD) and subsequently xanthurenic acid was injected. γ -CD is not charged and will therefore not influence the conductivity of the BGE. In the absence of the EOF in a neutrally coated capillary the migration velocity of these molecules will be negligible. When varying the concentration of γ -CD between 0-70mM, the migration time of xanthurenic acid changed, indicating interactions between the two compounds. However, no linear relationship between xanthurenic acid and γ -CD concentration was found, see Figure 13. One possible explanation could be that two xanthurenic acid molecules bind simultaneously to one molecule of γ -CD, when the concentration of the CD is high, i.e., 2:1 association. Therefore, the experiment was repeated using other concentrations of γ -CD. By doing so a linear relationship between the migration time and the CD concentration was obtained, which is shown in Figure 14. The association constant was determined to be approximately 60 l/mol. In other words, xanthurenic acid interacted weakly with γ -CD.

4.5.2 2-hydroxypropyl β -cyclodextrin

The same experiment as above was repeated but this time 2-hydroxypropyl β cyclodextrin was used instead of γ -Cyclodextrin. 2-hydroxypropyl β cyclodextrin has several residues of 2-hydroxypropyl and is therefore more likely to interact stronger with xanthurenic acid than it did with γ -cyclodextrin. The higher the concentration of cyclodextrin in the background electrolyte the longer migration time was found to be, see Figure 16. This result confirmed interactions between the two compounds. A linear correlation between the effective electrophoretic mobility of the acid and the CD concentration was obtained, Figure 15. The association constant was determined to be 90 l/mol.

To explore how the 2-hydroxypropyl groups on the cavity of the CD facilitate the binding of xanthurenic acid, experiments were performed using native β cyclodextrin. The migration time increased with 0.6 min when 5mM β cyclodextrin was present in the background electrolyte, which is shown in Figure 17. This was almost 1.5 min less than when the same concentration of 2-hydroxypropyl β cyclodextrin was

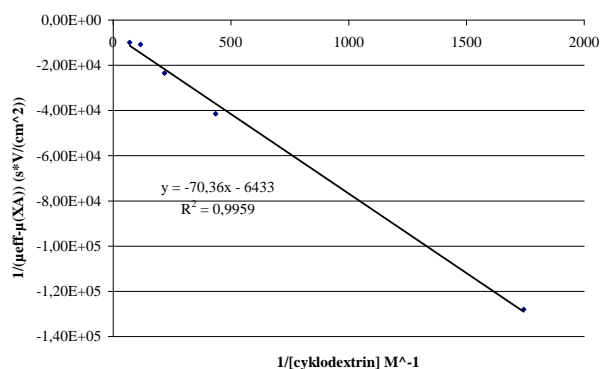


Figure 15. Determination of association constant between xanthurenic acid and 2-hydroxypropyl β -cyclodextrin by using equation 19 under 2.7.2. For experimental conditions see sections 3.1.1 and 3.5.

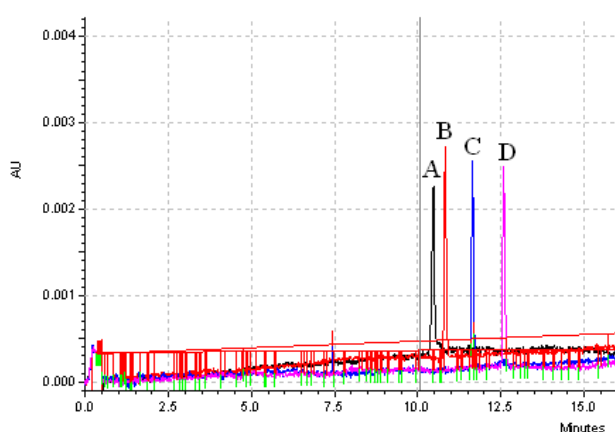


Figure 16. Xanthurenic acid analyzed in BGE containing different concentrations of 2-hydroxypropyl β cyclodextrins. The concentration of CD was 0, 0.5, 2.3 and 4.5 mM in A, B, C and D, respectively. For experimental conditions see sections 3.1.1 and 3.5.

present in the BGE, see Figure 17. The results indicated that the 2-hydroxypropyl groups are involved in the interaction with xanthurenic acid through hydrogen bonding and/or hydrophobic interactions.

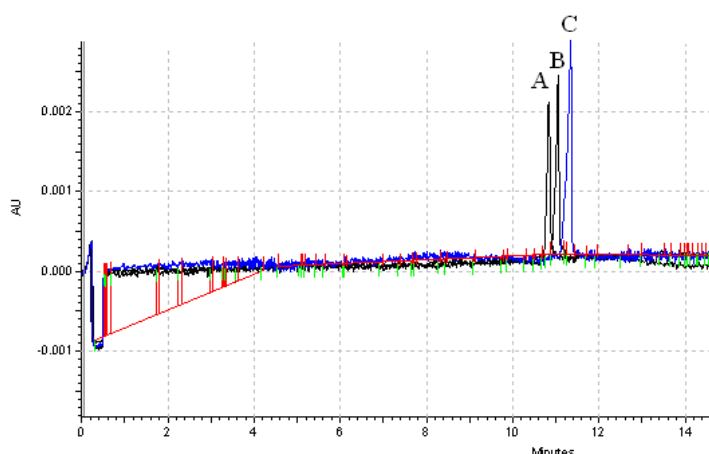


Figure 17. Xanthurenic acid analyzed in BGE containing different concentrations of β cyclodextrins. The conc. of the CD was 0, 2 and 5 mM in A, B and C, respectively. For experimental conditions see sections 3.1.1 and 3.5.

5. Conclusions

To determine the association constant between xanthurenic acid and insulin posed to be much more difficult than first anticipated. One problem was that the mobility of both insulin and xanthurenic acid are concentration dependent meaning that both concentrations should be held constant to the greatest possible extent. However, the major issue was the poor solubility of insulin. If the solubility of insulin was higher it would be much easier to study the interaction between insulin and xanthurenic acid at physiologic conditions. The concentration of insulin in most of the experiments was not optimal for studying the association. Still, if insulin and xanthurenic acid interact strongly, the interaction should have been detected. The conclusion from the experiments done was that xanthurenic acid and insulin seem to interact weakly at pH 7 and 8.4. Interestingly, when the capillary was filled with insulin chain β at high concentrations the migration time increased significantly for xanthurenic acid, which indicated interactions. This must however be verified by further studies.

In order to investigate whether xanthurenic acid interacts with positively charged proteins and with neutral compounds, the association of xanthurenic acid with calcitonin and cyclodextrins were studied. There was no change in the migration time of xanthurenic acid in the presence of calcitonin, which means that xanthurenic acid does not interact with calcitonin. As to cyclodextrins, xanthurenic acid bound to γ -cyclodextrin, 2-hydroxypropyl β -cyclodextrin and native β -cyclodextrin, and the association constants were determined to 60 M^{-1} and 90 M^{-1} , respectively. Furthermore, it was shown that the 2-hydroxypropyl attached to the cavity of the cyclodextrin facilitated the binding of xanthurenic acid to 2-hydroxypropyl β -cyclodextrin.

6. Acknowledgement

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Appendix 1

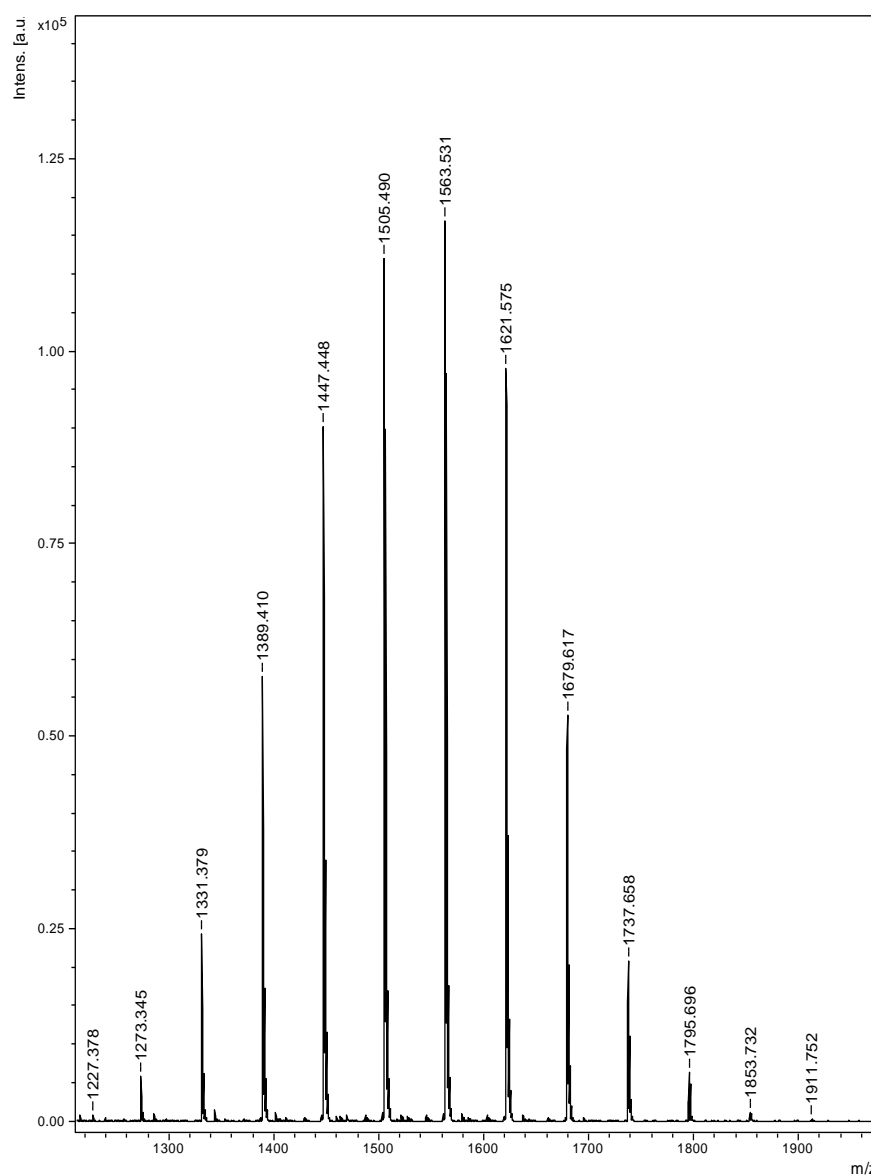
The molecular mass of 2-hydroxypropyl β -cyclodextrin depends on the number of 2-hydroxypropyl attached to the cavity of β -cyclodextrin. It is impossible to determine an exact molecular mass since some molecules will not have any 2-hydroxypropyl whereas others might have seven. An average molecular mass is however possible to determine by taking into account the relative number of molecules having one 2-hydroxypropyl, two 2-hydroxypropyls and so on. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-ToF MS) was used to determine the molecular weight, for experimental conditions see section 3.1.2. The average molecular mass was obtained by taking the sum of the relative MS signal intensity for each molecule multiplied with the corresponding molecular mass, using the following equation:

$$M = \sum_i \frac{I_i}{I_{tot}} M_i$$

The data in the table was used for calculating the relative molecular mass. The average molecular mass was evaluated to be 1509g/mol.

m/z	Intens.	Int%	rel Mmas
1273,345	5472,36	0,009699	12,13704
1331,379	22653,27	0,040151	52,57234
1389,41	54698,81	0,096948	132,5677
1447,448	89339,44	0,158345	225,7125
1505,49	112249	0,19895	295,14
1563,531	113880,6	0,201842	311,1452
1621,575	91192,73	0,16163	258,5389
1679,617	48333,66	0,085666	142,0022
1737,658	18973,68	0,033629	57,69569
1795,696	5593,5	0,009914	17,58425
1853,732	990,4	0,001755	3,21539
1911,752	170,92	0,000303	0,572478
summa			1508,884

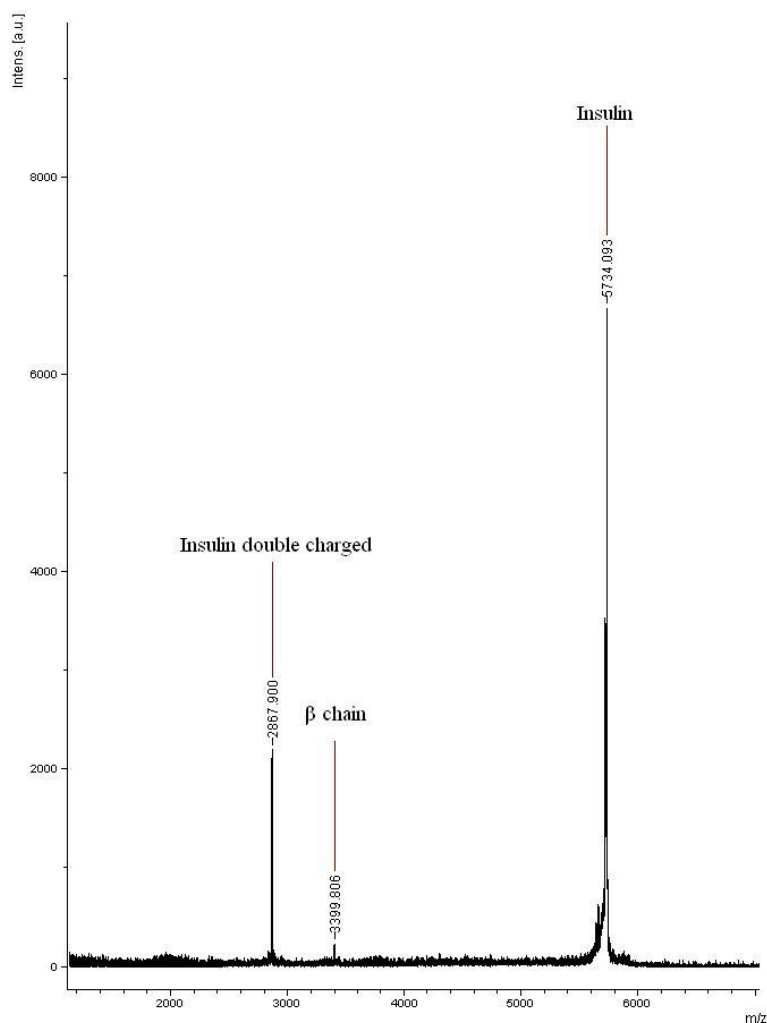
The mass difference between two peaks is exactly 58m/z, which is the molecular weight of one 2-hydroxypropyl. The molecular mass of β cyclodextrin is 1135g/mol. For experimental conditions see section 3.1.2.



MALDI spectrum of 2-hydroxypropyl β cyclodextrin. The difference in mass between two adjacent peaks corresponds to the mass of one hydroxypropyl molecule. For experimental conditions see section 3.1.2.

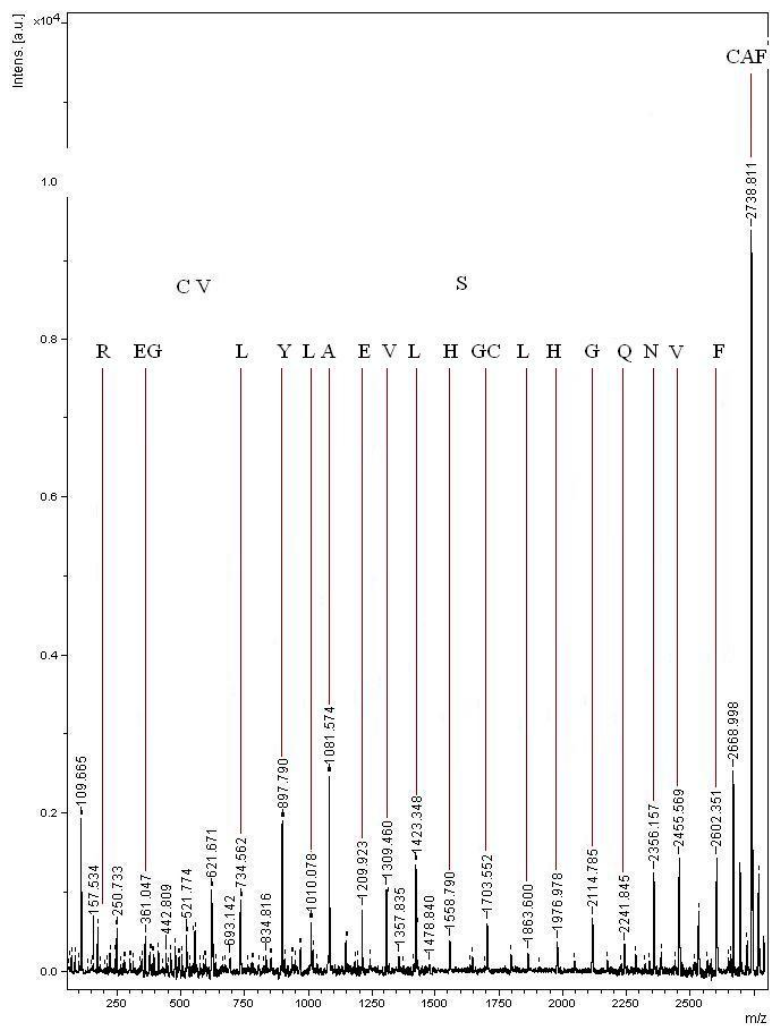
Appendix 2

To determine the quality of the bovine insulin being used, a MALDI spectrum of insulin was obtained using CHC as matrix.

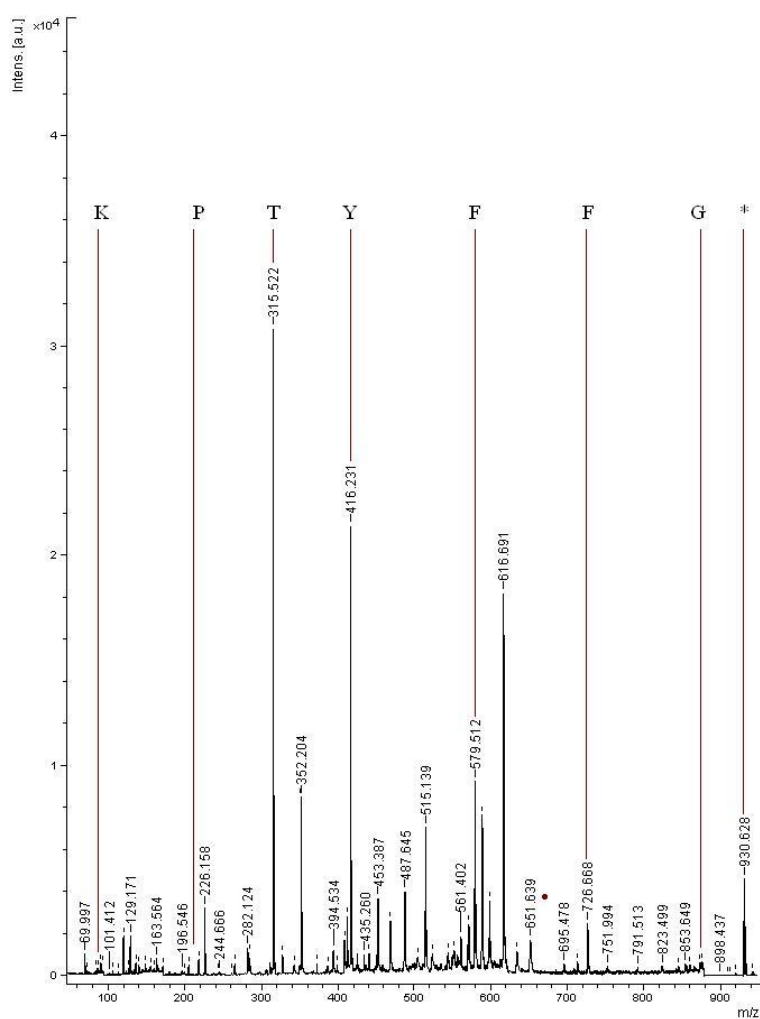


MALDI-spectrum of bovine insulin. For experimental conditions see section 3.1.2.

The amino acid sequence of the β -chain was also determined by MALDI working in the PSD mode (Post Source Decay). It is not possible to determine the amino acid sequence of a protein without cutting it down to smaller pieces of peptides. Insulin was first treated with DTT to break the disulfide bonds between the two chains and iodine acetate was then added in order to alkylate the free thiol groups. The modified insulin was finally digested with trypsin which breaks down the β -chain into three fragments. However, α chain remains intact since it does not contain any lysine or arginine residues. The α chain is very difficult to detect by MALDI since the chain does not contain any basic amino acid residues and determination of its amino acids was thus not possible. Still, this chain was detected in this experiment, though the amino acid sequence could not be determined. Chemical assisted fragmentation was used to facilitate the fragmentation of the peptide in the post source decay. The sequence of the whole β -chain was successfully determined, see MALDI spectra below.

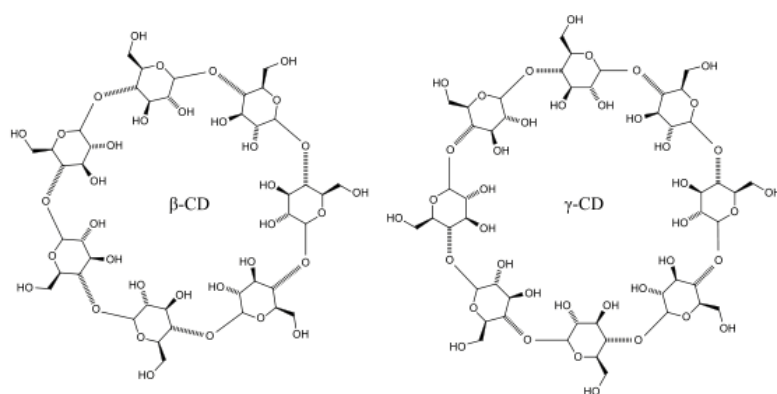


The amino acid sequence of one of the two pieces of the β -chain. The sequence is read from the right, where the C-terminal is found, to the left. For experimental conditions see section 3.1.2.



The amino sequence of the second piece of the β -chain. For experimental conditions see section 3.1.2.

Appendix 3



Structures of β - and γ -cyclodextrin