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Separation and determination of prostate-specific antigen isoforms in urine

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Abstract

Prostate-specific antigen (PSA) in urine from healthy participants (ages between 24 and 64 years old) and from patients with malign prostate cancer was separated in different isoforms by using two chromatography technologies and an ultra sensitive immunochromato-graphic test (IKR). The results are showing different isoforms with different amount of sialic acid in the antennary complex, which can have mono-, di- and multi-antennary structure. Other results are that the normal range of PSA in urine is somewhere between 100 and 1000 µg PSA/L in urine and that there is a leakage of PSA to the urethra between the urine rounds but also that the prostate has a continuous secretion of PSA during an urine round.

Keywords

Prostate-specific antigen (PSA), isoform, urine, human, Lectin affinity chromatography, Concavalin A, Ion exchange chromatography (IEC), Immunochromatographic test (IKR).

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Separation and determination of prostate-specific antigen isoforms in urine

Niclas Rollborn

Sammanfattning

Prostatacancer är västvärldens vanligaste manliga cancerform och den upptäcks idag genom att mäta nivåerna av prostataspecifikt antigen (PSA) i serum. Tyvärr föreligger det bekymmer med dagens tester, som inte är tillräckligt känsliga. De kan inte heller reda ut om det är en godartad eller elakartad cancer.

I detta projekt har PSA detekterats i urinprover från friska försökspersoner och från patienter med prostatacancer med ett nytt ultrakänsligt immunokromatografiskt test. PSA har även studeras efter kromatografisk separation med hjälp av affinitetsseparation på lektin och jonbyteskromatografi för att kunna särskilja PSA-isoformer.

Resultaten från detta projekt visade att koncentration av PSA i urin (uPSA) hos friska män ligger i området mellan 100 och 1000 µg /L PSA och att det antagligen ansamlas en liten mängd PSA i urinröret mellan urineringsomgångarna, men att prostatan även har en kontinuerlig utsöndring av PSA under en urineringsomgång. uPSA tycks även finnas i olika isoformer. Dessa isoformer tycks ha olika mängder sura molekyler (sialinsyra) i glykoantennkomplexet, som kan ha en förgrenad struktur med en, två eller flera förgreningar. uPSA hos patienter med prostatacancer tycks ha isoformer med en lägre andel sialinsyra i antennkomplexet. Friska män över 60 år verkar ha en större andel sialinsyra i antennkomplexet.

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Contents

1. Abbreviations

aa	Amino Acid
α-MM	α-Methyl Mannoside
BPH	Benign Prostate Hyperplasia
BSA	Bovine Serum Albumin
ConA	Concavalin A lectin
CV%	The Coefficient of Variation in percent
Δbl/pix	Delta blackness / pixel - Unit for the signal from immunochromatographic (IKR) system
e-o-e	End-Over-End
fPSA	Free form of Prostate-Specific Antigen
GalNAc	N-acetylgalactosamine
GB004	IKR absorbent sink in form of a blotting cellulose paper
IEC	Ion Exchange Chromatography
IKR	Immunochromatographic test
PCa	Prostate Cancer
pI	Isoelectric Point
pPSA	Precursor of Prostate-Specific Antigen
PSA	Prostate-Specific Antigen
tPSA	Total amount of Prostate-Specific Antigen
sPSA	Prostate-Specific Antigen in Serum
uPSA	Prostate-Specific Antigen in Urine

2. Introduction

Prostate cancer (PCa) is the most common type of cancer among men. The number of Swedish men who get PCa as diagnose increased from 7636 (year 2000) to 9881 (year 2005) [1]. This doesn't mean that there are more men who get PCa today than a few years ago. No, today it's more common to take a blood sample and check the physiologic condition of the prostate than it was a few years ago [2]. Small clusters of malignant cells have been discovered in a study (Soos, G., 2005 [3] of post-mortem Hungarian men in the age between 30 and 40 years old. There are geographical differences in the spread of PCa and year 2002 Hungary had fewer cases than Sweden [4]. However, when men have passed this age, there is an increased risk of PCa which is increasing with the age [5]. In Sweden 1/3 - 1/2 of all men in the age between 50 and 80 years old have small clusters of malign tumours [6], but mostly these wouldn't give any symptoms. PCa is an illness which develops very slowly and most of the middle-aged men don't know that they have it [6].

2.1. Today's PSA test

Today a tumour marker called Prostate Specific Antigen (PSA) is used to diagnose PCa. This tumour marker is mostly produced in the prostate and is secreted in high concentration (0.2 - 5 g PSA/L) in prostate secretes [7]. There is a natural leakage to the blood circulation that is responsible for the concentration of PSA in blood. The concentration of PSA in serum (sPSA) is normally quite low (<3 µg/L) but under some circumstances it can increase to 10 µg/L and sometimes even higher [7]. If the concentration of sPSA is 10 µg/L or more the risk for PCa is high, but with a concentration of 4 µg/L[1] or less the risk is low. There is a zone between these levels (4 µg/L and 10 µg/L), called the grey zone. In this zone there are men who have PCa without knowing it, because they don't have any symptoms. It's really important to discover a tumour at an early stadium [8] but then it isn't necessary that the patient have PCa[2]. The patient can have a benign prostate hyperplasia (BPH) which is a normal age-related enlargement of the prostate [7]. When an enlargement of the prostate exists, the concentration of sPSA will be increased. Unfortunately there is no test today which easily can clarify if the tumour is a benign or malign hyperplasia if the concentration of sPSA is over 3 µg/L [9]. The concentration of sPSA must be higher than 50 µg/L before the doctors can be 98.5 % sure that it will be a malign hyperplasia if the patient doesn't have a urinary tract infection or prostate inflammation when the blood-sample was taken [10].

Today different methods are used to diagnose PCa when sPSA is over the threshold level. One of these methods is palpation of the prostate gland and another is the ultrasound-aided [6]. With these methods it's possible to examine the size and the form of the prostate and also to discover if there are any spots of malignant cells. Another method is biopsy of prostate tissue that is examined by microscope. Mostly this method will be used when a patient has a sPSA concentration of 4-10 µg/L but also if a possible tumour has been discovered under the palpation [6]. The first biopsy-set is 10 samples (sextant biopsy and 2 lateral biopsies from each side of the gland) from the prostate tissue [11]. If no tumour has been discovered in the first set then another set will be done in a short period of time. If the concentration of sPSA has been stable and if no tumour has been discovered the biopsy is stopped here, but if there is an increasing concentration

[1] There exists a threshold level for sPSA approximately at 4 µg/L, some studies have used 3 µg/L [9, 14, 15] and others have used 4 µg/L [5, 11, 12]

[2] There are about 22% of these men who will get the diagnose of PCa [12]

of sPSA or a high suspicion of cancer 1-2 more sets will be done [11].
A third method is to use the ratio of free and total sPSA (fPSA/tPSA). This method will increase the diagnose specificity [6]. The scientists don't really know why, but the proportion of complex-bound PSA is higher when there is a malign tumour than a benign tumour[3]. Unfortunately there isn't any normal value of this quotient but the risk for PCa is around 50% when the ratio is under 0.10 while the risk is around 20 % when the ratio is over 0.20 [13, 14, 15].

It's very hard to find a PCa-test which is specific and sensitive [9]. Mostly the focus will be at the concentration of sPSA but it's very important to know the physiologic condition of the prostate when the sample was taken. The variation in the actual condition of the prostate causes a large variation in the concentration of sPSA. For example, when the gland is involved in a urinary tract infection the leakage of PSA is high and it takes months before the gland will go back to normal again [16].

2.2. Active monitoring of men

Today, a PSA-test will cost as little as 100 Swedish crowns but unfortunately, it isn't clarified if the test has an adequate specificity and sensitivity [6]. There is a study [8] which has discovered that treatment for PCa is most effective when it starts before the symptoms exist, but even then the 10-year survival gain is small. There are approximately 30-50% of the Hungarian men in the age between 50 and 70 years old, who have one or several clusters of malignant cells in the prostate [3]. Probably these clusters would have been discovered in an active monitoring of men, but wouldn´t necessary have giving any symptoms [6].

There are some demands which have to be satisfied before an active monitoring of men can be started: The illness must be significant and the test must be effective and favourable for both individuals and society. The illness must be found at an early stadium. There must be a treatment that decreases the mortality of the illness and this treatment must be more effective in an earlier stage than in a stage where symptoms already exist [17].
Most of these demands are already satisfied for active monitoring of PCa. The illness is very common and it brings mortality which influences both individuals and society. The treatment for PCa is very effective if it starts before any symptoms exist. Today, the test is cheap, simple and rather effective but it has disadvantages like the sensitivity and the specificity, which isn't clarified yet. With an active monitoring, more men will get PCa as diagnose but some of these diagnoses will be false. False positive diagnoses will exist because of the problem to discriminate between a benign and a malign tumour with the current test. Anyhow, if a tumour has been discovered a treatment will be started, even if it isn't mortal for the patient. So, there will be more treatment of patients than necessary, but on the other hand there will be very hard to explain why treatment isn't necessary if a tumour has been discovered [6].

Swedish national board of health and welfare has rejected active monitoring of men because they haven't found enough basic data that pointed out that the positive effects of an active monitoring would cover the negative effects [6].

[3] If cPSA is high, fPSA will be low (since tPSA = fPSA + cPSA) and the quotient (fPSA / tPSA) will also be low.

2.3. Prostate-specific antigen (PSA)

Prostate-specific antigen in its free and active form is a 33 kDa chymotrypsin-like serine protease [7], with 237 amino acids (aa) in its active form [18], belonging to the kallikrein family [7]. The inactive form of PSA has 261 aa, the first 17 aa are a signal peptide and the following 7 aa are the amino terminal end of a precursor form (pPSA) [18]. First the signal peptide is spliced from the polypeptide and then the 7 aa precursor peptide is spliced with a close related protease called human kallikrein 2 (hK2) [7]. PSA contains approximately 7-12% carbohydrates in the form of one N-linked oligosaccharide chain. PSA has three active sites and 5 stabilizers in form of disulphide bonds [18]. PSA is mainly produced in the human prostate but also in breast, thyroid, pancreas, uterus, placenta and salivary glands [19]. PSA's normal function is to splice gel-proteins in the seminal fluid, which will release the sperms with progressive movement [7].

PSA exists in seminal fluid, urine (uPSA) and serum (sPSA). sPSA is either in free noncomplexed form (fPSA) or in complexed form (complex with protease inhibitors) [7], but in urine it's in free form [20]. There is a study (Peter J. et al., 2001 [21]) that regards fPSA as a proteolytic inactive form; otherwise it will be in complex with the protease inhibitors. The study described two alternative explanations for this; the first is about a nick in the PSA sequence and the second is about an alternative pPSA form (uncompleted splicing of the precursor peptide). Peter et al. has investigated the second explanation of fPSA in serum from patients with PCa. They discovered two things; the first was that fPSA has different precursor forms (have probably parts from the signal sequence) and the second was that pPSA is in a greater extent found in PCa tissue than in BPH.

As mentioned before, sPSA is mostly in complex with protease inhibitors as alpha-1-antichymotrypsin and alpha-2-macroglobulin [7] but uPSA is in free active form. The knowledge about PSA in urine [22] simplifies the studies of PSA isoforms and of their different carbohydrate compositions. In normal patients the concentration of sPSA <3 µg/L (~20% is in free form) [7], in urine it's about 30-400 µg/L [20] and in seminal fluid it's about 0.2-5 g/L [7].

2.4. Glycosylation of PSA

PSA is a glycoprotein with a single N-oligosaccharide chain attached to aa 69, Asparagine [18]. Glycoproteins can be exposed to oncogenesis which leads to changes in the carbohydrate structure [23]. The structure difference can for example be an increased branching, an increased sialylation or an increased fucosylation[4] of the oligosaccharide chain at the exposed molecule [24]. N-glycans, like in PSA, often have structural differences as branching and sialylation [24]. PSA has in a study (Okada et al., 2001 [25]) been described to consist of two different forms, PSA-A and PSA-B (main form). The two forms have sialic acid at the antennae but PSA-A has less sialic acid than PSA-B, so PSA-A has an isoelectric point (pI) of 7.2 while pI for PSA-B is 6.9. When the sialic acid was removed from these two forms they have pI values of approximately 7.7. PSA-A and PSA-B in this study have N-oligosaccharide chains that were as a mono- and biantennary complex. Another study (Peracaula et al., 2003 [26]) compared the glycosylation pattern of PSA from normal seminal fluid and from prostate cancer cells (LNCaP cell line). They described PSA from normal patients as a biantennary complex with both disialylated antennae (which corresponds to low pI) and monosialylated antennae (which corresponds to high pI). On the other hand they also described LNCaP PSA as a biantennary

[4] Will be a higher concentration of sialic acid and of fucose in the antennary complex

complex but it wasn't sialylated. They observed that the oligosaccharide chain was neutral and it had a higher concentration of fucose. They also observed that GalNac was more frequent in the oligosaccharide chain in PSA from prostate cancer cells (65% presence) than from normal seminal fluid (25% presence). A third study (Prakash et al., 2000 [23]), that had a similar LNCaP PSA as the study above, observed structural differences between normal PSA from seminal plasma and LNCaP PSA. They described that PSA from normal patients only has a biantennary oligosaccharide chain while LNCaP PSA has a mixture of biantennary, triantennary and also possibly tetraantennary oligosaccharide chains. In a fourth study (Tabarés et al., 2006 [27]), they have observed differences in glycosylation between PSA from seminal plasma from PCa patients and LNCaP PSA, which can indicate that the cell lines (LNCaP) not always represent the physiological conditions. They have also observed different pI at sPSA from healthy donor and from patients with PCa, the healthy donor had a lower pI than the patients with PCa. On the other hand the LNCaP PSA had a higher pI than the patients with PCa, which again can indicate that the cell lines (LNCaP) not always represent the physiological conditions.

Peracaula et al., 2003 [26] wrote a comment in their paper about that it could be differences between PSA from seminal fluid and serum regarding the pI. So there are probably differences between different kinds of PSA. One paper (Jankovic et al., 2005 [19]) describes a study of uPSA that observed four isoforms and differences between PCa PSA and BPH PSA as far as it concerned lectin reactivities.

2.5. Previous Master's degree project

Before my project started another student did a Master's degree project in the same subject area at the Department of Physical and Analytical Chemistry, *Surface biotechnology*.
The title of that project was *"Development of Methods for Characterization of Prostate Specific Antigen in Urine"[20]* and the aims were:

> *" … to develop an immunochromatographic test for measuring the total concentration of PSA in urine and to verify, by using size exclusion chromatography, if PSA was free or complexed with other proteins. However, the first issue was to deal with the variable urine composition and the occurrence of precipitation in urine which can involve several proteins"[20].*

The results and conclusions from the project were summarized in some items. The first item was about the most sensitive immunochromotographic system. The second item was about uPSA and the existence of precipitates. The study discovered that more than 99% of PSA could be left in the precipitate, which could be dissolved by adjusting pH to neutral and adding a detergent and a chelator. The third item was about the uPSA concentration in men. The median concentration in normal men was 106 µg/L and in patients with PCa it was 11 µg/L. By the 9 samples from different patients with PCa 3 samples had an uPSA concentration under 1 µg PSA /L, 4 had between 1 and 10 µg/L, 1 had a very high concentration of 991 µg/L and 1 was undetectable. The author's comment of the results was as follows:

> *"The highest value of 991µg/L was obtained in urine from a patient with prostate cancer but several of the urine specimens from patients showed non-detectable values. The unexpected low PSA concentration in urine from patient with prostate cancer can depend on medical treatments of the patients, the handling of the urine*

samples during collection from patients and different forms of PSA in these samples that the chosen antibodies do not recognize"[20].

The forth item showed that PSA was in free form in urine, both for urine from normal men and urine from patients with PCa. The fifth and last item was that PSA might not be stable after purification [20].

2.6. Aims of the present project

The first aim of the present project was to determine the concentration of PSA in urine from normal men and from patients with PCa. A new ultra-sensitive immunoassay called immunochromatographic test (IKR) was set up for these determinations.
Another aim was to separate and detect different isoforms of PSA and see if there are any differences between PSA in urine from normal men and uPSA from patients with prostate cancer. Two different chromatographic methods, ion exchange chromatography (IEC) and lectin affinity chromatography, were used to separate uPSA isoforms.

2.7. Technologies of the present project

2.7.1. Immunochromatography test (IKR)

The test principle for immunochemical quantification has been developed in different directions and steps since 1977 when Glad C. and Grubb A.O. [28] presented a new technique called immunocapillarymigration. In the present project a method called immunochromatography test (IKR), has been used. This test is very fast (the whole procedure takes 15 minutes), simple and show good sensitivity. IKR uses a nitrocellulose membrane with a capturing zone (immobilized antibodies) and an absorbent sink, and the procedure can be seen in figure 1. The transport of liquid through the tiny pores of the membrane gives short diffusion distances between analyte and immobilized antibodies, which contributes to an efficient reaction. However, the fast flow leads to a requirement of high affinity between the interacting molecules since the interaction time will be extremely short [24].

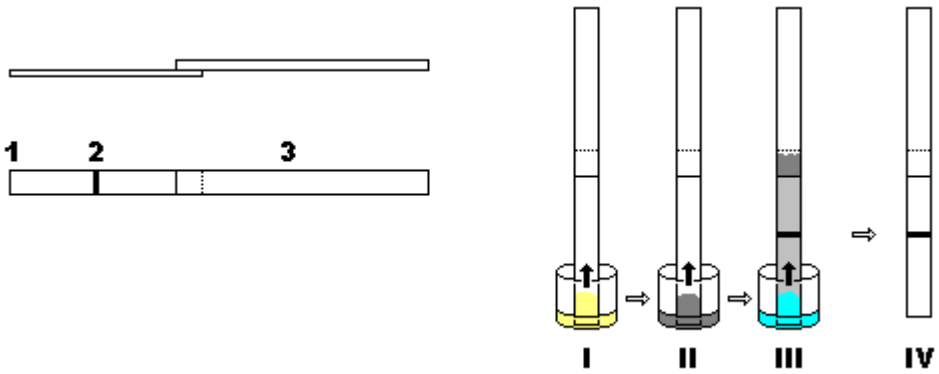

Figure 1: A schematic figure over a strip and the procedure of IKR.
The strip, 5 mm wide and 50 mm long, has an application zone (1), a capturing zone with antibodies (2) and an absorbent sink (3). The first well (I) contains sample, the second (II) contains carbon black labeled antibodies and the third (III) contains a washing buffer. The procedure (I – IV) took 15 minutes to carry out.

The IKR strip, with a layer of porous polymer applied onto a plastic backing, has an immobilized antibody line across the strip where the analyte is captured and an absorbent sink downstream collects the liquid surplus as seen in figure 1. A strip is placed in a microtiter well with sample (I), which migrates along the strip by capillary force. After the sample has been sucked off, the strip is placed into another well with carbon black labeled antibodies (II). When the labeled antibodies are passing the capturing zone they are bound to the analyte at a different epitope than the immobilized antibodies and this immunocomplex could be seen by a naked eye (2). Finally, the strip is placed into a third well with a washing solution (III). This solution washes non-bound antibody-carbon black to the absorbent sink and the strip is then glued at a template as shown in the image in figure 2.

The marker, carbon black, has been used as a pigment for printing inks but also as a marker in immunological tests. The carbon black particles are during the production in a primary form (one by one), which later becomes fused to cluster-like branched aggregates. These aggregates are what we call carbon black, which contains more than 96% of carbon and low concentrations of oxygen, hydrogen, nitrogen and sulphur. There are about 100 different grades of carbon black at the market where each grade has its own special characteristics[5] [24].

A flatbed scanner was used for quantitation of the detection line on each strip. Flatbed scanners have since 1994 been used for different detection approaches within the area of biochemistry [24]. Maria Lönnberg and Jan Carlsson presented in 2001 a new and quantitative detection approach for a flatbed scanner;

> *"Although, to the best of our knowledge, such equipments has not been evaluated with regard to precision profile and detection limit for the label, as is normal for immunoassay detection instrumentation" [24].*

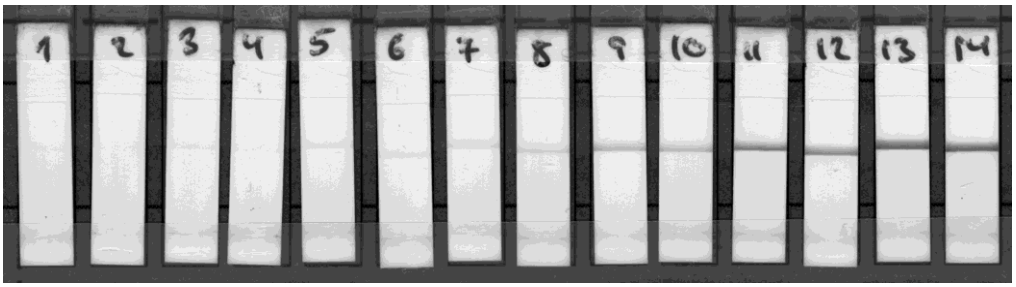

Figure 2: Scanned strips for immunochromatography test (IKR).
The scanned strips (without the absorbent sink) have been tested with 7 different PSA concentrations (50 µL of 0, 0.03, 0.1, 0.3, 1, 3 and 10 µg PSA/L) in duplicate.

2.7.2. Lectin affinity chromatography

The principle for affinity chromatography is that under a certain condition only the analyte will reversibly bind to the special ligand and all other molecules will be washed away. By introducing changes of the buffer conditions (like changing the pH, ionic strength or using a competitive compound) the analyte can be eluted. So, this technique requires a pre-study about the structure

[5] For example, these profiles can have different size of primary particle and different length and branching of the aggregates

and biological specificity of the ligand and the involved molecules. This technique was from the beginning developed for purification of enzymes, but has since the beginning been developed to purify many different types of molecules (such as immunoglobulins, nucleic acids, membrane receptors etc.). Affinity chromatography is a technique which theoretically can do an absolute purification, or separation, in a single run [29].

In the present study a tetrameric metalloprotein, Concavalin A (ConA), was used as lectin. ConA was coupled to NHS activated Sepharose 4 Fast flow[6] with a spacer arm of approximately 10 atoms which gives a better reversible binding to the analyte. This lectin requires $Mn^{2+}$and Ca^{2+} in the binding buffer with neutral pH; otherwise ConA wouldn't be stabile and active. It was important to avoid or use low concentration of detergents in the binding buffer, because they can give a negative effect on the reversible binding of the glycoprotein [30].

ConA would bind to two different kinds of sugar, a-D-mannopyranosyl[7] and a-D-glucopyranosyl at the glycoprotein through a reaction with the hydroxyl groups at the terminal sugar residues[8] [30]. According to a study (Hughes R.C. and Mills G. (1983) [31]) would ConA not bind to glycans with higher antennary complex (tri- or tetra-) than biantennary complex, to which it would bind weakly.

2.7.3. Ion-exchange chromatography

IEC is commonly used for purification and separation of charged molecules (like proteins, peptides, nucleic acids etc.) [29]. Ion-exchange chromatography (IEC) is based on the fact that opposite charged ions are attracted to each other. Many biomolecules have one or more ionisable groups in the structure, which can have a positive or negative charge. An ion exchanger can either have positively charged groups, called anion exchanger or basic ion exchanger, or negatively charged groups, called cation exchanger or acidic ion exchanger. Anion exchanger attracts molecules with negatively charged ions while cation exchanger attracts molecules with positively charged ions. It is important to choose the correct initial buffer with right pH and right ionic strength related to the molecules to be separated and the charge of the ion-exchanger. If these parameters are correct it wouldn't be any problem to eluate the charged molecules with a small change in pH or in ionic strength by the use of a continuous or a stepwise buffer gradient.

[6] The matrix consists of 4% highly cross-linked agarose [30].
[7] ConA prefer to bind to a-D-mannopyranosyl [30].
[8] The terminal sugar residues which have to be presence are C3, C4 and C5 [30].

3. Materials and Methods

3.1. Materials

A selected pair of anti-PSA antibodies, binding to different epitopes of PSA, was supplied by MAIIA Diagnostics, Uppsala, Sweden. The carbon black (CB1, 10 mg carbon /mL) which was used for label was provided by MAIIA Diagnostics. Enzymatic active human seminal plasma PSA (Calbiochemical, Merck KGa, Dramstadt, Germany) was used as calibration standard for the immunochromatographic test. 125 µm thick microporous nitrocellulose membranes, with an optical clear polyeten sheet backing, with a nominal pore size diameter of 3 µm were purchased from Whatman International Ltd, Maidstone, UK. As absorbent sink a blotting cellulose paper (GB 004) from Schleicher and Schuell GmbH, Dassel, Germany was used. Bovine serum albumin (BSA), Tween 20 and α-methyl mannoside (α-MM) were obtained from Sigma (St. Louis, USA). Nap5-desalting columns and Q-Sepharose high performance were purchased from GE Healthcare, Uppsala, Sweden. ConA was purchased from Medicago, Uppsala, Sweden. All other chemicals were of highest analytical quality (puriss pro analysis, p.a.).

3.2. Urine specimen

Four healthy volunteers, men at the age of 24, 54, 62 and 64 years participated in this study. All volunteers had a normal intake of liquid under the collection time. Each urination was divided into start urine (about the first 50 mL of one urine round) and remaining urine (urine which was left from the same urine round). Each man contributed with urine specimens from 2 to 4 urinations during different times of the day. All samples were kept in refrigerators until transport to laboratory, but not longer than for 3 days. The conditions (pH, conductivity, colour, precipitates) of all urine samples were documented at the laboratory and all urine samples were then stored in a refrigerator or in freezers (mostly in freezers).
Urine samples from patients with prostate cancer were used under the experimental procedures and these were a gift from University Hospital, Uppsala, Sweden. The laboratory didn't have any information about the time these urine samples were collected or if the patients were under treatment.

3.3. Dissolvation of urine precipitate and desalting with Nap5

It's very important to dissolve the precipitate in the urine since large amounts of PSA can be bound in the precipitates, commonly occurring in urine samples. All 500 µL urine samples were warmed up, in a water bath and at the laboratory desk, to a temperature of 21°C and were then prepared with 50 µL dissolvation buffer (MAIIA AB). The solution (urine sample and dissolvation buffer) was e-o-e incubated under 10-15 minutes. Meanwhile, the Nap5 columns[9] were washed and equilibrated with 10 mL of Nap5 buffer (20 mM Tris buffer pH 7.5 with 75 mM NaCl, 0.1 % Tween 20 and 0.02 % NaN_3). The incubated sample was then added to the column and after the sample had completely entered the gel, the proteins were eluted with 1 mL of Nap5 buffer. The eluate was collected in an Eppendorf tube with 6 µL 5% BSA in Nap5 buffer and stored in a refrigerator.

[9] Nap5 columns are pre-packed columns with Sephadex G-25 DNA grade and are used for purification, desalting and to change buffer environment.

3.4. Immunochromatography test

3.4.1. Preparation of anti-PSA membranes

PSA antibodies (antibody A) in 1 mg /mL in borate buffer were deposited (1 µl/cm) in a thin line approximately 10 mm up along the 30 x 2.2 cm sheet with microporous nitrocellulose membrane by equipment called Biodot XYZ 3000. The membranes were after incubation dried and prepared according to a confidential manufacturing procedure. Before the membrane could be used, a 5 mm wide absorption sink (GB004) was mounted and the membranes were cut into 5 mm wide strips by equipment called Guillotine Biodot model XYZ3050.

3.4.2. Adsorption of anti-PSA with carbon black

Carbon black (CB1) was suspended in a borate buffer to 500 µg CB1/mL and incubated with 35 µg/mL anti-PSA (antibody C) e-o-e for 1h. 20% BSA in borate buffer was then added to a final concentration of 1% BSA in the Ab solution, and incubated for another 30 minutes. After these 30 minutes the suspension with CB1, Ab and BSA was washed four times with a dilution buffer (20 mM phosphate buffer pH 7.5 with 1 % BSA, 0.05 % NaN_3) by repeated centrifugations (20800 g, 5 min). The concentration of CB1 in the final solution (~1 mg/mL) was determined by measuring the absorbance at 400 nm with a spectrometer.

3.4.3. IKR procedure

PSA standards (0, 0.03, 0.1, 0.3, 1, 3 and 10 µg PSA /L) were either diluted in 20 mM Tris buffer pH 7.5, 75 mM NaCl, 0.1 % Tween 20, 0.03% BSA and 0.02 % NaN_3 or in 20 mM Tris pH 7.5, 0.1 M NaCl, 0.1 % Tween 20 and 0.02 % NaN_3 (was only used for IEC fractions). The samples and the PSA standards were warmed up, at a laboratory desk, to 21°C and diluted with individual factors (for the samples see section 4.2) before addition into a microtiter well in a volume of 25 µL or 50 µL. 50 µL sample or PSA standard was used for determination of the concentration of the fractions from lectin affinity chromatography and IEC; otherwise 25 µL sample was used. The added volume of sample was absorbed for 5 (volume of 25 µL) or 13 minutes (volume of 50 µl) by the 5 mm wide strips with anti-PSA capturing line (see section 3.4.1). The strips were then placed in another microtiter well of 25 µL carbon black labeled anti-PSA antibodies (see section 3.4.2) dilution for 5 minutes. After these 5 minutes, the strips were again placed in another well, containing 20 µL washing buffer, for 5 minutes. The strips were then glued on templates and the absorbent sink was removed.

3.4.4. Detection with a flatbed scanner

When all strips were dried (approximately after 30 minutes) the templates were scanned with a flatbed Epson Expression 1680 Pro scanner. This scanner has an optical resolution of 1600 dpi (which was set to 600 dpi under the experimental procedure), a greyscale depth of 16-bits per pixel (both internal and external) and a xenon gas cold cathode fluorescent lamp with an operating temperature from 5°C to 35°C [32]. When the templates with the strips have been scanned and converted to digital pictures a software called MACRO (created of Mikael Lönnberg, MAIIA AB, Uppsala, Sweden) was used to calculate the signal from each strip's detection line. MACRO divides the pictures into smaller parts (one strip in each part) and tries to find a maximum and a minimum value of blackness in each part. The maximum and minimum values are mean values of 3 pixels. The difference between maximum and minimum values is

used to estimate a signal in delta blackness /pixel (Δbl/pix). If a determination of PSA concentration in unknown samples had to be done a software called Workout 2.0 was used. Input for this software were the signals (Δbl/pix) from a set of known PSA values, the standard curve, which were compared to the signal for the samples. The concentration values from Workout 2.0 were, for all the samples, corrected by calculation for dilution of the samples (individual factor for each sample, see section 4.2), adding of dissolving buffer (9 %), dilution in Nap5 desalting (50 %) and for the Nap5 column recovery of 85%.

3.5. Separation chromatographic technologies

3.5.1 Lectin affinity chromatography

The matrix with ConA was packed in a Pasteur pipette with a small wad of glass wool in the point of the pipette. The packing buffer was a 20 mM Tris buffer pH 7.4 with 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.02% Tween 20 and 0.02% NaN_3 (ConA buffer). The column was approximately 23 x 6 mm and had a calculated bed volume of 0.75 mL and a flow rate of 0.6 mL/min.

The experimental procedure begins with a desalting step of the samples by using Nap5 columns (see section 3.3). The desalted samples were in a 20 mM Tris buffer pH 7.5 with 75 mM NaCl, 0.1 % Tween 20, 0.03 % BSA and 0.02 % NaN_3. ConA wouldn't be stabile and active without Mn^{2+} and Ca^{2+}. All the samples were for that reason diluted with ConA buffer, which gave them a similar environment of 1 mM Mn^{2+}, 1 mM Ca^{2+} and a pH of 7.4. The column was washed with 4 mL of 100 mM α-MM separation buffer[10] and with 8 mL of ConA buffer. Approximately 3 ng PSA in 0.5 mL sample was then added to the column and the collection of the approximately 32 fractions was started when the sample had completely entered into the matrix. The column was washed with 2.8 mL ConA buffer and then PSA was eluted with a stepwise gradient in three steps of 2.8 mL α-MM separation buffer with 1 mM, 3 mM and 100 mM α-MM, respectively. The PSA concentration of each fraction was determined with the IKR method (50 µl solution in single run). The PSA value was divided with the total amount of PSA to get %PSA per fraction. All the fractions and the column were stored in a refrigerator.

3.5.2 Ion-exchange chromatography

In the present study an anion exchanger was used and the matrix (Q-Sepharose High Performance) was packed in a column using a Bis-Tris buffer with pH 6.4. The exchanger was 46 x 5 mm and had a bed volume of 0.90 mL and 1 mL solution was pumped through the exchanger in 1 min. The exchanger was installed to an ÄKTA™ Explorer 10S from GE Healthcare with 1 mL injecting loop and a Frac 950 fraction collector. Under the whole procedure the absorbance (mAU) at 280 nm and the conductivity (mS/cm) were measured. All solutions were 0.22 µm filtered and sonicated for 3 minutes in an ultrasonic bath of 50 Hz, before the first connection with the instrument.

The samples were prepared according to section 3.3. by a desalting step with Nap5 columns and afterwards they were diluted (with a range between 1/19 to 1/62, except for UP7 which wasn't diluted since it had a very low concentration of PSA) in 20 mM Tris buffer pH 7.5 with 0.1 % Tween 20 and 0.02 % NaN_3 (IEC separation buffer A). 0.5 mL diluted sample was then 0.22 µm

[10] α-MM separation buffer is ConA buffer with different concentration of α-methyl Mannoside (α-MM).

filtered and the final sample volume, which was injected into the injecting loop[11], was roughly 0.3 mL and contained about 1.3 ng PSA. When the sample has been injected into the injecting loop, the program Q-sepharose HP (see table 1) was started. This program gave a continuous gradient of 0 - 40 % of IEC separation buffer B (sep. buffer A with 400 mM NaCl). Finally the PSA concentration in each fraction was determined with the IKR method (50 µl solution in single run), which was divided with the total amount of PSA to get %PSA per fraction. All the fractions were stored in a refrigerator.

Table 1: An overview of the program Q-Sepharose HP for the instrument ÄKTA™ Explorer 10S. With help of this program an ion-exchange chromatography (IEC) could be done for prepared urine samples. The 0.3 mL sample was loaded into the 1 mL injection loop and IEC separation buffer B contained NaCl.

Step	Event	Volume (mL)	Total volume (mL)
1	I) Equillibration of the column with IEC separation buffer A II) The Absorbances are set to zero	2	2
2	I) Collection of fractions is begin (0.35 mL/fraction) II) Empting of injection loop with IEC separation buffer A	5	7
3	Start of the continous gradient i) Startconcentration of IEC separation buffer B: 0% ii) Endconcentration of IEC separation buffer B: 40%	14	21
4	Gradient delaying: Endconcentration of IEC separation buffer B	2	23
5	Washing the column with 100% of IEC separation buffer B	2	25
6	I) Collection of fractions is ending II) Washing the column again with 100% of IEC separation buffer B	3	28
7	Equillibration of the column with IEC separation buffer A	5	33
Pump rate under the whole procedure: 1 mL/min Total time of the run: 33 minutes			

[11] It was not allowed to inject more 50 % of the loop volume of sample at ÄKTA™ Explorer 10S.

4. Results and Discussion

4.1 PSA immunochromatographic test

The results from the PSA IKR test (see section 3.4.3) using 25 µl of 0, 0.03, 0.1, 0.3, 1, 3 and 10 µg /L of PSA (Calbiochem) is shown in figure 3. The concentration levels of PSA in the standard curve are plotted against obtained signal in ΔBl/pix. All values in the figure are mean values from 5 different runs within 20 days. These runs had a mean detection limit[12] of 4.7 ng PSA /L (range between 3.0 and 7.0 ng /L) and a mean median CV (coefficient of variation[13]) of 3.5% between the duplicates (range between 0.1 and 10 µg PSA /L).

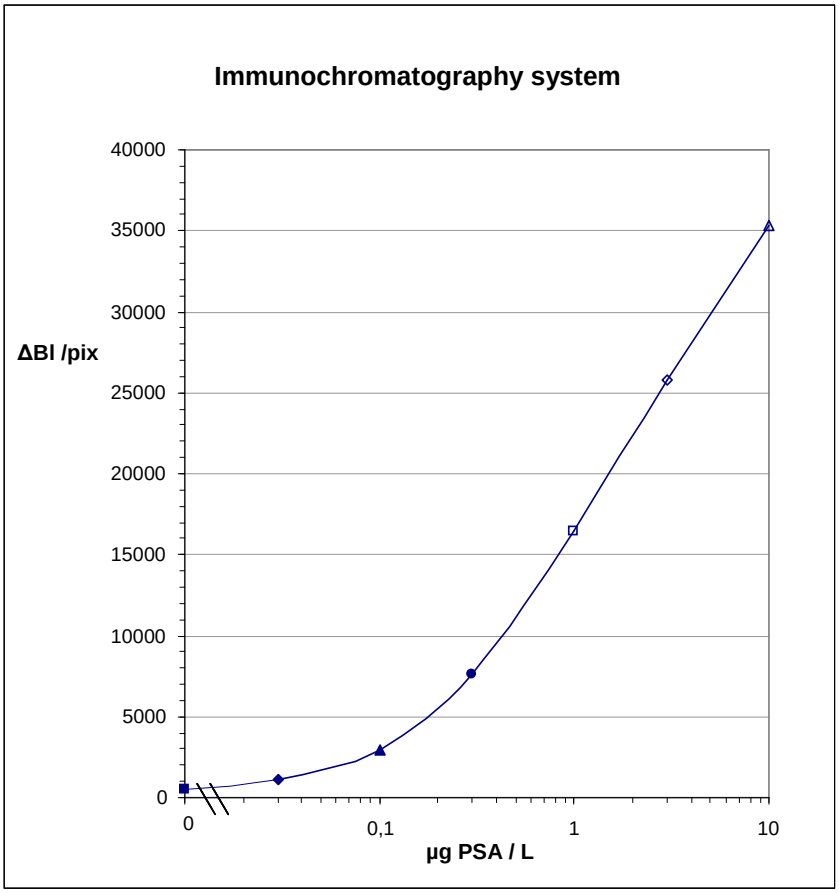

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Figure 3: An average standard curve for PSA IKR.
The mean signal obtained from five different IKR testing during a period of 20 days, is shown when 25 µl of 0.03 to 10 µg PSA/L was tested.

This system has a low CV (mean median value of 3.5%) and a low detection limit (mean value of 4.7 ng /L) which is very good. The test which is used today, called PSA-EIA, has a median CV of 2.2% and a detection limit of 160 ng /L [20]. This just confirms that IKR is a very good technology with a good precision and with a very low detection limit.

[12] The detection limit is defined as the concentration calculated from the standard curve for the signal obtained at two standard deviations from the signal of point zero

[13] Coefficient of variation is a ratio of the standard deviation and the mean value

4.2. Determination of PSA concentration in urine specimen

The concentration of PSA in urine samples from four healthy volunteers in the ages 24 to 64 years old and urine from two patients with PCa are presented in table 2 and figure 4. The urines were at furthest stored in a refrigerator for 7 days[14] and treated in accordance with 3.3 by addition of dissolvation buffer and Nap5 buffer. All Nap5 urines were determined by IKR (see section 3.4.3) and by using series of 25 µL PSA standards (0, 0.03, 0.1, 0.3, 1, 3 and 10 µg PSA /L) diluted in 20 mM Tris buffer pH 7.5, 75 mM NaCl, 0.1 % Tween 20, 0.03% BSA and 0.02 % NaN_3. The urine samples were diluted between 20 (UP7) to 500 (UPN7) times with Nap5 buffer and a final sample volume of 25 µL was used.

Table 2: PSA in urine for a 24-year, a 53- year, a 62- year and a 64- year old man and patients with PCa. The concentration of PSA in urine is measured by IKR (µg/L) and the amount PSA obtained for each urine round is calculated. The collecting time, the total volume and the date of collection of the urine sample are showed in the table.

	Code name	Date (collection)	**Time** (collection)	Tot. volume col. **mL**	**Urine type** (start / remain)	**µg PSA/L**	µg PSA / urine round
Man 24 year	**UPN1**	2007-01-30	**22:40**	60	**start**	**815**	49
	UPN2			450	**remain**	**375**	169
	UPN3	2007-01-31	**07:10**	90	**start**	**824**	74
	UPN4			320	**remain**	**284**	91
	UPN5	2007-01-31	**12:30**	110	**start**	**218**	24
	UPN6			420	**remain**	**227**	95
	UPN7	2007-01-31	**17:30**	50	**start**	**1235**	62
	UPN8			450	**remain**	**309**	139
Man 53 year	**UPN9**	2007-02-20	**00:50**	39	**start**	**417**	16
	UPN10			100	**remain**	**330**	33
	UPN11	2007-02-20	**06:25**	47	**start**	**428**	20
	UPN12			500	**remain**	**106**	53
Man 62 year	**UPN17**	2007-02-24	**08:00**	55	**start**	**1824**	100
	UPN18			250	**remain**	**1270**	318
	UPN19	2007-02-24	**12:20**	49	**start**	**741**	36
	UPN20			280	**remain**	**170**	48
Man 64 year	**UPN13**	2007-02-20	**18:00**	42	**start**	**311**	13
	UPN14			95	**remain**	**184**	17
	UPN15	2007-02-21	**06:30**	47	**start**	**473**	22
	UPN16			130	**remain**	**110**	14
Patient urines, prostate cancer, under treatment?							
	UP6	2006-11-09				**459**	
	UP7	2006-11-09				**14**	

The mean value for all healthy participants was 533 µg PSA /L but it was a large variation between the urine samples in this study (see table 3). The urine samples from a 24-year old man, for example, had a mean value of 536 µg /L and variation between 218 and 1235 µg /L while the urine samples form a 64-year old man had a mean value of 270 µg /L and variation between 110 and 473 µg /L.

[14] Most of the sample had only been stored for 3 days, but UPN17 – UNP20 had been stored for 7 days.

Table 3: A summarized table over normal urine.
There is a high concentration of PSA in urine, compared to PSA in serum, but there is also a large variation between different urine samples.

Urine from healthy participants in µg PSA / L	Mean	2xSD	Range
All participants	533	915	106 - 1824
24 years old man	536	751	218 - 1235
53 years old man	320	299	106 - 428
62 years old man	1001	1418	170 - 1824
64 years old man	270	318	110 - 473

As seen in figure 4, most of the normal samples have a PSA concentration between 100 µg /L and 1000 µg /L. The value from the PCa patients (UP7) is a mean value over 4 different IKR runs (3 runs for UP6) of the same sample. There was also a large variance here of the PSA concentration between the runs (from 280 to 659 µg /L for UP6 and from 8 to 18 µg /L for UP7).

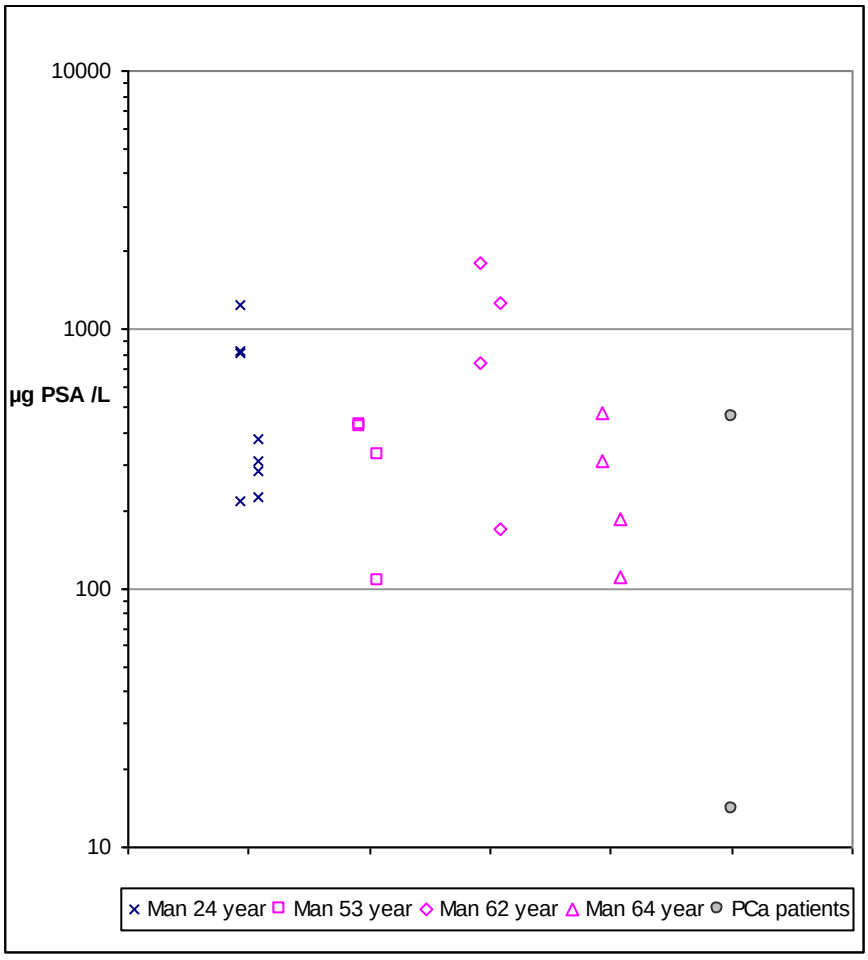

Figure 4: The concentration of PSA in urine samples.
The concentration of PSA in urine samples from healthy men, 24 to 64 years old, and from two patients with PCa (ev. treated) show considerable varying PSA levels in the range from 100 to 2000 µg/L. One of the PCa urines has low PSA levels which can be due to medical treatment.

There are two samples from patients with PCa in table 2, but no information about these urines (urine from a treated patient or a start or remain urine etc.) was available. One of these urines (UP6) was in the same area as the normal urines but the other (UP7) had a mean PSA value of only 14 µg /L. A reasonable explanation for this low mean value is that the patient was under medical treatment when the sample was collected.

The urine in the present project was divided in two classes (start and remaining portion) to investigate if PSA will be collected in urethra and flashed out by urine or if the prostate has continuous secretion of PSA during the urine round. PSA would be collected in the urethra if the largest amount and a high concentration of PSA were found in the start portion; otherwise the prostate seems to have a continuous secretion of PSA.

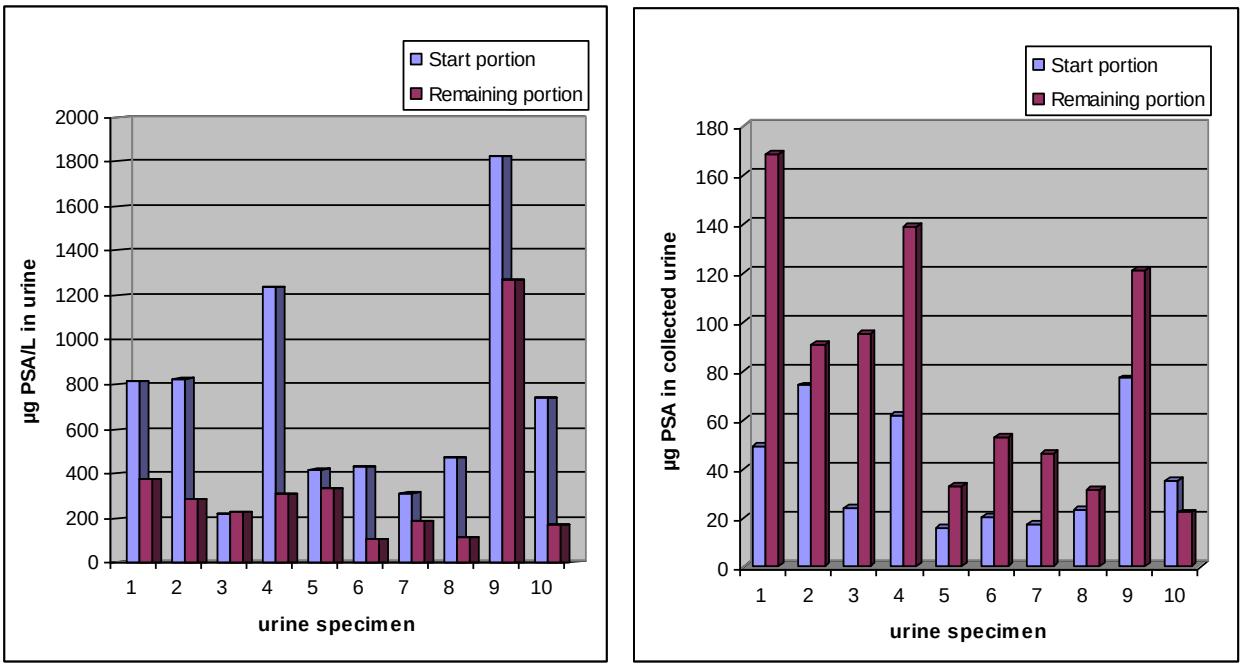

Figure 5: Continuous secretion of PSA.
The prostate seems to have continuous secretion of PSA during the urine round since it's a larger amount of PSA in the remaining portion than in the start portion. The concentration of PSA (in µg /L) is shown to the left, while the amount of PSA (in µg / urine round) is shown to the right.

The mean value of PSA concentration for start portion of urine from all healthy volunteers is 729 µg/L while its 337 µg /L for the remaining portion, but the mean value for the amount of PSA is 40 µg for the start portion and 80 µg for the remaining urine. This is also shown in figure 5. So, there is a higher concentration of PSA in start urine than in the remaining urine, but the amount of PSA is larger in the remaining urine than in the start urine. This is very interesting because the high concentration in the start urine shows that PSA is in a small extent collected in the urethra but the prostate has a continuous secretion of PSA during the urine round.

4.3. Lectin (ConA) affinity chromatography

4.3.1. To find a strategy to separate isoforms of PSA.

First a pre-study was started to increase the knowledge about the molecules ConA and PSA. The aims of this pre-study were to investigate which concentration of ConA was necessary in the matrix and also to investigate which kind of step was necessary in the stepwise gradient.

The pre-study was started with three different columns; the first had a purchased matrix with 10 mg ConA per ml Sepharose (4B) from GE Healthcare, Uppsala, Sweden, the second and the third were coupled at the laboratory with respectively 7.5 mg and 15 mg ConA per mL NHS-activated Sepharose 4 fast flow gel from GE Healthcare, Uppsala, Sweden. A sample called Nap5 UPN1 was then separated on these different columns. The stepwise gradient was started with four different steps (0, 10, 100 and 500 mM) of α-methyl-mannoside (α-MM), the inhibiting sugar for ConA.

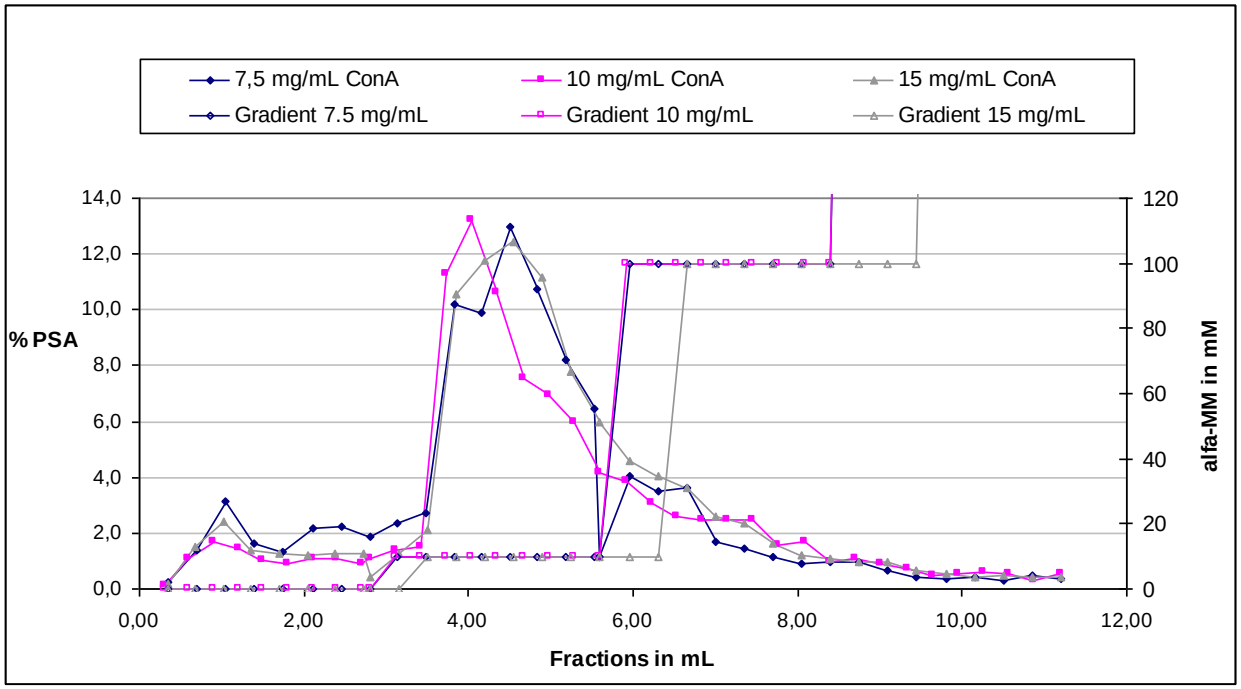

Figure 6: Results from a pre-study with lectin (ConA) affinity chromatography.
One desalted sample (UPN1) and three different columns with different ligand density have been used to evaluate a strategy to separate isoforms of PSA with a stepwise elution gradient (0, 10, 100 and 500 (not showed) mM) of α-MM.

A similarity between the columns with different substitution grade of ConA is shown in figure 6. Less than 15% of PSA of the total amount did not bind to the matrix and went right through the columns and the largest amount of PSA (around 63 %) was eluted with 10 mM of α-MM. Approximately 20% of PSA was then eluted at 100 mM. However, as can be seen in figure 6 and as mentioned before it was only some small differences between the columns and the column with 7.5 mg ConA /mL was chosen for further investigation of the stepwise gradient (see figure 7) since it had a low substitution grade and seemed to fit the analyte very well.

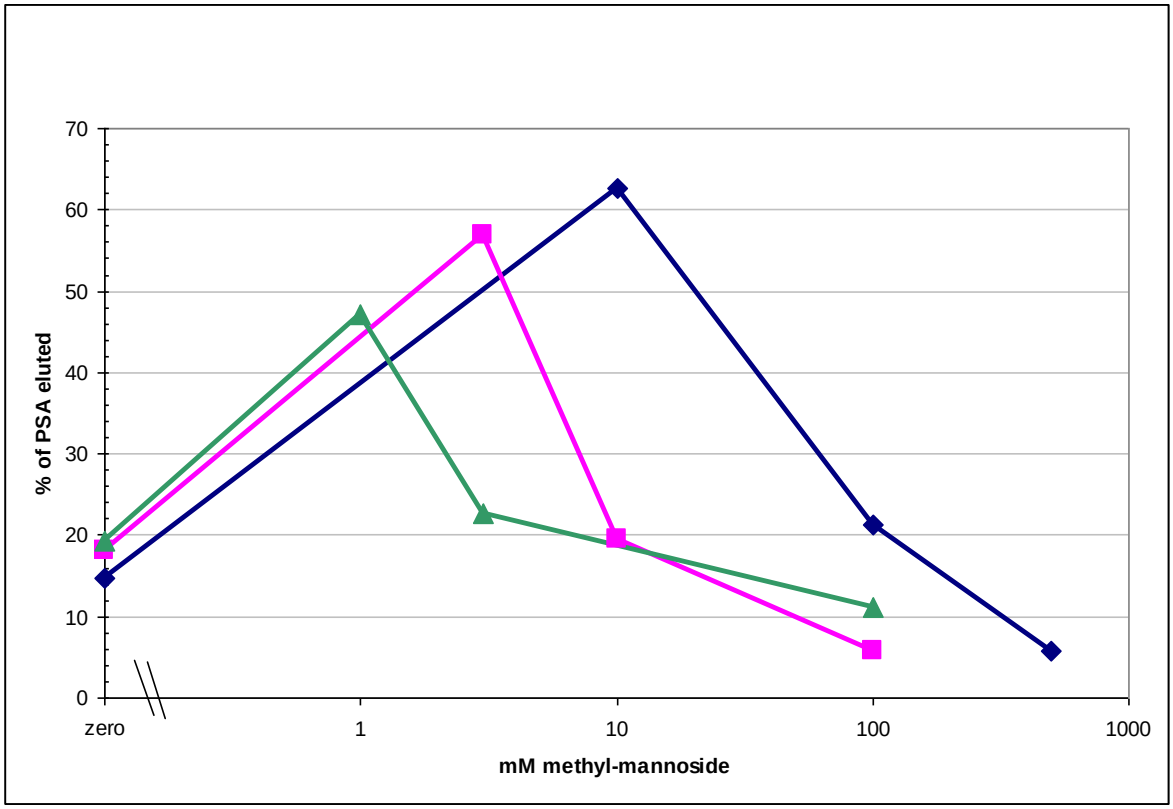

Figure 7: Different stepwise gradients of methyl-mannoside (α-MM) were used to investigate how hard PSA bound to a 7.5 mg ConA /mL -column. The gradient of 0-1-3-10 mM α-MM [▲] was choosen since it generated a peak smaller than 50 %PSA (47%) of the total amount (the other two gradients (0-3-10-100 mM α-MM [■], 0-10-100-500 mM α-MM [♦]) had a peak of 57 %PSA and of 63 %PSA) and for the first time a separation of PSA could be seen (not shown in the figure). Approximately 15-19 %PSA unbound to the matrix.

The results in figure 7 are showing that 15-19 %PSA will not bind to the matrix and goes right through the column and 63 %PSA of the total amount will eluate at 10 mM α-MM when a stepwise gradient of 0-10-100-500 mM α-MM was used. It's hard to do a good separation if more than 50% PSA will eluate in the first step, so steps with lower concentrations of α-MM was made (1 and 3 mM α-MM) and 500 mM α-MM was not used. The gradient with 0-3-10-100 mM α-MM generates a peak with 57 %PSA of the total amount at 3 mM α-MM while the gradient with 0-1-3-10 mM α-MM generates a peak smaller than 50 %PSA (47%) at 1 mM α-MM. When the peak was smaller than 50 %PSA a separation of PSA could be seen in the results and the pre-study had reached the aim and found a strategy to separate isoforms of PSA with lectin affinity chromatography.

4.3.2. ConA separation of urine samples

10 different urine samples from healthy men between 24 and 64 years old and 2 urines from patients with PCa were separated by using the 7.5 mg /mL ConA column. The PCa urines (UP6 and UP7) were separated twice. These samples have been stored in freezers and refrigerators and treated in accordance with 3.3 by addition of dissolvation buffer and ConA buffer. The desalted urines were prepared according to 3.5.1 and added on the ConA column. Profiles over the results are shown in figure 8 where the collected fractions in ml are plotted against the percent of PSA. The percent PSA of the total obtained amount of PSA in each gradient step is shown in table 4.

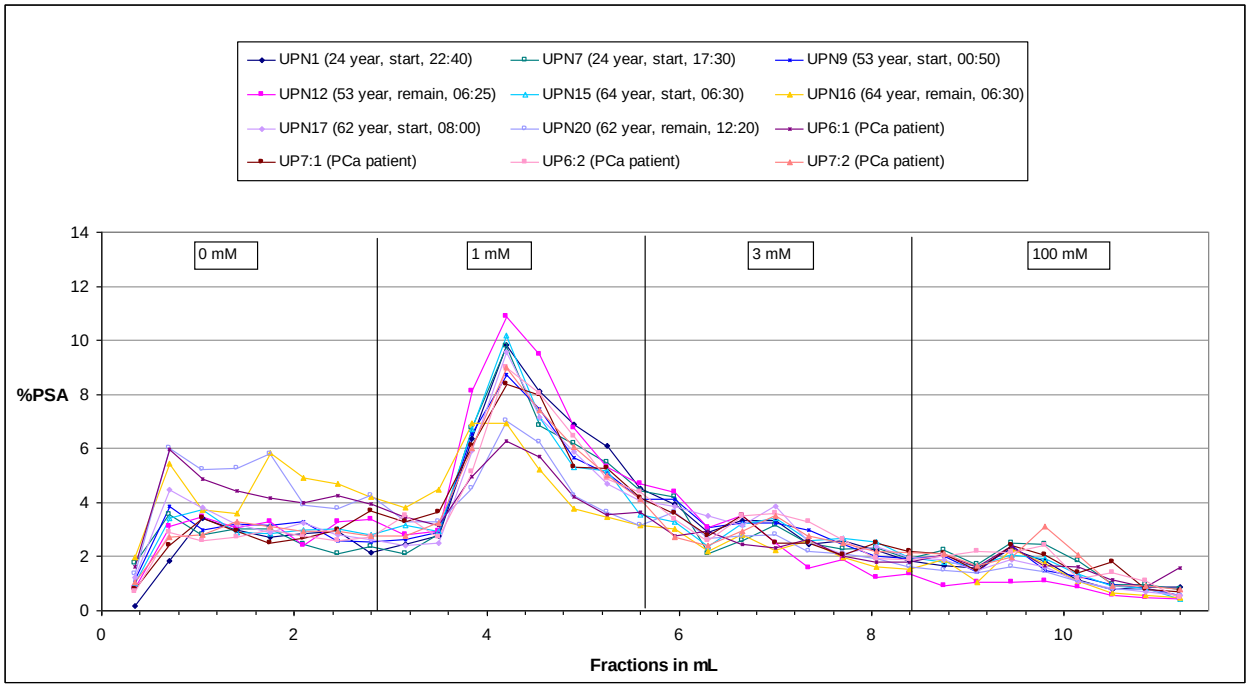

Figure 8: Eluation profiles for different urine samples applied to the 7.5 mg /mL ConA column. A stepwise gradient with 4 steps was used and the collected fractions in mL are plotted against the percent of the total PSA amount.

In figure 8 and table 4 it's shown that three samples (UPN16, UPN20 and UP6:1) show large percent of unbound PSA, which either can be isoforms containing no carbohydrate at all or isoforms with a multiantennary carbohydrate structure. According to Hughes and Mills (1983) [31] ConA would not bind to glycans with a higher antennary complex (tri- or tetra-) than bi-antennary complexes. This indicates that these samples might contain more of high antennary complexes. A multiantennary factor was calculated as the ratio between %PSA eluted with 0 mM α-MM and at 1 mM α-MM. This ratio is shown in the lower part of table 4 and for the three samples with a large amount of unbound PSA the mean value of the ratio was 95.5 whereas the remaining samples had a mean value is 48.7. However, UP6 (one of the PCa samples) was tested at both of the two test occasions and showed different results which has to be studied in more detail later.

Table 4: Percent PSA of the total obtained amount of PSA after lectin affinity chromatography runs with a 7.5 mg /mL ConA column. The multiantennary factor is the ratio of the percent PSA isoforms eluted at 0 mM α-MM and 1 mM α-MM and indicates if there can be any tri- or tetra-antennary complex.

% PSA of total amount	Code name	UPN1	UPN7	UPN9	UPN12	UPN17	UPN20	UPN15	UPN16	UP6:1	UP6.2	UP7:1	UP7.2	
	Age, year	24	24	53	53	62	62	64	64					
	uPSA, µg/L	791	1198	417	106	1824	170	473	110	630	630	18	18	
	Urine type (pre/mid	pre	pre	pre	mid	pre	mid	pre	mid					
	Time (collection)	22:40	17:30	00:50	06:25	08:00	12:20	06:30	06:30					
	Conc. alfa-MM													
Fractions in mL	in mM													**Average values**
0 - 2.80	**0**	19,2	21,1	22,8	22,9	24,3	35,6	22,7	34,4	33,3	20,0	21,5	21,7	25,0
2.80 - 5.60	**1**	47,1	44,4	43,2	51,0	42,2	35,4	44,2	37,9	35,0	44,1	44,2	43,6	42,7
5.60 - 8.40	**3**	22,7	21,1	23,0	19,6	23,4	19,8	22,1	17,9	18,7	22,9	21,6	21,2	21,2
8.40 - 11.20	**100**	11,1	13,4	11,0	6,5	10,1	9,2	11,0	9,8	12,9	13,0	12,7	13,5	11,2
Summation:		100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0
Quotient 0/1 mM multiantennary factor		**40,7**	**47,5**	**52,7**	**44,9**	**57,7**	**100,5**	**51,4**	**90,8**	**95,3**	**45,4**	**48,6**	**49,7**	**58,5**

Since the three samples with high multiantennary factor were in the same test set up (see figure 9) it is possible that there are some differences between the two test occasions. However, these results encourage to further investigation with a larger group of urine samples. Two parameters can have influenced these results, the preparation of the sample and the instability of the sample (the obtained concentration of this sample has differed a lot between the different determinations).

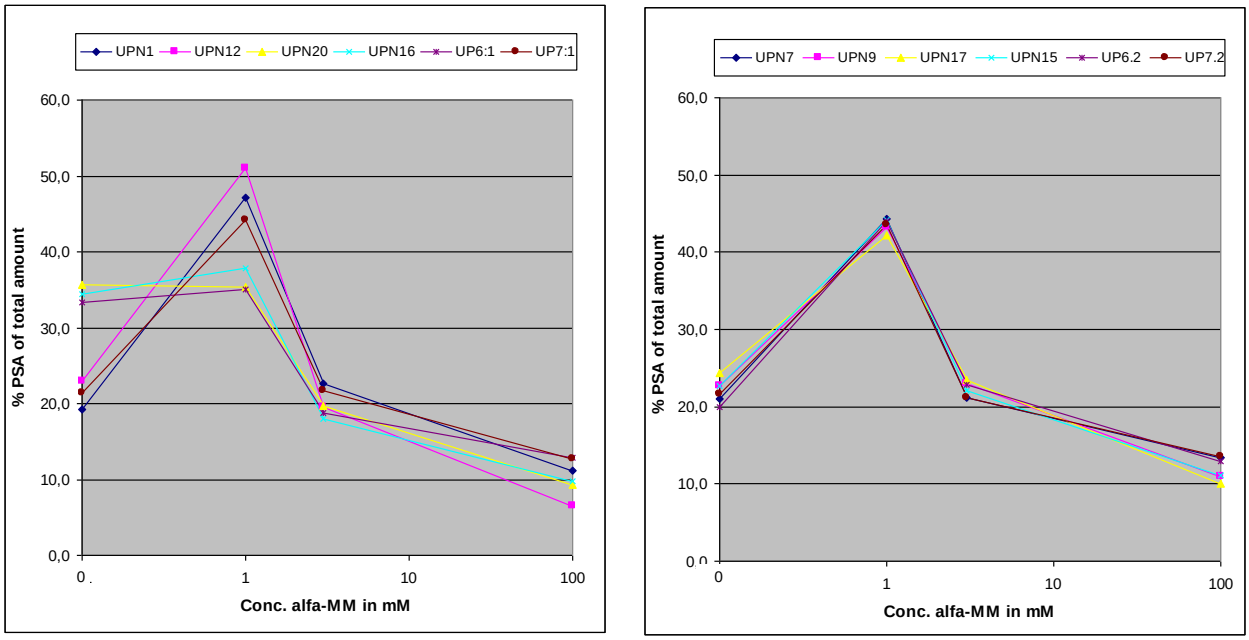

Figure 9: Differences in results between test occasions, the first set to the left and the second set to the right. %PSA for each sample was calculated after lectin affinity chromatography runs using a 7.5 mg /mL ConA column. Three samples showed a high elution of PSA isoforms when elution with no inhibiting sugar was performed, which indicates that these samples have no glycans or multiantennary glycans. However, when the separation was repeated for one of the PCa samples (UP6) this pattern was not obtained.

4.4. Ion-exchange chromatography

Ion-exchange chromatography (IEC) has been used to separate the differentially charged uPSA isoforms. The tested urine samples were from four healthy participants and two patients with PCa. All urines had been stored in freezers from 1 month (UPN17) to 5 months (UP6) and treated in accordance with 3.3 by addition of dissolvation buffer and IEC separation buffer A. The desalted urines were prepared according to 3.5.2 and were one by one injected into the anion exchanger Q-Sepharose mounted in an ÄKTA™ Explorer 10S. A continuous gradient of 0 – 40 % of IEC separation buffer B with 400 mM NaCl, was used in the program (table 1).

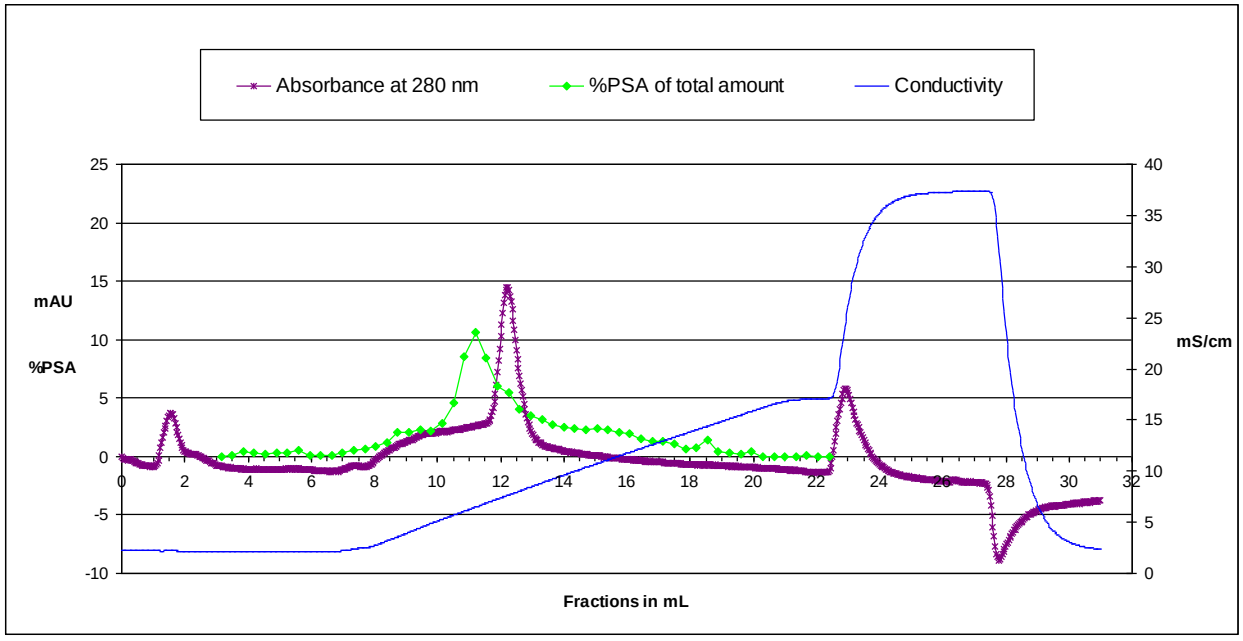

Figure 10: A schematic plot over a run with continuous gradient (0-160 mM NaCl [See conductivity in the figure]) for sample UPN1. UPN1 was desalted by Nap5 and diluted with IEC separation buffer A (1/50). About 0.9 ng PSA was injected into the injection loop at ÄKTA™. Three Abs 280nm peaks were shown in different proportions in all the applied samples. PSA was measured by IKR.

A schematic plot over a run is shown in figure 10. There are three different curves in the plot; the absorbance at 280 nm showing 3 peaks, the conductivity obtained from the continuous gradient from 0 mM to 160 mM NaCl (the column is then equilibrated with 400 mM NaCl) and the measurement of PSA in percent of total measured amount. PSA in percent has been determined with the sensitive and specific IKR method as the PSA content in the diluted urine samples were about 1.3 ng which is to low for the detection technology in the instrument as well as that several other proteins appear in higher amounts. The second peak in Abs 280 nm most probably shows the content of other urine proteins together with the applied amount of BSA which due to its more acidic charge will elute later than PSA.

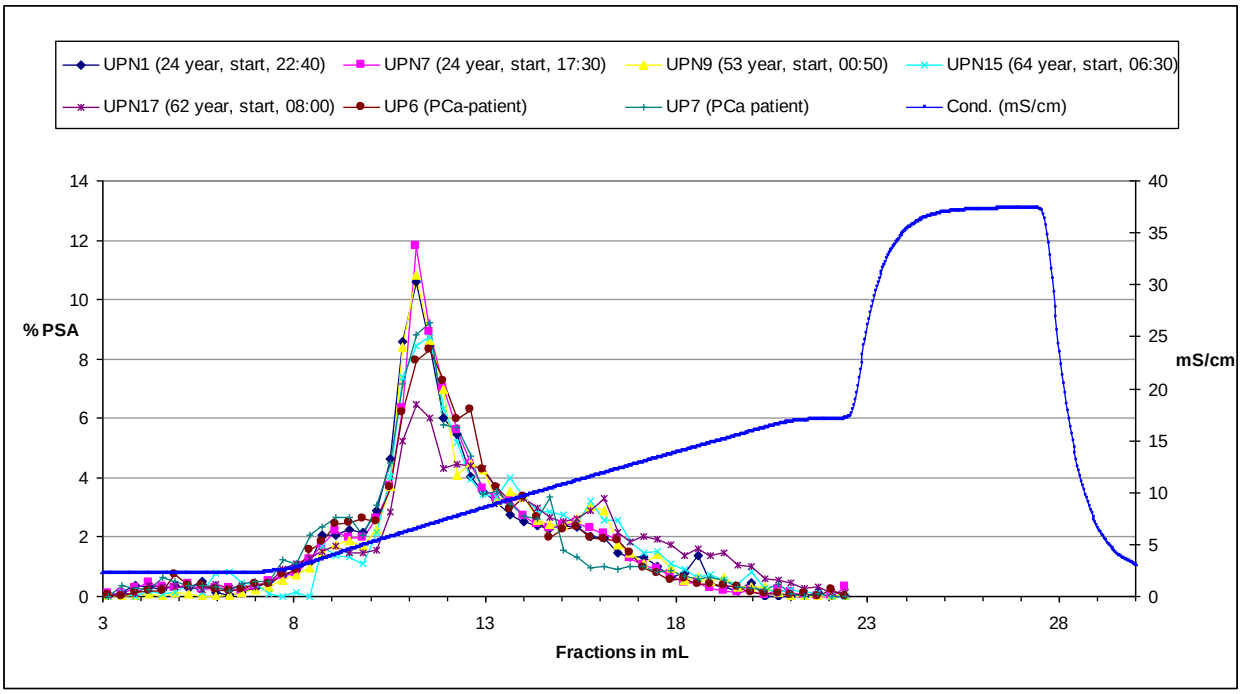

Figure 11: A profile over the distribution of PSA for all the applied urines and the continuous gradient between 0 – 160 mM of NaCl. It's seemed to be 2 forms of PSA, PSA-A (pre-peak) and PSA-B (main peak).

A profile over all the results and the continuous gradient are shown in figure 11, where the collected fractions in ml are plotted against the percent of PSA of the total amount and against mS/cm. In figure 11 it seems as the uPSA isoforms are distributed into two clearly distinguish peaks, a minor peak with less acidic PSA, a major large peak and some smaller with more acidic PSA. 3 urines (UPN1, UPN7 and UPN9) have a steep main peak, UPN17 has a characteristic composition with a low and very wide peak and the other urines have an appearance between these. The calculated figures of percent PSA of the total amount of PSA from the separations in figure 11 is shown in table 5.

Table 5: Percent PSA calculated for IEC separation of desalted urines.
The results from the separation are shown in figure 10 and have here been used to calculate the percentage of PSA distributed in different fractions.

% PSA of total amount	Code name	UPN1	UPN7	UPN9	UPN17	UPN15	UP6	UP7	
	Age, year	24	24	53	62	64			
	uPSA, µg/L	815	1235	417	1824	473	630	18	
	Urine type (pre/mid)	**pre**	**pre**	**pre**	**pre**	**pre**	**Pca**	**Pca**	
	Time (collection)	22:40	17:30	00:50	08:00	06:30			
	Added PSA, ng	0,808	1,075	1,052	1,613	1,545	1,502	1,537	
Fraction in mL	Conc. NaCl in mM								Average values
0 - 7,35	0 - 28	3,2	3,8	1,2	3,6	3,7	3,5	**4,7**	3,4
7,35 - 9,80	28 - 56	11,2	10,6	9,0	9,5	5,7	**12,5**	**14,1**	10,4
9,80 - 14,35	56 - 108	65,0	65,6	66,3	52,8	63,2	65,1	64,5	63,2
14,35 - 22,40	108 - 400	20,5	19,9	23,5	34,0	27,4	19,0	16,9	23,0
Summation:		100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0
Summation	0-56	14,5	14,4	10,2	13,1	9,3	**16,0**	**18,7**	13,7
Summation	56 - 108	65,0	65,6	66,3	52,8	63,2	65,1	64,5	63,2
Summation	108 - 400	20,5	19,9	23,5	34,0	27,4	19,0	16,9	23,0
Acidic groups >108 mM		**20,5**	**19,9**	**23,5**	**34,0**	**27,4**	**19,0**	**16,9**	**23,0**

Both figure 11 and table 5 show that a small amount (mean value of 3.4 %) of PSA will passes through the column when the concentration of NaCl is very low (0 – 28 mM) and the pH is 7.5. It's then shown in figure 11 that it seems to be a pre-peak of PSA (mean value of 10.4%) which elute when the concentration of NaCl is between 28 and 56 mM and a major peak (mean value of 63.2%) seems to eluate between 56 and 108 mM NaCl. But there is also some PSA that have more negative charge and elute at higher NaCl concentrations(>108 mM), which means that there are more acidic groups (probably more sialic acid) in these PSA molecules than in the others. In average, 23% of the PSA isoforms elute at a NaCl concentration higher than 108 mM NaCl, but UPN17 and UPN15 are exceptions with a mean value of 31 %.

As mentioned before, PSA is a glycoprotein with a single N-oligosaccharide chain attached to asparagine (aa 69) [18]. The N-oligosaccharide chain can show different antennary complexes [23,24, 26] but the most common one is bi-antennary complex. This bi-antennary complex can be both mono- and di-sialylated which corresponds to a high pI and a low pI respectively [26]. Okada et al. [25] describe that PSA consists of two different forms; PSA-A and PSA-B. 5-10% of total PSA in seminal fluid is PSA-A [25] which has less sialic acid than PSA-B, which means that PSA-A has a higher pI (7.2) than PSA-B (6.8).

The results in table 5 show that the urines from patients with PCa have a larger pre-peak of PSA than the other urines; this indicates that PCa PSA has higher pI and a structure of mono-sialylated bi-antennary complex. The structure with less sialic acid in the antennary complex seems to be the major structure for patients with PCa [27]. The major peak in figure 11 (mean value of 63.2%) is according to Okada et al. [25] PSA-B and will be the main form. PSA-B has probably a structure of a di-sialylated bi-antennary complex and a lower pI than the mono-sialylated structure. It´s also shown in table 5 that the urines from volunteers over 60 years old have PSA with a larger amount of acid groups (approximately 31% which elutes at a NaCl concentration above 108 mM NaCl), which indicates a higher amount of sialic acid in probably a multi-antennary structure with more than di-sialylated antennary complex.

5. Conclusions and future development

PSA in urine can be detected in low concentrations and with a low CV by using an immunochromatography test (IKR). This was also what Therése Tengstrand found out in her master's degree project about development of methods for characterization of PSA in urine, just before this project. The results obtained in this project show a large variation in PSA concentration between the urine samples (see table 6); the mean PSA value for all healthy participants was 533 µg /L (range 106-1824 µg /L).
The results also show a variance in concentration and amount of PSA in start and remaining portion of urine (see table 6). Start urine had a higher concentration of PSA than the remaining urine (729 µg/L versus 337 µg/L), but the amount of PSA was larger in the remaining urine than in the start urine (80 µg versus 40 µg). This was very interesting since the high concentration in the start urine indicated an enrichment of PSA in the urethra, but it seems like the prostate also has a continuous secretion of PSA during a urine round.

PSA isoforms in urine can also be separated by using ion exchange chromatography and the results show two clear peaks, a minor pre-peak with less acidic PSA (probably a structure of mono-sialylated bi-antennary complex) and a major peak with more acidic PSA (probably a structure of di-sialylated bi-antennary complex). Different urines show different distributions of the major peak of PSA isoforms, from a steep peak to a low and very wide peak. The urines from patients with PCa have a larger pre-peak of PSA isoforms than the other urines (see figure11 and table 6), this indicates that PCa PSA has more isoforms with higher pI and less sialic acid in the antennary complex. Less sialic acid in the antennary complex seems to be the major structure for patients with PCa acccording to Tabarés et al. [27].

The PSA isoforms in urine can be separated by using lectin (ConA) affinity chromatography. Some urines had a large precentage of PSA isoforms that were not binding to the ConA column. This can either be isoforms containing no carbohydrate at all or isoforms with a multiantennary carbohydrate structure. Assuming that the presence of isoforms without carbohydrates is low, as indicated from the ion-exchange chromatography results, a multiantennary factor was calculated by the ratio between %PSA eluted with 0 mM and at 1 mM α-MM. Six of the tested urines had a multiantennary factor of 48.7, while the remaining three had a mean value of 95.5. However, there seems to be some inconsistence between the results and therefore retesting is necessary.

Other urines, which are from volunteers over 60 years old, have PSA with a larger amount of acid groups (approximately 31% which elutes at a NaCl concentration above 108 mM NaCl, see table 6). This result indicates a higher amount of sialic acid, probably in a multi-antennary structure with more than di-sialylated antennary complex.

The results in this study encourage to further investigations with a larger group of urine samples from volunteers of different ages, especially volunteers over 60 years old, together with urines from PCa patients. It will also be very interesting to further optimise the separation conditions for IEC, to see if it possible to better distinguish the mono-sialylated bi-antennary complex.

Table 6: A summarized table over PSA concentration in urine samples and the separation of uPSA isoforms using anion-exchange and ConA (lectin-affinity) chromatography. Patients with PCa seem to have PSA isoforms with less sialic acid in the antennary complex (less % basic forms), while volunteers over 60 years old seems to have a higher amount of sialic acid in the antennary complex (more % acidic forms), according to the ion exchange chromatography. Some urines seem to have uPSA isoforms with more than bi-antennae, multiantennary complex.

	Code name	Time (collection)	Tot. volume col. mL	Urine type (start / remain)	µg PSA/L	µg PSA / urine round	Con A Multiantennary	Ion exchange chromatography % basic forms	% acidic forms
Man 24 year	UPN1	22:40	60	start	815	49	40,7	14,5	20,5
	UPN2		450	remain	375	169			
	UPN3	07:10	90	start	824	74			
	UPN4		320	remain	284	91			
	UPN5	12:30	110	start	218	24			
	UPN6		420	remain	227	95			
	UPN7	17:30	50	start	1235	62	47,5	14,4	19,9
	UPN8		450	remain	309	139			
Man 53 year	UPN9	00:50	39	start	417	16	52,7	10,2	23,5
	UPN10		100	remain	330	33			
	UPN11	06:25	47	start	428	20			
	UPN12		500	remain	106	53	44,9		
Man 62 year	UPN17	08:00	55	start	1824	100	57,7	13,1	34,0
	UPN18		250	remain	1270	318			
	UPN19	12:20	49	start	741	36			
	UPN20		280	remain	170	48	100,5		
Man 64 year	UPN13	18:00	42	start	311	13			
	UPN14		95	remain	184	17			
	UPN15	06:30	47	start	473	22	51,4	9,3	27,4
	UPN16		130	remain	110	14	90,8		
Patient urines, prostate cancer, under treatment?									
	UP6				459		95,3/45,4	16,0	19,0
	UP7				14		49	18,7	16,9

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