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Investigation of biomarkers for noninvasive early diagnosis of cancer

Sanjeewa Bandara Gamagedara

Department: Chemistry

INVESTIGATION OF BIOMARKERS FOR NONINVASIVE EARLY DIAGNOSIS OF CANCER

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SANJEEWA BANDARA GAMAGEDARA

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Yinfa Ma, Advisor
Philip D. Whitefield
Paul K. Nam
Jeffrey G. Winiarz
David J. Westenberg

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ABSTRACT

Cancer is the second leading cause of death worldwide, and deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030. Cancer occurs when cells in a part of the body begin to grow out of control. It develops very rapidly, and early diagnosis offers a greater chance of successful treatment and a higher survival rate. Biomarkers are good potential tools for early diagnosis. The main hypothesis of this study is that the levels of certain metabolites present in human urine can indicate the current or future behavior of cancer. Therefore, these studies focused on metabolomics biomarker analysis in urine. Eight pteridine molecules were analyzed using a house-built, high-performance capillary electrophoresis with laser-induced fluorescence detection (HPCE-LIF) in the first study. The results showed that 5 pteridine molecules were elevated in cancer compared to non-cancer urine samples. The second study investigated the levels of proline, kynurenine, uracil, and glycerol-3-phosphate in 126 patients with genitourinary malignancies (63 prostate cancers & 63 bladder cancers) and compared them to 68 normal samples using a validated LC/MS/MS method. Statistical analysis showed that the above biomarkers were not reliable enough for prostate cancer detection or for differentiating the aggressiveness of prostate cancer. The third study describes a validated, reliable, fast, and simple HPLC-MS/MS method to simultaneously separate and detect potential urinary bladder cancer biomarkers such as taurine, L-phenylalanine, hippuric acid and creatinine in urine samples. The final paper is a review article that discusses the applications of different LC-MS/MS and CE-MS techniques in prostate biomarker discovery.

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1. INTRODUCTION

Hippocrates (460 BC – 370 BC) first described several kinds of cancers, referring to them with the Greek word *carcinos* (crab or crayfish). This name came from the appearance of the cut surface of a solid malignant tumor, which has veins stretched on all sides, appearing much like a crab's feet. Cancer is the second leading cause of death worldwide. The disease accounted for 7.9 million deaths in 2007, which is around 13% of all deaths worldwide [1]. The main types of cancer leading to overall cancer mortality each year are lung cancer (1.4 million deaths/year), stomach cancer (866,000 deaths/year), liver cancer (653,000 deaths/year), colon cancer (677,000 deaths/year) and breast cancer (548,000 deaths/year) [1]. About 72% of all cancer deaths in 2007 occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 [1]. The most common types of cancer among men are lung, stomach, liver, colorectal, esophagus and prostate. Among women, the most common types are breast cancer, lung cancer, stomach cancer, colorectal cancer and cervical cancer. These statistics allow us to gauge the intensity and magnitude of this problem. Many research organizations and scientists are focused on cancer research because it is one of the most serious health issues in the modern world.

1.1 CANCER

Normal cells divide and grow in an orderly fashion, but cancer cells grow in a more sporadic manner. Cancer occurs when cells in a part of the body begin to grow out

of control and crowd out normal cells. Although many different kinds of cancer exist, they all have in common this out-of-control cell growth. Cancer cells invade and metastasize. Invasion refers to the intrusion upon and destruction of adjacent tissues and metastasis refers to spreading to other locations in the body via the lymphatic system or blood. A normal cell transforms into a tumor cell through a multistage process instigated by the interaction between a person's genetic factors and three categories of external agents. These three categories include physical carcinogens, such as ultraviolet and ionizing radiation; chemical carcinogens, such as asbestos, components of tobacco smoke, aflatoxin, and arsenic; and biological carcinogens, such as infections from certain viruses, bacteria or parasites. Cancer develops rapidly, and early diagnosis and treatment greatly improve the chances that the patient will survive and live an active and productive life. For example, lung cancer begins with changes in some of the cells in the lungs. As the cancer grows, some of the cells may spread from the lungs to other parts of the body. If the cancer cells are only in the lungs and have not spread to other parts of the body, it is likely that they can be removed completely and not be life threatening. Also, if cancer is diagnosed at an early stage, the patient will have a much greater chance of successful treatment and more treatment choices. According to World Health Organization, about one-third of the cancer burden could be decreased if cancer was detected and treated at an early stage, but early-stage diagnosis is a major challenge to modern cancer research [1]. Currently, cancer diagnosis is based largely on radiological evaluations, such as mammography, X-ray computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and morphological examination of tumor biopsy specimens, as it has been for decades. This approach has significant limitations for

predicting a given tumor's potential for progression and response to treatment. These methods also may fail to detect cancer early in its development. Biomarkers, however, are good potential candidates for this early diagnosis [2, 3].

The second and fourth papers in this dissertation provide detailed discussions of prostate cancer. A healthy prostate in the male reproductive system is a small, soft gland located under the bladder [2]. Its main function is to help produce and store seminal fluid. The process of cell mutation in the prostate glands results in prostate cancer, which has become the most common cancer among American men. Unfortunately, in its early stages, prostate cancer does not show any symptoms; however, it can cause elevated prostate specific antigen (PSA) levels that can be detected in routine medical examinations [4]. The major drawback of PSA is that it leads to false-negative or false-positive test results [4]. Also, other diagnostic methods, such as digital rectal examination and radiological techniques, have their own side effects. Thus, an urgent need exists for reliable biomarkers to detect prostate cancer.

1.2 BIOMARKERS

A biomarker is any biological, chemical, or biophysical indicator of an underlying biological process. Biomarkers are used in many fields, such as biology, medicine, geology, astrobiology, and genetics. In medicine, a biomarker can be a substance whose detection indicates a particular disease state. We can specifically define a cancer biomarker as *"A molecular, cellular, tissue, or process-based alteration that provides indication of current, or more importantly, future behavior of cancer"* [5]. These biological and physiological indicators could include a broad range of biochemical entities, such as nucleic acids, proteins, sugars, lipids, and small metabolites, as well as

whole cells, in either specific tissues of interest or in the overall circulatory system. Biomarkers can be detected, either individually or as larger sets or patterns, using a wide variety of methods, ranging from biochemical analysis of blood or tissue samples to biomedical imaging. Modern technological developments in genomics and proteomics have made it much easier to examine a large number of potential markers simultaneously. However, progress remains limited by the sensitivity and specificity of the current technologies, as well as the methods and tools used to analyze the enormous pools of data generated by high-throughput technologies. The need remains for new and improved technologies to discover potential biomarkers.

Biomarkers offer many advantages compared to other traditional clinical diagnosis techniques. Biomarkers can be used as non-invasive diagnosis methods. People typically do not like to spread or damage their organs and tissues to give samples during the diagnostic process, nor do they like to give blood for such tests [3]. Non-invasive diagnosis is a medical diagnosis procedure that does not penetrate mechanically or break the skin or any body cavity. It does not require an incision into the body or the removal of biological tissue. Therefore, many researchers have begun to focus on non-invasive cancer diagnosis, primarily through analyzing urinary cancer biomarkers because donors tend to give urine more willingly than tissue or blood samples. Furthermore, biomarkers often are less expensive and easier to measure than 'true' endpoints. Also, they can be measured expeditiously at an earlier stage. Given these advantages, the studies presented in this dissertation focus on non-invasive biomarker analysis in human urine.

1.3 URINE ANALYSIS

Urine is a typically sterile liquid by-product of the body that is secreted by the kidneys. Urine is produced through a process of filtration, reabsorption, and tubular secretion. The kidneys extract soluble wastes, as well as excess water, sugars, and a variety of other compounds, from the bloodstream. In addition to these major components, urine contains proteins and other metabolites in relatively low concentrations. These proteins and metabolites can be valuable indicators of the status of an underlying disease. Urine also is a popular medium for biomarker discovery because its collection is not invasive. Normally, centrifugation was done in urine sample preparations to remove sediments. Sometimes, cutoff ultracentrifugation was conducted in the urinary protein analysis to remove high-abundance proteins. In most cases, solid phase extraction [6] was incorporated as a metabolite pre-concentrate technique, as well as a sample clean-up procedure. Creatinine was included as an analyte in all of these studies to account for the renal dilution of urine [7]. A creatinine concentration corresponds closely to urine dilution, so its levels must be monitored in any urinary biomarker analysis, with a biomarker-to-creatinine ratio representing the biomarker levels in urine [3, 7]. After collection, urine should be refrigerated to minimize bacteria growth. Samples were stored long-term at -80°C to minimize the degradation of urinary metabolites.

1.4 ANALYTICAL TRENDS

Urinary biomarkers have been analyzed by various methods, such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and liquid chromatography tandem mass spectrometry (LC/MS/MS). The following papers

primarily utilized CE and LC/MS/MS techniques for urinary cancer biomarker analysis. CE separates ions based on their electrophoretic mobility with the use of an applied voltage. Many factors, such as the charge of the molecule, the viscosity, and the atom's radius, affect the electrophoretic mobility. The rate at which the particle moves is directly proportional to the applied electric field-the greater the field strength, the faster the mobility. Although neutral species are not affected, ions move with the electric field. Therefore, even if two ions are the same size, the one with the greater charge will move the fastest. For ions with the same charge, the smaller particle has less friction and an overall faster migration rate. CE offers faster results and provides high-resolution separation. It has been used widely as a highly-efficient analytical technique in biomedical research and clinical and forensic analysis [8, 9] . CE has utilized different detection techniques, such as ultraviolet-visible absorption, conductimetry, mass spectrometry, patch clamp, electrochemical detection, and laser-induced fluorescence, based on the nature of the analytes [10]. CE has been used to study a variety of analytes from smaller molecules (inorganic ions and organic molecules) to larger biomolecules (DNA and proteins). CE is used often in bioanalysis due to a number of distinct advantages it offers compared to other analytical methods. CE requires only a very small sample volume for a single run (nL), making it an ideal analytical technique for applications that require analysis of low nanoliter samples. Recently, CE reportedly has been used in single-cell analysis [11] and even subcellular-level analysis [12]. CE generates a very small amount of waste compared to HPLC. Other advantages include rapid analysis, great resolution, and low cost. All of these advantages designate CE as an ideal tool for biomarker analysis [13].

Liquid chromatography-mass spectrometry (LC/MS) is a powerful, widely-used tool for quantitative and qualitative biomarker analysis. It is very useful due to its high sensitivity and ability to identify chromatographically co-eluting peaks by mass selectivity independent of chromatographic resolution [14]. The mass spectrum generates a chemical fingerprint of a compound, ensuring accurate peak assignment, even in the presence of a complex matrix. LC/MS also can determine or confirm known and unknown compounds using molecular weight. Furthermore, controlled fragmentation permits structural elucidation of unknown compounds. LC/MS permits rapid method development because it easily identifies eluted analytes without the need to validate retention time. Further, its sample matrix adaptability decreases sample preparation time. Using LC/MS, quantitative and qualitative data can be collected easily with limited instrument optimization. Tandem mass spectrometry coupled with electrospray ionization (ESI) was used for the studies presented in this dissertation . Tandem mass spectrometry uses two or more quadrupoles to separate ions based on a sample's electronic mass-to-charge ratio. In ESI, a sample solution is sprayed across a high potential difference from a needle capillary into an orifice in the interface. Heat and gas flows then are used to de-solvate the ions in the sample solution. These characteristics and advantages make LC/MS a promising tool for the identification and quantification of biomarkers.

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PAPER

I. INVESTIGATION OF URINARY PTERIDINE LEVELS AS POTENTIAL BIOMARKERS FOR NON-INVASIVE DIAGNOSIS OF CANCER

ABSTRACT

Background: Biomarkers are good potential tools for early cancer diagnosis. Here we have analyzed eight different pteridines in the urine samples of cancer patients and compared them with samples from healthy subjects. Pteridines are important cofactors in the process of cell metabolism, and they have recently become a focal point of cancer screening research because certain pteridine levels have been shown to reflect the presence of cancers.

Methods: This study analyzed eight pteridines; 6,7-dimethylpterin, 6-biopterin, D-(+)-neopterin, 6-hydroxymethylpterin, pterin, isoxanthopterin, xanthopterin and pterin-6-carboxylic acid using a house-built high-performance capillary electrophoresis with laser-induced fluorescence detection (HPCE-LIF). The levels of pteridines were reported as a ratio of pteridine to creatinine. Statistical hypothesis testing was conducted and P-values were calculated to analyze the data.

Results: Among the eight pteridines studied, 6-biopterin, 6-hydroxymethylpterin, pterin, xanthopterin, and isoxanthopterin levels were significantly higher in samples from cancer patients than in those from healthy subjects. Further, xanthopterin and isoxanthopterin levels were compared in breast cancer and lung cancer patients, and no significant difference was observed.

Conclusion: This work demonstrates that some pteridine levels can be used as biomarkers for noninvasive diagnosis of cancer; however, more data are needed to support this hypothesis.

KEYWORDS

Capillary Electrophoresis, Biomarkers, Pteridines, Cancer, Urine

1. INTRODUCTION

Cancer is the second leading cause of death worldwide, accounting 7.9 million deaths in 2007. It is the cause of about 13% of all deaths worldwide [1]. The types of cancer that contribute most to cancer mortality each year are lung cancer (1.4 million deaths/year), stomach cancer (866,000 deaths/year), liver cancer (653,000 deaths/year), colon cancer (677,000 deaths/year), and breast cancer (548,000 deaths/year) [1]. In 2008, there were an estimated 1,437,180 cancer cases in the United States. About 72% of all cancer deaths in 2007 occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 [1]. These statistics demonstrate the intensity and magnitude of the problem. Many research organizations and scientists focus on cancer research because it is one of the most serious health issues of the modern world.

Cancer occurs when cells in a part of the body begin to grow out of control. Normal cells divide and grow in an orderly fashion, but cancer cells do not, and they crowd out normal cells. Although there are many kinds of cancer, all have this trait in common. Cancer develops rapidly, and early diagnosis and treatment greatly improve the

chances that a patient will survive and live an active and productive life. Also, if cancer is diagnosed at an early stage, the patient will have a much greater chance of successful treatment and more treatment choices. Currently, as for the last several decades, the diagnosis of cancer is based largely on radiological evaluations such as mammography, X-ray computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and morphological examination of tumor biopsy specimens. This approach has significant limitations for both early diagnosis and for prediction of a tumor's potential for progression and response to treatment. Biomarkers, however, are good potential tools for early diagnosis.

Hayes et al. (1996) defined a cancer biomarker as "a molecular, cellular, tissue, or process-based alteration that provides indication of current, or more importantly, future behavior of cancer" [2]. These biological and physiological indicators could include a broad range of biochemical entities, such as nucleic acids, proteins, sugars, lipids, and small metabolites, as well as whole cells, in either specific tissues or in circulation. Today, circulating cancer cells are becoming a powerful tool in "micro-scopic" cancer screening. Detection of biomarkers, either individually or as larger sets or patterns, can be accomplished by a wide variety of methods, ranging from biochemical analysis of blood or tissue samples to biomedical imaging. This research investigated the urinary pteridine levels as potential biomarkers for noninvasive diagnosis of cancer.

Normally, patients are hesitant to damage their organs and tissues to give samples during the disease diagnosis process. They may also be reluctant to give blood for diagnostic tests. Therefore, development of a non-invasive diagnostic technique for early cancer screening is very crucial for all populations. Noninvasive diagnosis involves

procedures that do not penetrate the body mechanically, nor break the skin or involve penetration through a body cavity. It does not require an incision into the body or the removal of biological tissue. Currently, many researchers are focusing on noninvasive means to diagnose cancer by analyzing cancer biomarkers in urine, which is more easily collected than tissue or blood samples.

Pteridines have become a focal point of cancer screening research in the last two decades because certain pteridine levels have been shown to reflect the presence of cancer [3-9]. The present research took a noninvasive approach to the detection of cancer by analyzing eight pteridines in urine samples from cancer patients and comparing them from healthy objects with no evidence of cancer. Specifically the project tested for 6,7-dimethylpterin, 6-biopterin, D-(+)-neopterin, 6-hydroxymethylpterin, pterin, isoxanthopterin, xanthopterin, and pterin-6-carboxylic acid. These pteridines are well distributed in living organisms. Pteridine and its derivatives play important roles in the synthesis of some vitamins, and they are important cofactors in the process of cell metabolism. The pteridines are excreted in human urine and they can be potentially used as biomarkers in clinical diagnosis. Pteridine levels have been reported to be significantly elevated when the cellular immune system is activated by certain diseases such as cancer, viral infections, and renal disease [9-12,19]. Kaufman [13] noted the importance of various pteridines in the cell metabolism of higher mammals. Different pteridine derivatives can play various roles in tumor-related diseases. Each type of tumor is likely to result in a distinct pattern of changes in pteridine concentrations. High-performance liquid chromatography (HPLC) methods have been used for pteridine analysis [14-16]. However, it is time-consuming and expensive, and it results in unsatisfactory separations, especially

for real urine samples. On the other hand, high-performance capillary electrophoresis (HPCE) is fast and efficient and requires only a small sample size. Our group has developed and optimized a HPCE with laser-induced fluorescence (LIF) detection method (HPCE-LIF) for quantitative analysis of pteridines in urine samples [7, 8]. This study used optimized HPCE-LIF technique investigated pteridine level patterns in 38 urine sample from a variety of cancer patients. Some types of cancer were not studied mainly due to the availability of urine samples not the limitation of the technique. In order to assure that the pteridine levels representing for the physiological concentration, the amount of pteridines was reported here as a ratio of pteridine to creatinine [17].

2. MATERIALS AND METHODS

2.1 Chemicals

Six of the pteridines, 6-biopterin, D-(+)-neopterin, pterine, isoxanthopterin, pterine-6-carboxylic acid, and xanthopterin, were purchased from Sigma-Aldrich® (St. Louis, MO, USA). The other two, 6,7-dimethylpterin and 6-hydroxymethylpterin, were purchased from Schircks Laboratories™ (Jona, Switzerland). Boric acid and tris-(hydroxymethyl) aminomethane were also purchased from Sigma-Aldrich® (St. Louis, MO, USA). Sodium hydroxide, EDTA disodium salt, potassium iodine, iodide, and sodium phosphate dibasic were purchased from Fisher Scientific™ (Fair Lawn, NJ, USA). Ultrapure water was obtained using a Milli-Q Advantage® A10 and Millipore Elix® water purification system. All pH measurements were performed on an Accumets® Excel XL-15 pH meterthat was standardized using pH standards 4, 7, and 10 (Fisher

Scientific™). Standard creatinine for creatinine analysis was purchased from Sigma-Aldrich® (Milwaukee, WI).

2.2 Buffer Preparation

A 0.1 M Tris-0.1 M borate-2 mM EDTA running buffer was prepared and pH was adjusted to 9.63±0.02 using 2.0 M NaOH. A 50mM aqueous solution of Na_2HPO_4 sample buffer was prepared and pH was adjusted to 7.70±0.02 using concentrated phosphoric acid. This was used to dilute both standards and samples. A 15 mM KH_2PO_4 solution was prepared and pH was adjusted to 6.4 using 1.0 M NaOH. This was used as the running buffer for creatinine analysis. The creatinine sample dilution buffer was prepared in exactly the same way as the running buffer except that 2% EDTA was present in sample dilution buffer. EDTA was used to complex the metal ions in the urine sample so that these ions would not form complexes with creatinine. All the buffers were filtered with a 0.45μm membrane and degassed before use.

2.3 Standard Preparation

To prepare pteridine standard solutions, 2mg of pteridine standard was dissolved in a solution consisting of 0.3 mL of 1.0M sodium hydroxide and 9.7 mL of sample buffer. A standard mixture was then prepared by combining equal-molar concentrations of each standard and diluted to a final concentration of 5.0×10^{-5}M with the sample buffer. A dilution series of this stock solution was used to generate calibration curves.Standard creatinine stock solution was prepared by dissolving 50 mg creatinine into 50 mL sample dilution buffer to make the creatinine concentration 100 mg/dL. This standard stock solution was diluted to an appropriate concentration with the sample dilution buffer.

2.4 Oxidizing Solution Preparation

The oxidizing solution consisted of 4.0% potassium iodide and 2.0% iodine (w/v). It was prepared by dissolving 0.6008 g of KI in 15 mL of Milli-Q water. Once the KI was dissolved, 0.3025 g of I_2 was added to the solution. The solution was then stirred (for about 1 hr) until the I_2 was completely dissolved. The final solution was stored in an amber glass vial, and the cap was wrapped with Parafilm. The vial was wrapped with aluminum foil to avoid photo degradation of the iodine.

2.5 Urine Samples Preparation

Urine samples from cancer patients who didn't undergo chemical or radiation therapy were obtained from Ellis Fischel Cancer Center, Columbia, Missouri. The normal urine samples were collected from student volunteers at Missouri S&T who did not take any medicine including vitamin supplements. The demographic distribution of cancer patients and normal subjects are mainly from the near by cities like Columbia, Jefferson City, Rolla, and St.Louis. The cancer patients age distribution varies from 26 to 70 years and normal subjects age range varies from 22 to 45 years. There were no dietary or exercise restriction performed among the control group and cancer patients. The urine samples from the cancer patients represented breast cancer (12), lung cancer (9), colon cancer (4), rectal cancer (2), pancreatic cancer (1), Ovarian Cancer (3) non-hodgkins lymphoma (4), esophageal cancer (1), bladder cancer (1), and kidney cancer(1). All the samples were stored in a freezer at -80°C. Prior to analysis, the samples were removed from the freezer and brought to room temperature. A 1000 μL aliquot of urine sample was taken using a micropipette and placed in a 1.5 mL yellow microcentrifuge tube. Then 400 μL of the oxidizing solution and 100 μL of 2.0M sodium hydroxide were added to

the sample and mixed well. The sample mixture was incubated at 4°C for 30 min and centrifuged at 5000 rpm for 20 min at 4 °C. Then 500 μL of the supernatant was placed in a new vial, and 500 μL of sample buffer was added to it. It was mixed thoroughly and injected directly into the CE for analysis. Further dilutions were made in some samples.

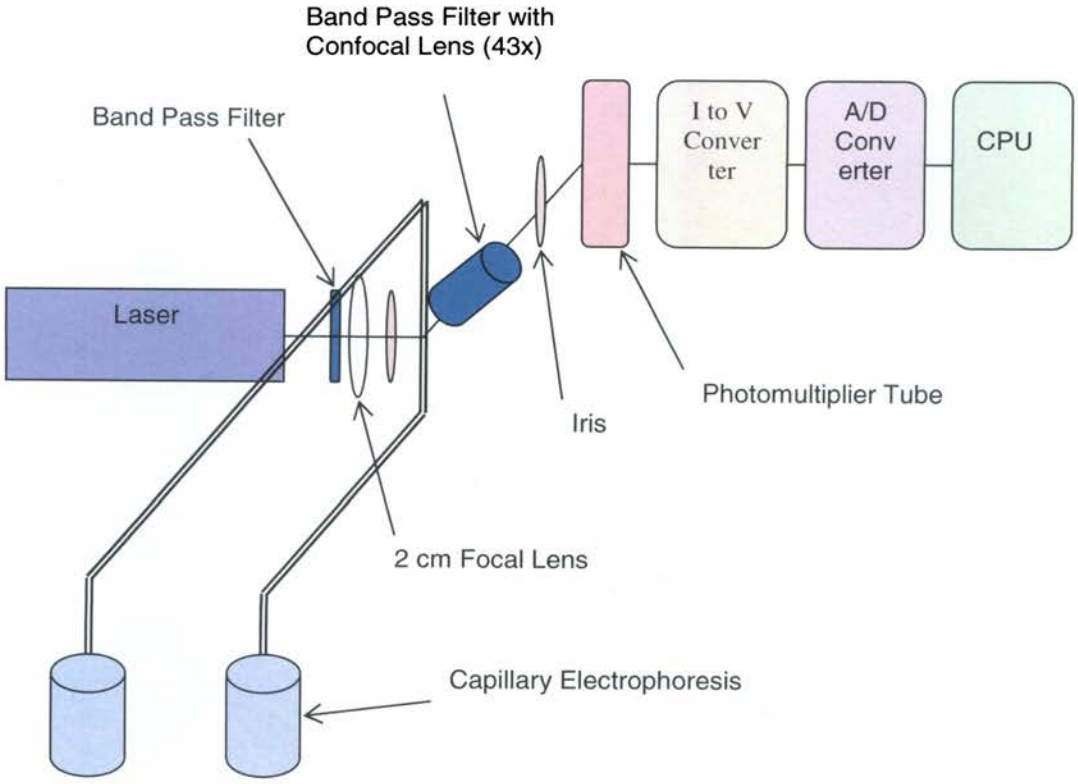

Figure 1. Instrumental design of the home-built CE-LIF system.

2.6 Capillary Electrophoresis Laser-induced Fluorescence System

A home-built capillary electrophoresis LIF system was used for this study as shown in Figure 1 [18]. A Milles Griot Omnichrome Series-74, 325 nm laser (35mW

power, Carlsbad, CA, USA) was used for excitation. The stray and scattered light from the nonpolarized laser were removed using a 325 nm band-pass filter (Ealing, Holliston, MA, USA; model UG-11). The laser beam passed through an iris and was focused on the capillary window with a 2.0 cm focal length lens. The fluorescence emission was collected by a microscopic objective and focused onto a R982 Hamamatsu photomultiplier tube (Bridgewater, NJ, USA). Background noise was minimized using a band-pass filter (400–539 nm, Ealing, model 35–532). The resulting output current from the photomultiplier tube was converted to a voltage signal through a home-built current-to-voltage converter. The analog signal was then digitalized though a Logger Pro analog-to-digital converter (Verniers Software and Technology, Beaverton, OR, USA). The data were collected using Logger Pro 3.1 data collection software.

2.7 Creatinine Analysis

Creatinine analysis was performed following a method that was previously developed in our group. A Beckman P/ACE capillary electrophoresis instrument equipped with ultraviolet absorbance was used for creatinine analysis. The detection wavelength was set at 214 nm. The capillary column (Polymicro Technologies, Phoenix, AZ) used was 60 cm x 50 μm inner diameter fused silica tubing with an effective length of 35 cm. Electrophoresis was carried out at 20 kV. Urine samples were diluted with sample dilution buffer and directly injected into the HPCE column. The data were collected and processed by 32 Karat Software version 5.0 (Beckman, Inc.). Peaks were identified by both retention time and standard addition.

2.8 Pteridine Analysis

A 70 cm x 50 μm inner diameter fused silica capillary (Polymicro Techniques, Phoenix, AZ, USA) was used for separation. First, the new capillary was rinsed with 1.0M NaOH for 15 min, followed by MilliQ water for 5 min. Next, it was rinsed with 1 M HCl for 15 min, with MilliQ water for 5 min, and finally with running buffer for 15 min. A 1 cm section of the polymer coating was burned off at 35 cm from the cathode end, forming the detection window and leaving an effective capillary length of 35 cm. Samples were injected into the capillary by the gravimetric method. Samples were injected 17.5 cm from the top of the sample to the instrument table with an injection time of 10 s. Electrophoresis was carried out at 26 kV (371 V/cm) for 20 min. After each injection, the capillary was regenerated by flushing with 0.2M NaOH for 1 min, followed by a 2 min water rinse, and finally by a 2 min running buffer rinse. The individual pteridine peaks in the urine samples were identified by retention time comparisons and standard addition and obtained from the Logger Pro software.

Table 1. Calibration data for each pteridine.

Pteridine	Limit of detection	Linear equations of calibration curves	R^2 Values
6,7-dimethylpterin	2.5×10^{-10}	$y = 1.2383 \times 10^6 X + 0.9642$	0.9828
6-biopterin	2.5×10^{-10}	$y = 9.2704 \times 10^5 X + 0.3174$	0.9911
6-hydroxymethylpterin	2.5×10^{-10}	$y = 7.6386 \times 10^5 X + 0.3339$	0.9923
D(+)neopterin	2.5×10^{-10}	$y = 9.2866 \times 10^5 X + 0.2902$	0.9947
Pterin	2.5×10^{-10}	$y = 7.9057 \times 10^5 X + 0.3014$	0.9936
Isoxanthopterin	2.5×10^{-10}	$y = 3.6299 \times 10^5 X + 0.0897$	0.9987
Xanthopterin	2.5×10^{-10}	$y = 6.5796 \times 10^5 X + 0.0863$	0.9988
Pterin-6-carboxylic acid	4.72×10^{-10}	$y = 1.6335 \times 10^6 X + 0.3788$	0.9988

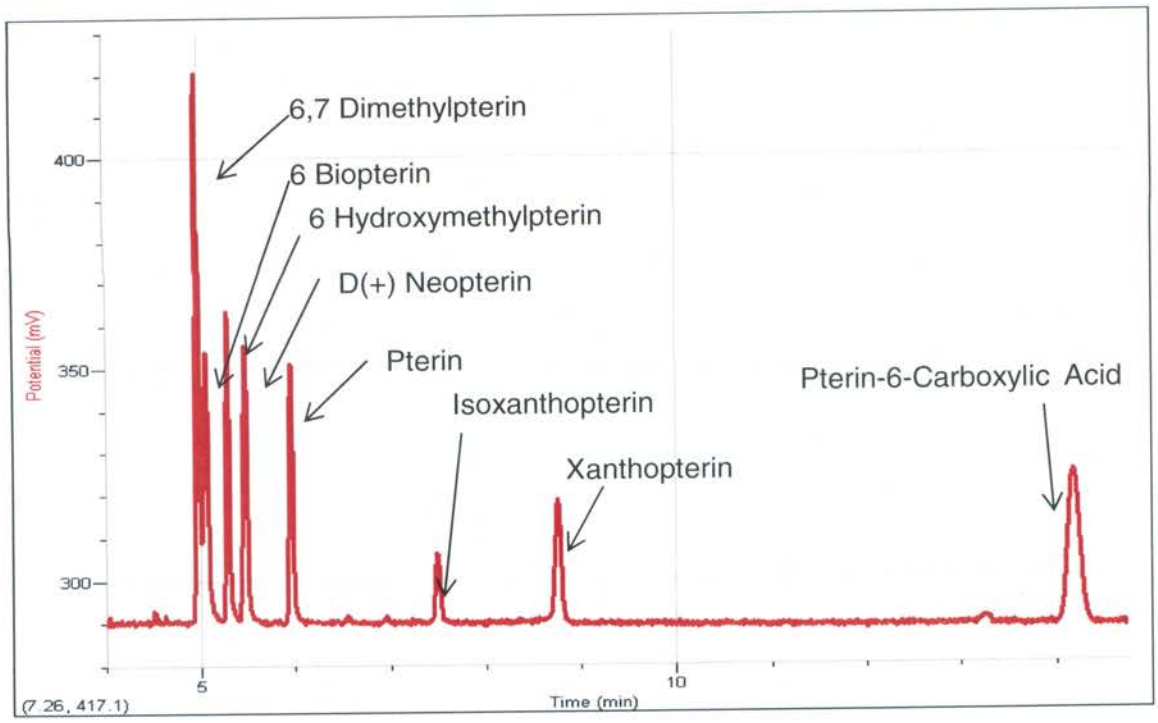

Figure 2. Electropherogram of the eight pteridine standards. The experimental conditions were described in the experimental section.

2.9 Statistical Analysis

Pteridine concentrations were determined by linear regression against experimentally generated calibration curves. The basic statistical information such as mean, variance, and pooled variance of each pteridine for both cancer and normal urine samples were calculated by MINITAB™ software . Statistical hypothesis testing was conducted to analyze the data. The null hypothesis, H_0, was that the mean pteridine levels in cancer samples are lower than or equal to those in normal urine. The alternative hypothesis, H_1, was that mean pteridine levels in cancer samples are higher than those in normal samples. The P values (last raw in Table 2) were calculated for all samples. The

null hypothesis was rejected, and the results demonstrate that the alternative is significant at a 5% significance level if the P value is set at ≤ 0.05.

3. RESULTS AND DISCUSSION

Pteridines exist in three oxidative states: tetrahydro-, dihydro-, and fully oxidized. These compounds require sample preparation to obtain accurate quantification that either fully oxidizes or reduces the pteridines [8]. For this project, full oxidization of pteridines in the urine samples was selected for ease of sample preparation and quantitation. Fully oxidized pteridines also produce a simpler sample matrix with less background interference [8].

The standard mixture of eight pteridines was first injected into the CE, and the elution order was obtained. The detection limit, linear equations and R^2 values are given in the table 1. Since the method detection limit, quantitation limit, and the linear range have been carefully studied and reported [7], they were not described in this study. Figure 2 showed that the 6,7- dimethylpterin eluted first and the pterin-6-carboxylic acid eluted last. Figure 3 through Figure 12 showed the box plots of various ptridines in samples from both cancer patients and healthy subjects. Creatinine calibration graph had a linear range from 0 to 20 mg/dL and R^2 was 0.9819. Creatinine levels in cancer patients and healthy subjects were shown in Figure 13.

Table 2 Basic statistical summaries of each pteridine for cancer and normal urine samples.

Pteridine	6,7-dimethyl pterin	6-biopterin	6-hydroxymethyl pterin	D-(+)-neopterin	Pterin	Isoxantho pterin	Xanthopterin	Pterin-6-carboxylic acid
Mean Cancer (n=38)	7.41E-04	8.96E-04	4.81E-04	0.001.14E-03	6.00E-04	3.37E-02	5.59E-03	2.54E-04
Mean Normal (n=17)	2.79E-05	1.14E-04	4.83E-05	2.37E-04	1.75E-04	1.06E-03	1.13E-03	1.25E-04
Variance Cancer	8.81E-06	2.67E-06	9.20E-07	1.16E-05	5.28E-07	1.10 E-03	3.94E-05	9.109E-08
Variance Normal	3.30E-09	1.14E-08	1.57E-09	1.20E-07	2.93E-08	5.66E-07	1.47E-06	1.98E-08
Pooled Variance	6.15E-06	1.87E-06	6.43E-07	8.16E-06	3.78E-07	7.68E-04	2.79E-05	6.957E-08
t Stat	0.986	1.958	1.849	1.079	2.373	4.040	2.891	1.6817877
P(T<=t) one-tail	0.164	0.028	0.035	0.143	0.011	8.67E-05	0.003	0.049247

Table 2 provided basic statistical information such as mean, variance, and pooled variance of each pteridine for both cancer and normal urine samples. The key objective of this work was to determine whether there is any significant difference between levels of pteridine in cancer urine samples and those in normal urine samples. Figure 3 showed the box plot of 6,7-dimethylpterin. In the urine samples of cancer patients three outliers are disregarded, there was no significant difference in levels of 6,7-dimethylpterin in cancer and normal samples. This was confirmed by the P value. The calculated P value for 6,7-dimethylpterin ($P = 0.16$) was greater than 0.05. The P value of 6-biopterin was 0.028. Since the latter was less than 0.05, the mean value of 6-biopterin in the cancer samples was significantly greater than that in the normal samples. The results were shown in Figure 4. Figure 5 indicated that, even after the outliers were removed, the 6-hydroxymethylpterin level in cancer patients was higher than the normal. Its P value ($P = 0.035$) was smaller than 0.05.

Figure 6 showed that the D-(+)-neopterin level was higher in cancer patients than in normal subjects. However, when the P value ($P = 0.14$) was considered, it was greater than 0.05. Therefore, the D-(+)-neopterin level in cancer urine samples was not significantly greater than normal because D-(+)-neopterin variance was higher in cancer. The P value of pterin was 0.011. Since this was less than 0.05, pterin levels in cancer were significantly higher than normal (Figure 7).

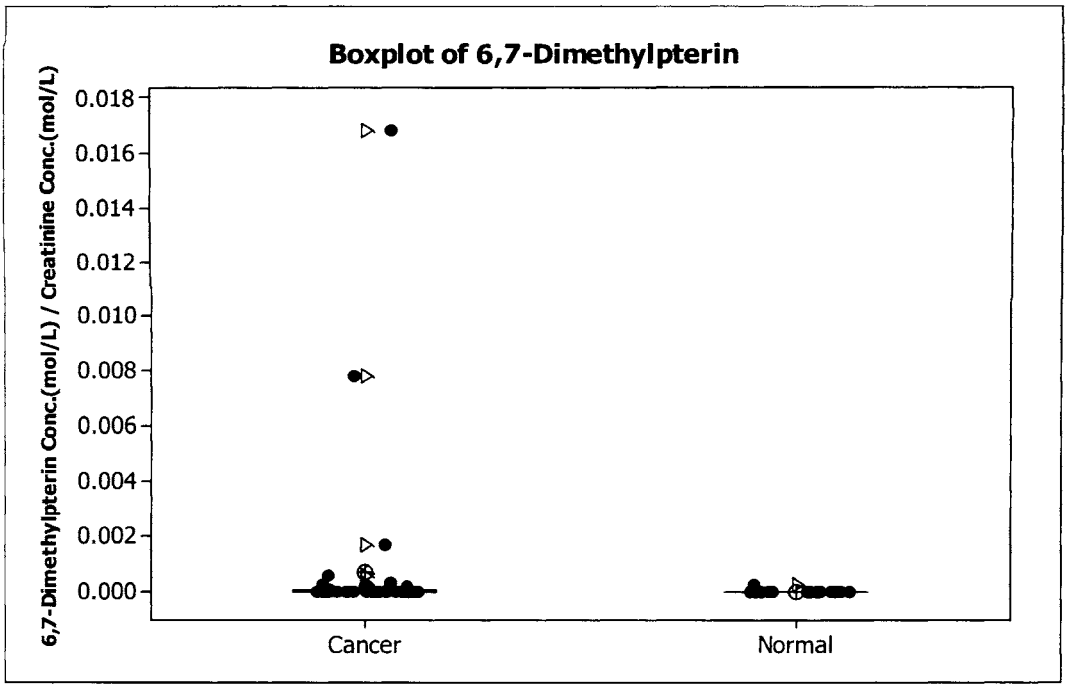

Figure 3 Box plot of 6,7-dimethylpterin levels in cancer urine samples (n=38) and normal urine samples (n=17).

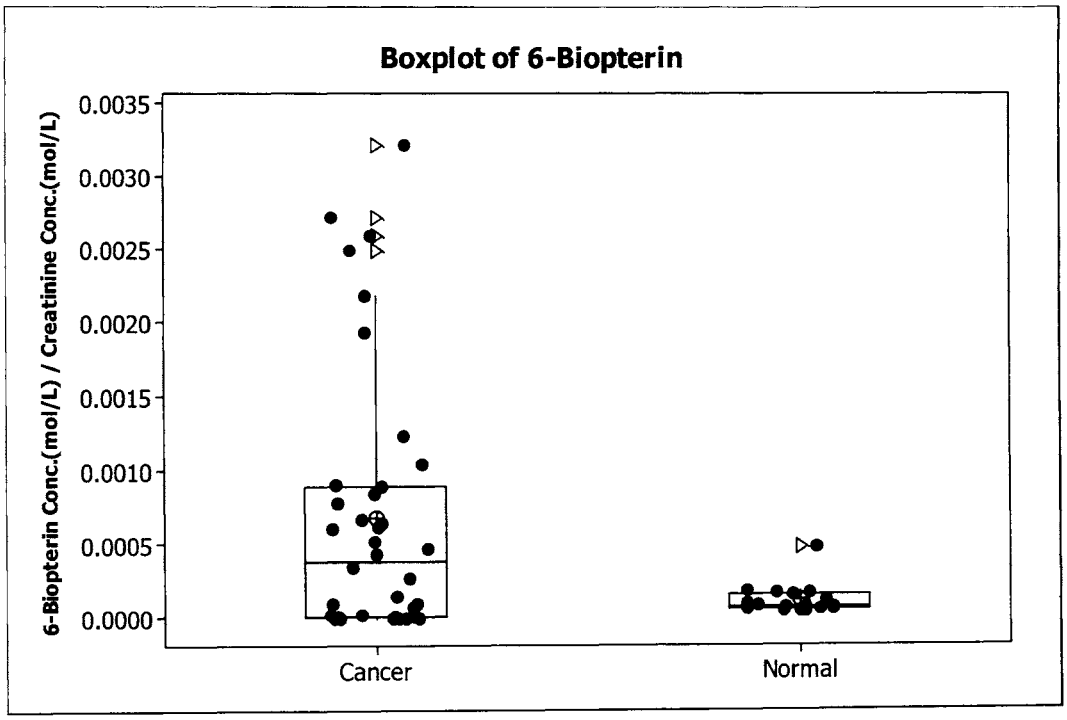

Figure 4 Box plot of 6-biopterin levels in cancer urine samples (n=38) and normal urine samples (n=17).

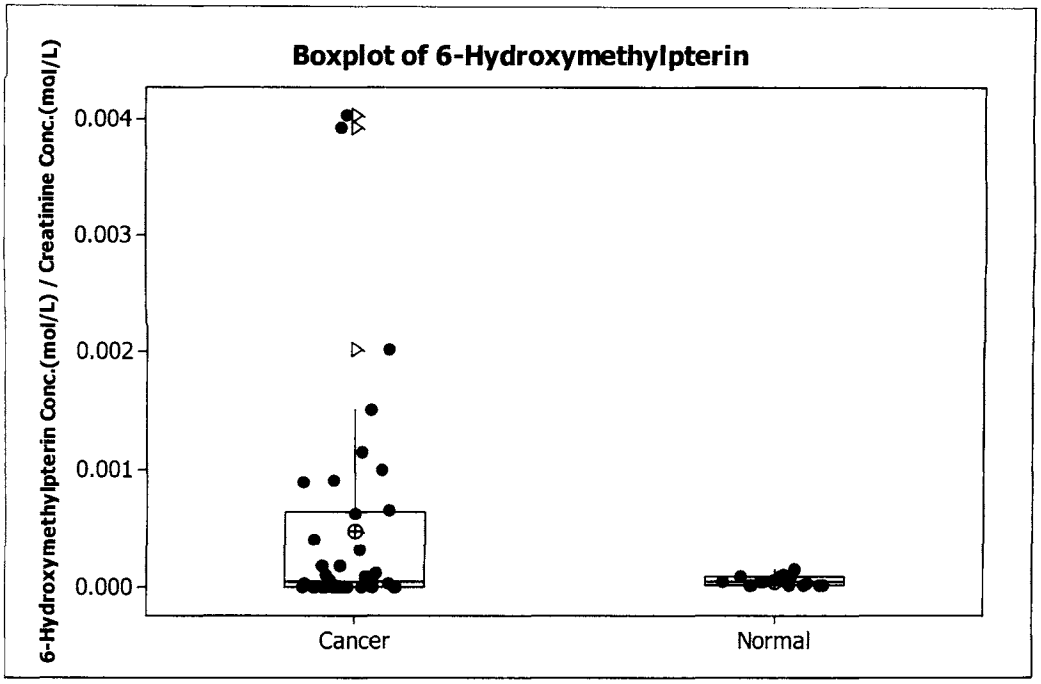

Figure 5 Box plot of 6-hydroxymethylpterin levels in cancer urine samples (n=38) and normal urine samples (n=17).

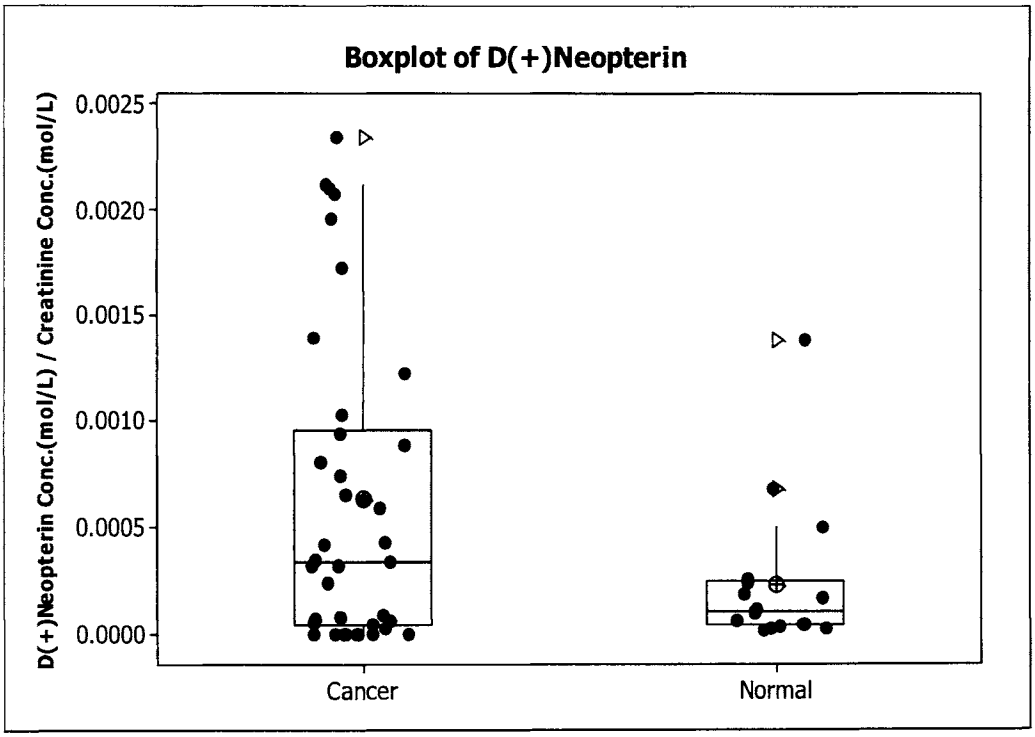

Figure 6 Box plot of D-(+)-neopterin levels in cancer urine samples (n=38) and normal urine samples (n=17).

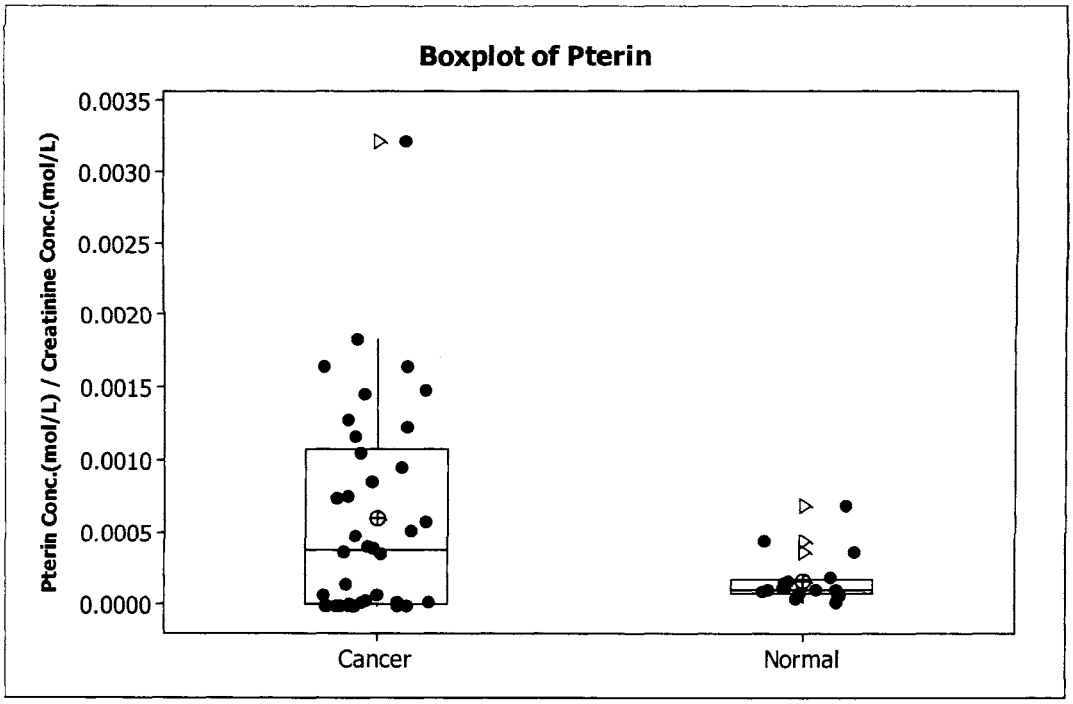

Figure 7 Box plot of pterin levels in cancer urine samples (n=38) and normal urine samples (n=17).

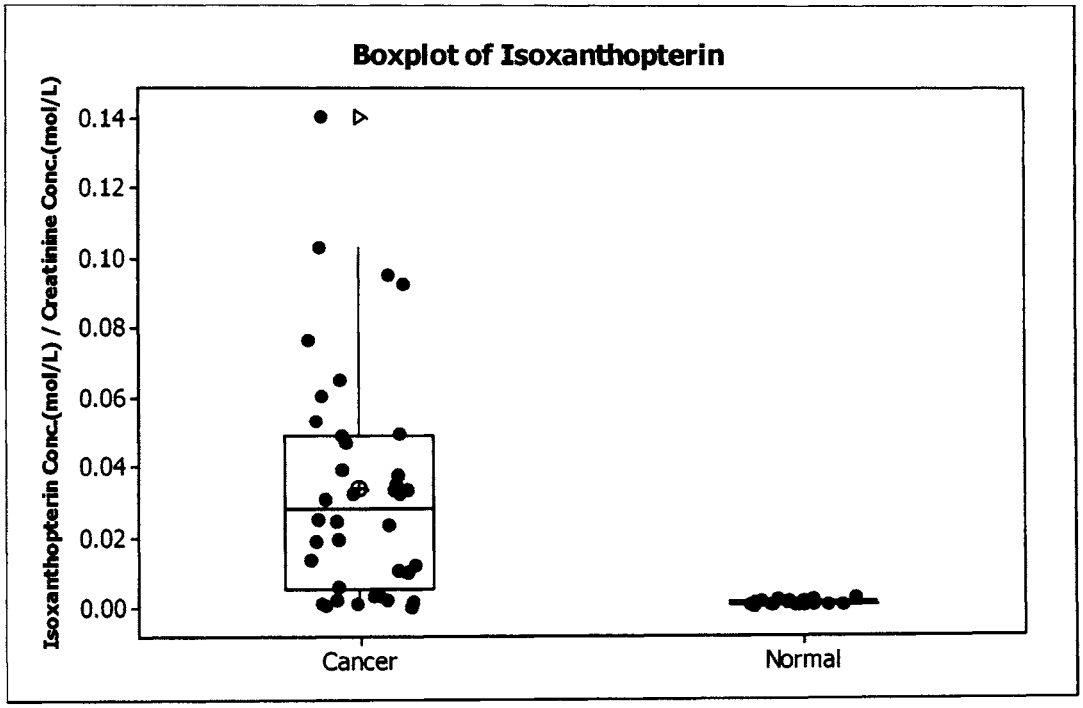

Figure 8 Box plot of isoxanthopterin levels in cancer urine samples (n=38) and normal urine samples (n=17).

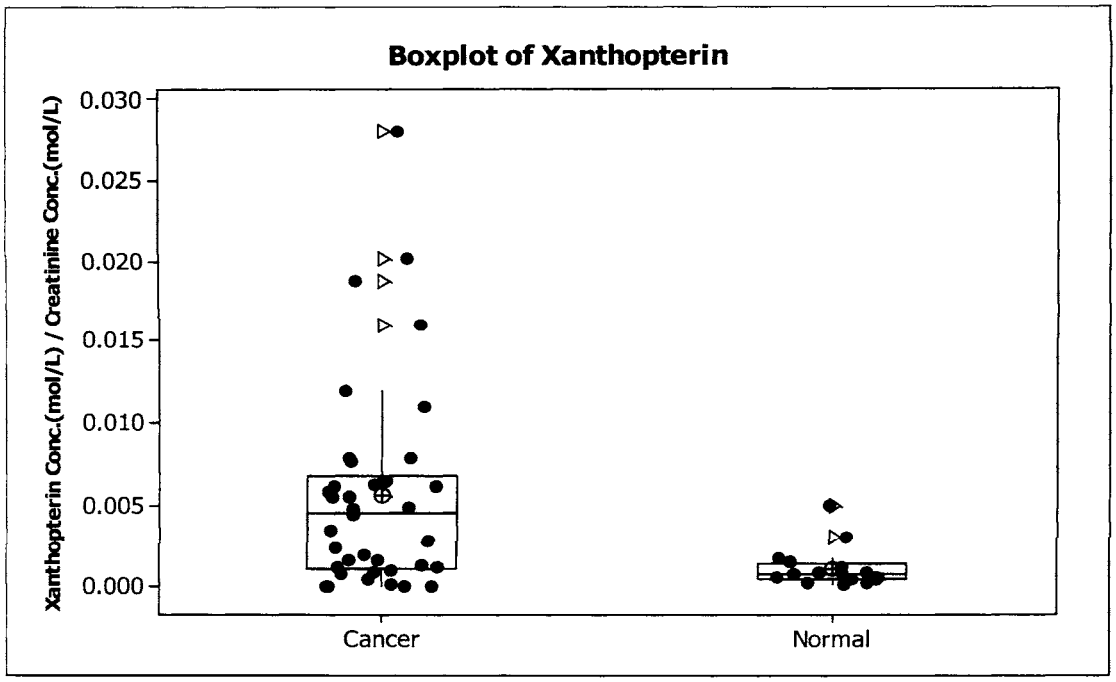

Figure 9 Box plot of xanthopterin levels in cancer urine samples (n=38) and normal urine samples (n=17).

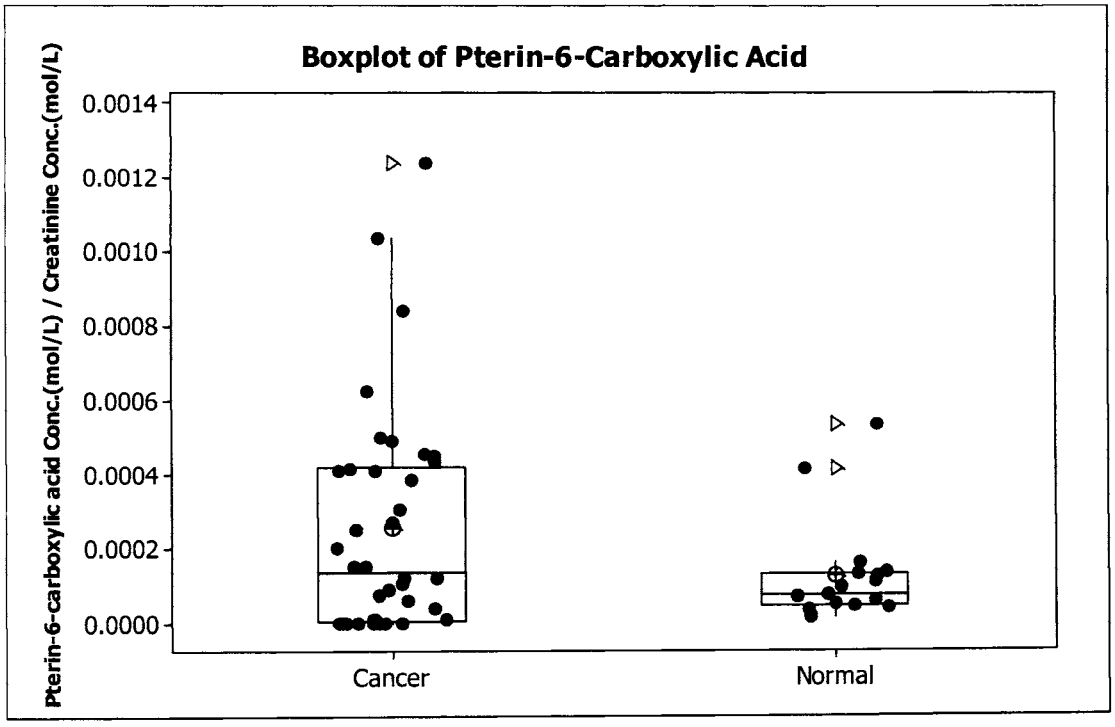

Figure 10 Box plot of pterin-6-carboxylic acid levels in cancer urine samples (n=38) and normal urine samples (n=17).

In both cancer and normal urine samples, levels of xanthopterin and isoxanthopterin levels were higher than those of other pteridines, as indicated by the y axes of the box plots. The box plot for xanthopterin (Figure 8) showed that its level in the cancer urine samples was much higher than in the normal urine samples. Its P value ($P = 8.7 \times 10^{-5}$) was significantly lower than 0.05. The P value of isoxanthopterin ($P = 2.8 \times 10^{-3}$), was also much lower than 0.05. Therefore, it is obvious that xanthopterin (Figure 8) and isoxanthopterin (Figure 9) levels are much higher in cancer urine samples than in the normal. It has been shown in Han et al. (1999) [7] that higher isoxanthopterin levels in cancer patients compared to the normal. But they have analyzed only nine cancer patients urine samples and this may be due to outliers present in the data. The P value of pterin-6-carboxylic acid was 0.049, nearly equal to 0.05. Therefore, thepterin-6-carboxylic acid level was not significantly higher than normal (Figure 10).

A comparison of the P values of each pteridine demonstrates that the P values of xanthopterin and isoxanthopterin were much lower than those of the other pteridines. Thus, their levels in breast cancer (n=12) and lung cancer (n=9) urine samples were checked (Figure 11 and Figure 12). Table 3 showed the P values calculated for various combinations. They were found to be higher than 0.05, therefore, xanthopterin and isoxanthopterin levels cannot be used to differentiate lung cancer from breast cancer. P-values for these two biomarkers in lung and breast cancer, however, were much lower than 0.05 and thus significantly different from normal.

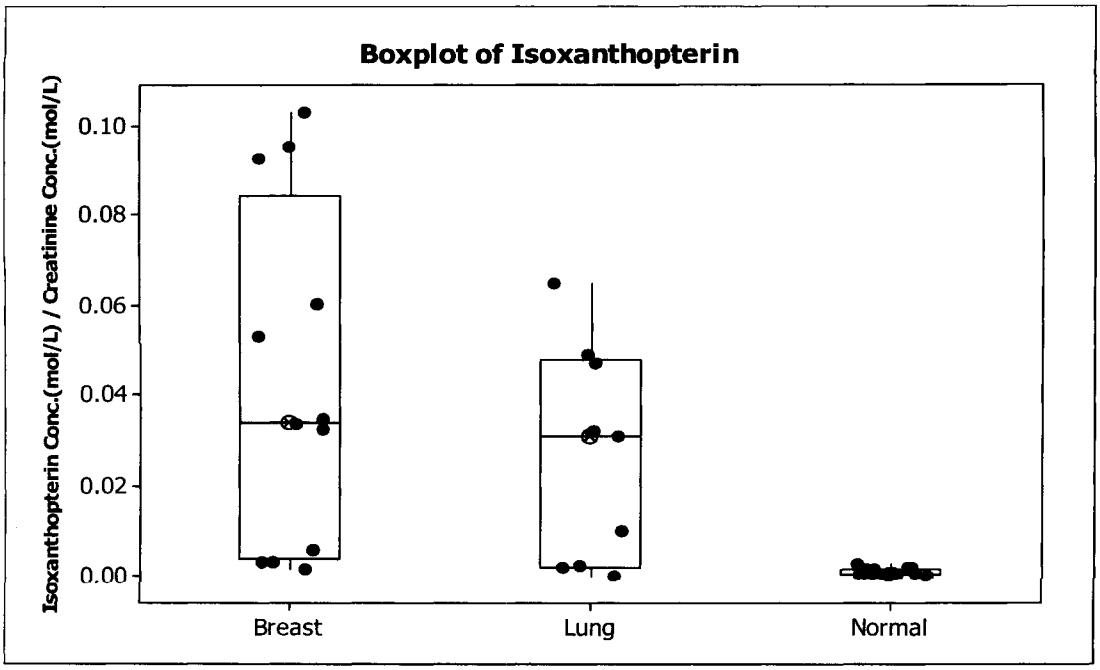

Figure 11 Box plot of isoxanthopterin levels in breast cancer urine samples (n=12), lung cancer urine samples (n=9), and normal urine samples (n=17).

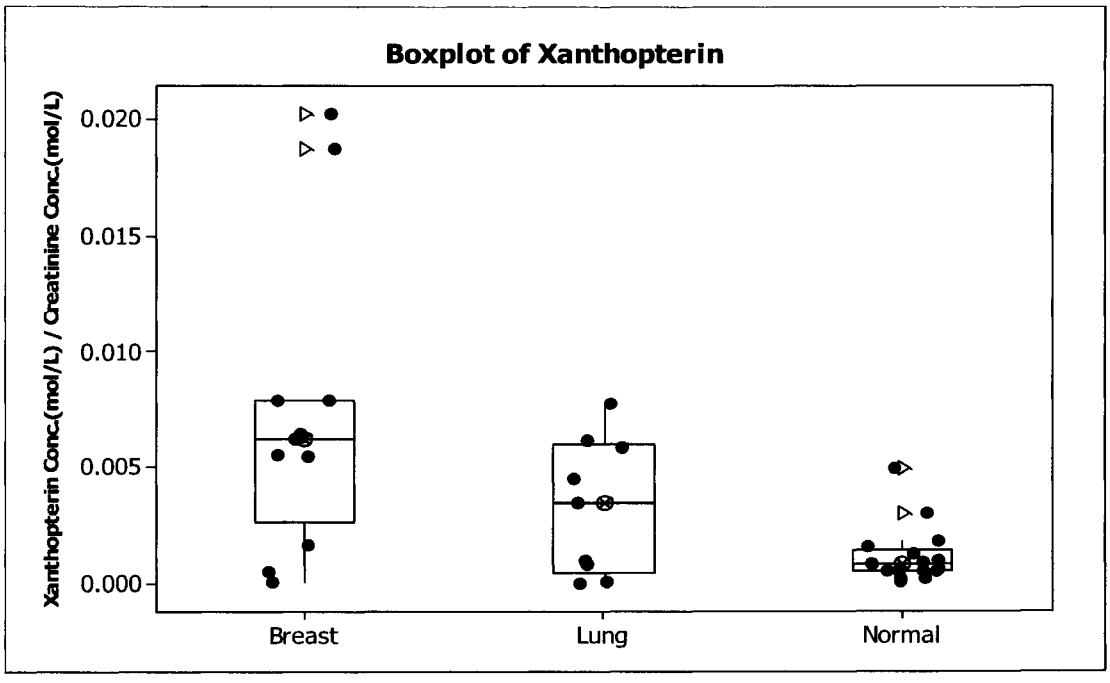

Figure 12 Box plot of xanthopterin levels in breast cancer urine samples (n=12), lung cancer urine samples (n=9), and normal urine samples (n=17).

Table 3. Xanthopterin and isoxanthopterin P values for various combinations of lung cancer, breast cancer, and normal urine samples.

Pteridine	**Combination**	**P Value**
Xanthopterin	Lung Cancer vs. Breast Cancer	0.131109
	Lung Cancer vs. Normal	8.51E-05
	Breast Cancer vs. Normal	4.18E-05
Isoxanthopterin	Lung Cancer vs. Breast Cancer	0.04898
	Lung Cancer vs. Normal	0.000287
	Breast Cancer vs. Normal	0.007288

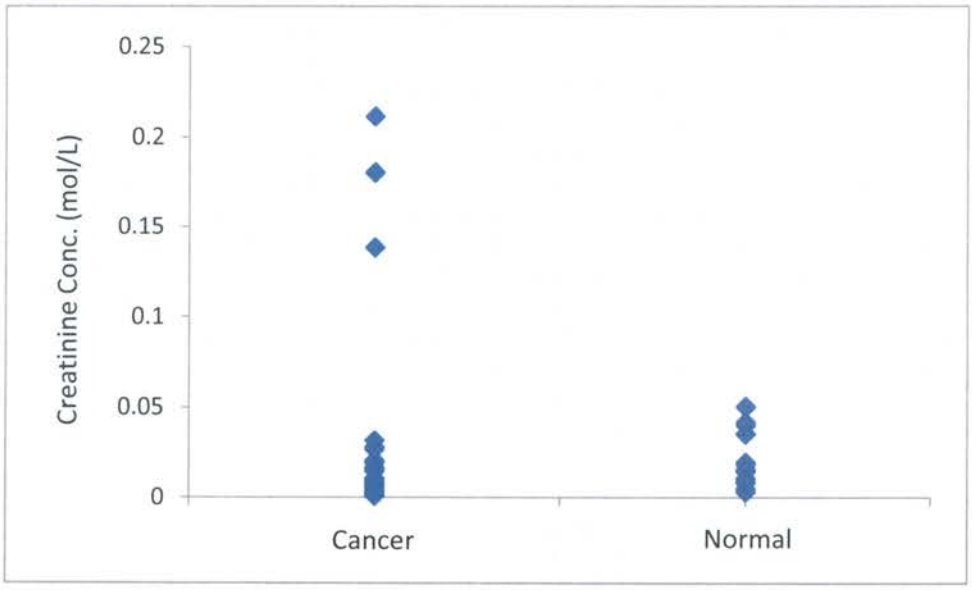

Figure 13 Scatter plot of creatinine levels in cancer urine samples (n=38) and normal urine samples (n=17).

4. CONCLUSION

This work analyzed eight pteridines in urine samples from thirty eight cancer patients and seventeen healthy subjects. Some pteridine levels in the urine samples of the cancer patients were higher than those of healthy subjects. More specifically, 6-biopterin, 6-hydroxymethylpterin, pterin, xanthopterin and isoxanthopterin levels in cancer samples were significantly higher than normal. Also, levels of xanthopterin and isoxanthopterin in both cancer patients and healthy subjects were higher than other pteridines. Then we conducted a comparison to determine whether we can use xanthopterin & isoxanthopterin to differentiate breast cancer from lung cancer. Tests determined that there was no significant difference in levels of xanthopterin and isoxanthopterin between breast cancer and lung cancer, therefore, levels of those two pteridines cannot be used to differentiate between these two cancers. More urine samples from patients with each type of cancer will be needed for further study of the potential of pteridines for differentiating among cancers. This research has demonstrated a great potential that pteridines can be used as biomarkers for early cancer screening even though further study is required for developing fingerprints for each individual cancer. Finally, more sophisticated instruments, such as HPCE-MS and LC-MS/MS, could be used to verify this.

ACKNOWLEDGEMENTS

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II. INVESTIGATION OF URINARY METABOLITES AS POTENTIAL BIOMARKERS FOR PROSTATE CANCER DETECTION

ABSTRACT

Background: Urinary metabolomic profiles recently drew a lot of attention because a debate regarding their possible role as potential clinical markers for prostate cancer. In this study, levels of proline, kynurenine, uracil, and glycerol-3-phosphate in 126 patients with genitourinary malignancies were analyzed using a validated method and compared with no evidence of malignancy.

Results and Discussion: The statistical results show that these biomarkers cannot differentiate prostate cancer from no evidence of malignancy or from other related cancer types, such as bladder cancer. Also for prostate cancer, there is no significant difference in biomarker levels for T1, T2 stages and Gleason scores <7, ≥ 7. From the correlation study, we can see that age or serum PSA levels do not influence these metabolite concentrations in urine. However, the strong correlation between these metabolites and urinary creatinine concentrations implies that their occurrence is mainly due to renal excretion.

Conclusions: This detailed study shows that above urinary metabolites are not reliable biomarkers for prostate cancer detection or for differentiating the aggressiveness of prostate cancer.

KEY TERMS

Biomarker: Any biological, chemical or biophysical indicator of an underlying biological process.

LC-MS/MS: An analytical instrument that combines the separation of analytes by liquid chromatography and detection by tandem mass spectrometry.

Prostate Cancer: A type of cancer that develops in the prostate gland in the male reproductive system.

Bladder Cancer: A type of cancer that originates in the bladder, an organ located in the pelvic cavity that stores and discharges urine.

PSA: Prostate specific antigen is a serine protease that purpose is semen coagulum liquefication.

1. INTRODUCTION

Adenocarcinoma of the prostate, transitional cell carcinoma of the urinary tract categorized as a genitourinary malignancies. The majority of genitourinary malignancies are from adenocarcinoma of the prostate and transitional cell carcinoma of the bladder, ureters and renal collecting system. Adenocarcinoma of the prostate is a hormonally mediated tumor that has a hereditary component as well as a sporadic genetic component to create malignancy. Dietary, environmental and genetic factors have all been implicated as potential causes. Prostate cancer (PCa) ranks as the third most common cancer in men in the world [1, 2] and it is the second leading cause of cancer death in the United States

and Canada. One out of every 10 men will develop this disease at some time in his life [101] and the disease incidence is increasing steadily over the years. Bladder cancer (BCa) is the fourth most common cancer among men and the ninth most common among women in the United States. Each year, more than 50,000 new cases of bladder cancer are diagnosed [101]. Unfortunately, PCa diagnosis is not a precise science yet. The screening of PCa is based on the triad of digital rectal examination (DRE), blood prostate specific antigen (PSA) level measurement, and trans-ultrasound guided prostate biopsy [3, 4] . PSA is a serine protease that purpose is semen coagulum liquefication. It is one of the few biomarkers that are widely used in the diagnosis and management of PCa. It is a normal constituent of prostatic fluid and therefore is neither specific nor sensitive for prostate cancer. Most doctors consider a PSA level below 4.0 ng/mL as normal, but in one large study prostate cancer was diagnosed in 15.2% of men with a PSA level at or below 4.0 ng/mL [102]. The use of PSA testing has led to an increased detection of clinically irrelevant tumors (over-diagnosis), followed by unnecessary treatment and monitoring of patients over long periods of time [5]. Also, PSA is unable to diagnose the aggressiveness of tumors, or to identify which tumors will become unresponsive to anti-androgen therapy at an early stage. In the United States, each year nearly 30,000 men are dying of prostate cancer and millions of others, who have the disease, are not even aware of it [6, 7]. Widespread uses of broad spectrum antibodies have increased the morbidity of trans-rectal ultrasound biopsy and in some studies the risk of the mortality from biopsy supersedes that of radical prostatectomy. Also it is reported that around 29,000 cases of malignancy will occur each year from radiologic imaging radiation exposure with 14,000 deaths [8]. So the need for accurate, noninvasive testing for genitourinary malignancies is

present. Therefore, there is an urgent need for clinically validated biomarkers that will improve the diagnosis, accurately predict staging and grade as well as follow for malignant recurrence.

Sreekumar et al.(2009) quantified 1,126 metabolites and found that a subset of five metabolites including sarcosine, proline (Pro), kynurenine (Kyn), uracil (Ura) and glycerol-3-phosphate (G3P) were significantly increased with disease progression, from benign to PCa, to metastatic prostate cancer [9]. Sarcosine was thoroughly studied compared to the other metabolites, but it was not included in this study due to the difficulties of analysis [10] such as interference with alanine and its contradictions [11, 12] as a biomarker. This motivated us to quantify other metabolites given less attention in the literature compared to sarcosine. A validated LC/MS/MS method [13] was used for the analysis of above metabolites and creatinine in urine samples. Because the changes in metabolic activity and cancer progression are highly interrelated events, we can hypothesize that these metabolites are elevated with cancer progression. Moreover, changes in the levels of metabolites reflect the inherent changes in the biochemistry of a tumor as it develops and progresses to a more advanced state [9]. Generally, patients are reluctant to be subjected to interventional procedures, in the process of disease diagnosis. Thus, noninvasive diagnostic techniques for early cancer screening are essential for people living all over the world. Presently, the attention of researchers is drawn towards such noninvasive cancer detection methods as the analysis of biomarkers in urine. This technique is comparatively easier to follow than other methods due to the fact that urine is more easily collected than tissue or blood samples [14].

Liquid chromatography, coupled with tandem mass spectrometry (HPLC/MS/MS), is a powerful and accurate technique because a combination of the specific parent mass and the unique fragment ion is used to selectively monitor the compound to be quantified [13, 15]. No studies have been reported up to date to investigate the levels of other biomarkers (in Sreekumar et al.'s study) such as proline, kynurinine, etc., in other types of cancer (e.g., genitourinary malignancies) and in normal urine samples. Therefore, we investigated these biomarker levels in urine samples of PCa, BCa and no evidence of malignancy (NEM). The primary goal of this study was to evaluate the potential of these urinary metabolites as biomarkers for early PCa detection, in relation to PSA, and see whether these biomarkers can differentiate PCa from other cancer types such as BCa. The aggressiveness of PCa was studied, in comparison to the classic prognostic parameters of Gleason score and tumor stage, and correlation with age and PSA level. Since the method development, quantification limit, HPLC optimization, MS optimization, and linear range were reported in our previous study [13], they were not described here.

2. MATERIALS AND METHODS

2.1 Chemicals

Five standards, including proline, kynurenine, uracil, glycerol-3-phosphate, and creatinine, were purchased from Sigma–Aldrich (St Louis, MO, USA). L-Glutamine (Glu) isotope ($C_5H_{10}{}^{15}N_2O_3$) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA); and it was used as the internal standard (IS) for LC-MS/MS analysis. Acetonitrile (LC-MS grade) and Formic acid (99%), which was used to prepare

the mobile phase, were purchased from Sigma-Aldrich (St Louis, MO, USA). Ultrapure water was obtained using a Milli-Q Advantage® A10 and Millipore Elix® water purification system (Millipore, MA, USA).

2.2 Patients and Urine Samples

Prostate cancer (n=63) and bladder cancer (n=63) urine samples were obtained from the Central Missouri Urology Clinic (Rolla, MO, USA). The no evidence of malignancy (NEM) urine samples (n=68) were collected from healthy volunteers at Missouri University of Science and Technology (Rolla, MO, USA) and Central Missouri Urology Clinic (Rolla, MO, USA). All samples were collected between December 2009 and September 2010 using the same sample collection protocol. The demographic distribution of cancer patients and normal subjects was mainly from nearby cities. The age distribution of cancer patients was from 53-94 y and the range for normal subjects was 18-87 y. There was no dietary or exercise restriction performed among the control group or cancer patients. All pathologic analyses were made at the Central Missouri Urology Clinic under the supervision of an experienced urologist. The prostate cancer urine samples were categorized as Gleason score < 7 (n=17) and Gleason score ≥ 7 (n=32). The main pathologic tumor stages of prostate cancer were pT1 (n=16) and pT2 (n=11). This project and study protocol was approved by the Institutional Review Board, Phelps County Regional Medical Center (Rolla, MO, USA). Serum PSA levels of the prostate cancer patients were measured at Oppenheimer Urologic Reference Laboratory (Nashville, TN, USA) using a Beckman Coulter UniCel Dxl 800 Access Immunoassay System. After collection, the samples were immediately frozen and stored at -80 °C. The samples were thawed at room temperature and then diluted five times using ultra-pure

water before analysis. A 10 μL of diluted urine was mixed with 10 μL of the internal standard solution (150 ppb) and 1480 μL of 0.1% formic acid in water. Then the sample was analyzed by LC/MS/MS.

2.3 High-Performance Liquid Chromatography (HPLC)

A phenyl-hexyl, 3.0 μm, 3.0×150 mm column (Phenomenex, USA) and Agilent 1100 series LC system (Santa Clara, CA) were used. Liquid chromatography was performed at 25°C under a flow rate of 250 μL/min using a gradient system with the mobile phase consisting of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile (100%). The gradient program was: initial 98% A and 2% B; linear gradient to 60% A and 40% B in 5 min; return to initial conditions in 0.1 min at a flow rate of 250 μl/min, followed by equilibration for 10 min. Run-to-run time was 15 min. The sample injection volume was 10 μL.

2.4 Tandem Mass Spectrometry

An API 4000Q trap MS/MS system (Applied Biosystems, Foster City, CA) was used for identification and quantification of the above metabolites in urine samples. The mass spectrometer was operated in multiple-reaction monitoring mode (MRM) with ESI-positive ionization. Turbo spray was used as the ion source. Nitrogen gas was used as curtain gas and cone gas. The cone gas flow was 50 L/h and the desolvatation gas flow was 800 L/h. Optimal detection conditions were determined by direct infusion of each standard solution (20 ppb) in solvent A, using a syringe pump. Source-dependent parameters: ion source gas 1, ion source gas 2, source temperature, curtain gas, and ion spray voltage were set at 30 psi, 40 psi, 500 °C, 25 psi, and 4500 V, respectively.

2.5 Statistical Analysis

Biomarker concentrations were determined by linear regression against experimentally generated matrix-based calibration curves. The basic statistical information of each metabolite, for both cancer and normal urine samples, was calculated by Minitab software (Minitab Inc., PA, USA). Statistical hypothesis testing was conducted to analyze the data. The null hypothesis was that the mean metabolite levels in cancer samples are lower than, or equal to, those in NEM urine. The alternative hypothesis was that the mean metabolite levels in cancer samples are higher than those in NEM samples. Since the sample size is large, the assumption about normality is satisfied by all of the hypothesis tests. However, the normality assumption was checked by plotting normal probability plots for each of the biomarkers; there were not any harmful violations of the data on the normality assumption. The *P* values (Figure 1) were calculated for all samples. The null hypothesis was rejected, and the results demonstrated that the alternative is significant at a 5% significance level if the *P* value ≤ 0.05. For prostate cancer, statistical hypothesis testing was conducted using Minitab to study the variation between tumor stage (pT1 and pT2) and tumor grade (Gleason score <7 and ≥ 7). A correlation study was conducted to identify statistical relationships between metabolites and other parameters, such as age, urinary creatinine level, and serum PSA level. The *P* values and correlation coefficients (R) were calculated using Matlab software (MathWorks Inc., MA, USA). Multivariate analysis of variance (MANOVA) was conducted using Matlab software to identify the differences in the mean values of biomarkers between PCa, BCa, and NEM. Principal component analysis (PCA) was conducted using Matlab software to classify cancer types based on biomarker levels. Finally, the classification and regression tree (CART) was used in our further analysis to

determine whether a complex relationship exists between cancer types and biomarkers. CART was used to classify (or predict) categorical response variables; it classified the response variables by determining the probabilities based on independent variables.

3. RESULTS AND DISCUSSION

The levels of urinary metabolites were calculated by using urine matrix-based calibration curves (Table 1). Glutamine (Glu) was used as the internal standard and all peak areas were divided by corresponding Glu peak areas. In order to assure that these metabolite levels represent the physiological concentration, the amount is reported here as a ratio of metabolite (nM) to creatinine (μM) [14]. Creatinine has been used in many clinical studies as an internal standard since its concentration strictly corresponds to urine dilution. In our study, we did not use any derivatization for any of the compounds. Since these metabolites are small molecules, we can assume that they rapidly release into the urine supernatants [11].

Table 1. LC/MS/MS information for five metabolites, creatinine, and glutamine (IS)

	Glycerol-3-phosphate	Proline	Kynurenine	Uracil	Creatinine	Glutamine (IS)
Q1	173.0	116.0	209.1	112.9	114.0	148.0
Q2	99.0	70.1	192.1	70	44.1	130.0
LOD (nM)	2	2	0.05	0.4	3	0.4
R^2	0.995	0.999	0.999	0.999	0.995	N/A
Retention Time (min)	5.3	3.2	9.6	5.1	2.3	2.1

Table 1 shows the parent (Q1) ions, daughter ion (Q2), limit of detection (LOD), linear equation, R^2 values, and retention times for each compound. Box plots were constructed for each metabolite in each cancer type (PCa and BCa) and compared them with NEM (Figure 1). The P values were calculated for each comparison to identify whether there were any significant differences between them. Sreekumar et al. reported that these metabolites, found in higher levels in PCa compared to NEM [9]. We therefore took our alternative hypothesis as mean biomarker levels in cancer samples were higher than those in NEM. Figure 1A shows the four metabolites in PCa compared to NEM. From that we can see that all metabolites, including G3P (p=0.874), Pro (p=0.906), Kyn (p=0.744), Ura (p=0.774), did not show any significant difference from NEM (p>0.05). Figure 1B compares BCa with NEM and G3P (p= 0.432), Pro (p= 0.497), Kyn (p= 0.286), Ura (p= 0.536), had no significant difference among PCa and NEM (p>0.05). For further analysis of prostate cancer, patients were grouped according to the tumor stage (pT1 and pT2) and tumor grade (Gleason score <7 and ≥7). Here, the alternative hypothesis is that there is no difference in mean biomarker levels among tumor stages and tumor grades.

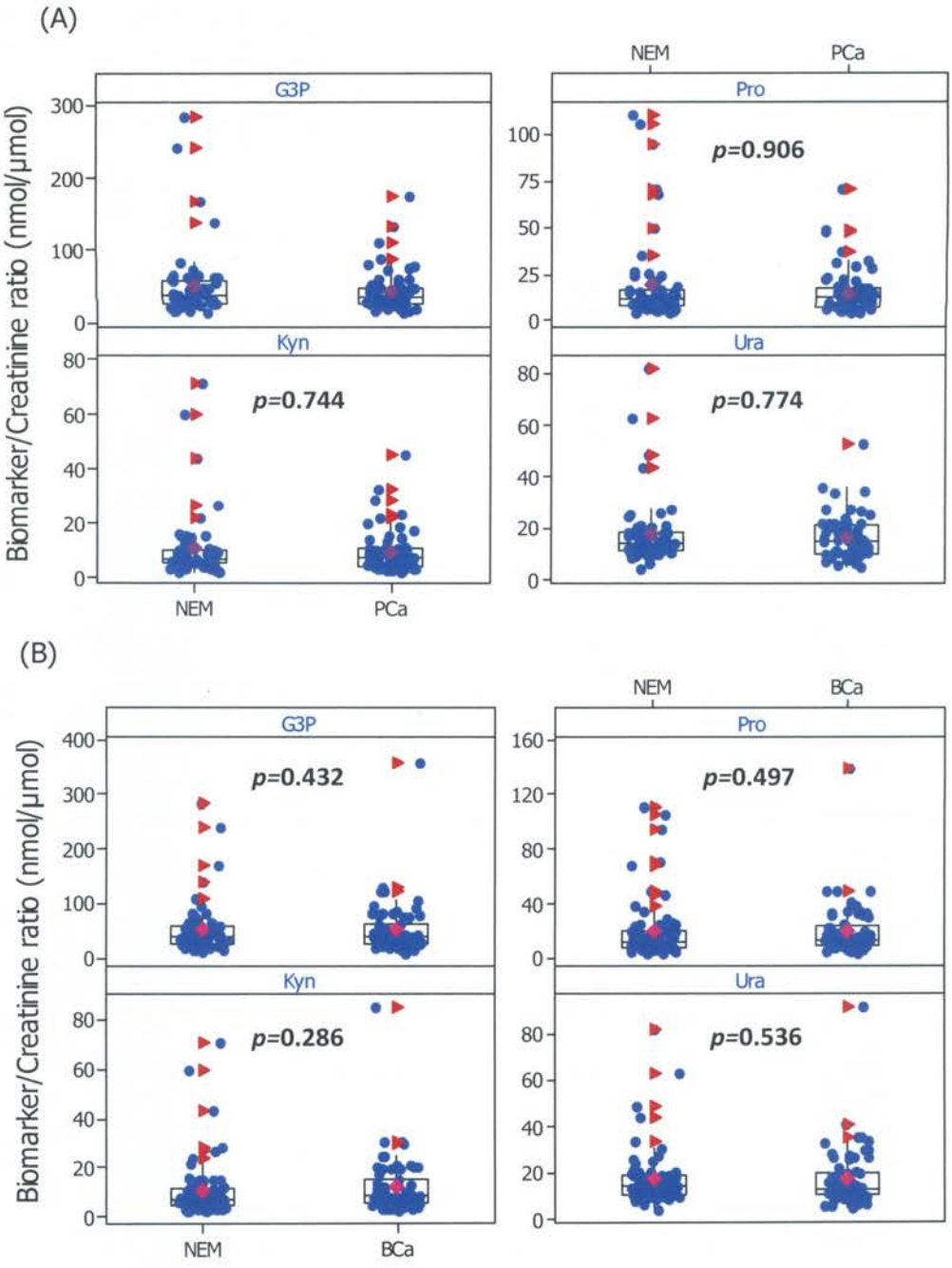

Figure 1. (A) Biomarkers to creatinine in urine ratio for prostate cancer (PCa)(n=63) and no evidence of malignancy (NEM) (n=57). (B) Biomarkers to creatinine in urine ratio for bladder cancer (BCa)(n=63) and no evidence of malignancy (NEM) (n=68).

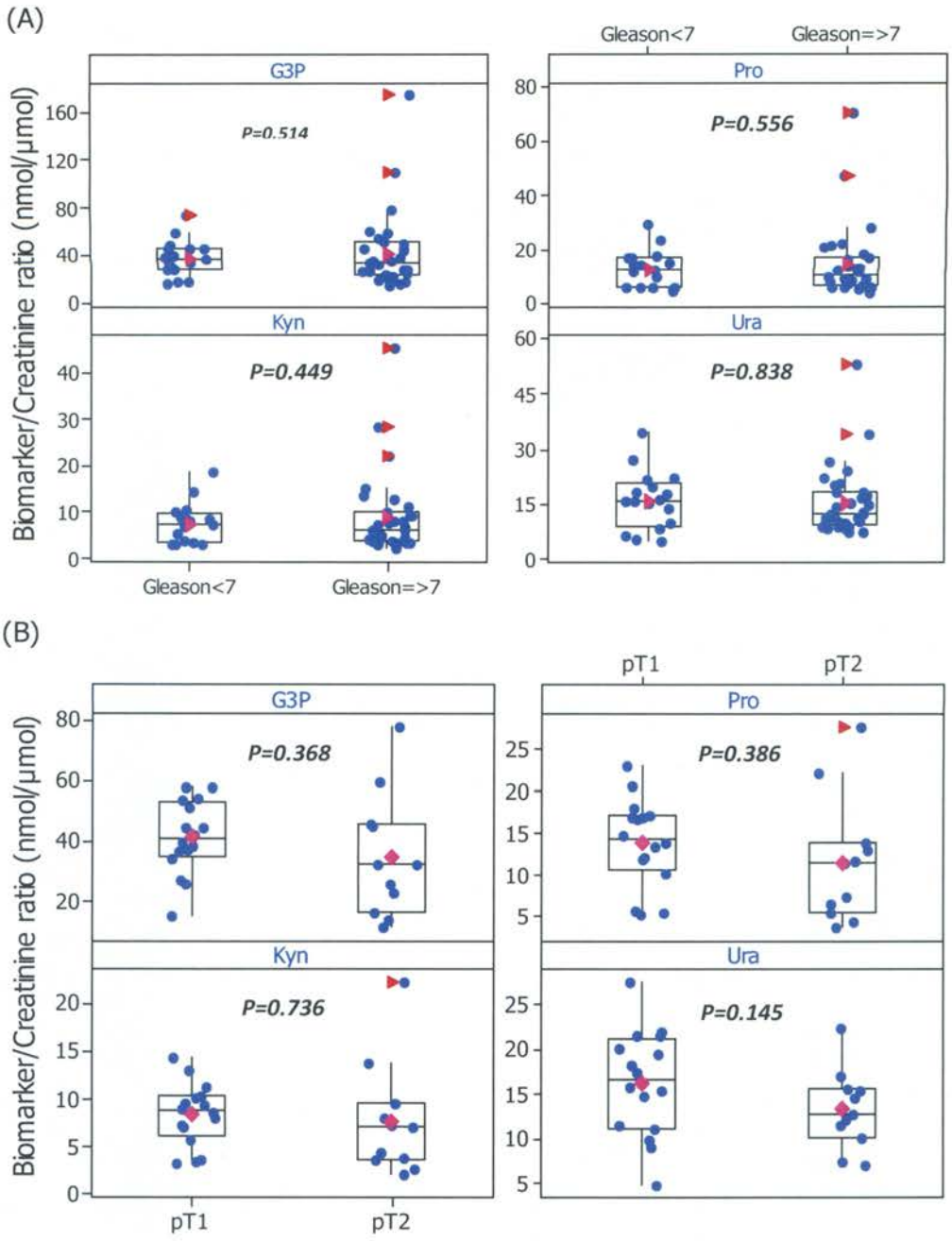

Figure 2. (A) Biomarkers to creatinine ratio in prostate cancer urine for Gleason score <7 (n=17) and Gleason score ≥7(n=32) (B) Biomarkers to creatinine ratio in prostate cancer urine for tumor stage pT1 (n=16) and tumor stage pT2 (n=11).

Neither tumor grades (Figure 2A) nor tumor stages (Figure 2B) were associated with the biomarker levels. These statistical hypotheses tests show that these metabolites were unable to distinguish cancer from NEM. Also these urinary metabolites are not able to differentiate between prostate cancer patients with stages pT1 and pT2, or between patients with a Gleason score of <7 and ≥ 7 (Figure 2).

Next, a correlation study was performed to see whether these biomarker levels correlated with the urinary creatinine level, age, and serum PSA level (only for PCa). The correlation coefficient and P values were calculated for each biomarker in the cancer and normal samples (Table 2). The table 2 shows that all biomarkers in the cancer and normal samples are highly correlated (except proline in the normal samples, $P= 0.58483$) with urinary creatinine levels because their p values are much less than 0.05. This can be interpreted as associated enzymatic regulation of both components [16]. This strong correlation implies that their occurrence is mainly due to renal excretion and we, therefore, cannot use them as reliable biomarkers for cancer detection. According to Table 2, biomarker levels in both cancer and normal samples do not correlate with the age ($P>0.05$). It also shows that these metabolites do not correlate with the serum PSA levels in prostate cancer ($P>0.05$). The MANOVA test was then applied to all three groups (i.e., PCa, BCa and NEM) because it is used in cases where there are two or more sub populations. We used this to determine whether cancer types have significant effects on biomarker levels.

Table 2. Correlation data for each metabolite with urinary creatinine, age, and serum PSA levels, and P values for MANOVA

Correlation with	Urine Type	Rs & P values	Glycerol-3-phosphate	Proline	Kynurenine	Uracil
Creatinine (0.25-26.71 µmol/L)	Cancer (n=126)	R value	0.68839	0.4741	0.62178	0.72749
		P value	2.8192E-20	6.3116E-9	8.4178E-16	1.6438E-23
		Correlation	Yes	Yes	Yes	Yes
	Normal (n=68)	R value	0.63009	0.066424	0.48021	0.76552
		P value	5.0841E-9	0.58483	2.5888E-5	1.1834E-14
		Correlation	Yes	*No*	Yes	Yes
Age (18-94y)	Cancer (n=126)	R value	-0.003506	-0.0014898	-0.0062564	-0.058903
		P value	0.96981	0.98717	0.94616	0.52456
		Correlation	*No*	*No*	*No*	*No*
	Normal (n=68)	R value	0.081719	0.14162	0.086461	0.030567
		P value	0.50443	0.24576	0.47994	0.80311
		Correlation	*No*	*No*	*No*	*No*
Serum PSA (0.008 – 7.53 ng/ml)	Prostate Cancer (n=48)	R value	-0.014236	-0.024407	-0.015674	-0.050546
		P value	0.92349	0.86921	0.91579	0.73297
		Correlation	*No*	*No*	*No*	*No*
MANOVA test for PCa,BCa and NEM		P value	0.4257	0.2848	0.4389	0.8186
		Sig. Dif.	*No*	*No*	*No*	*No*

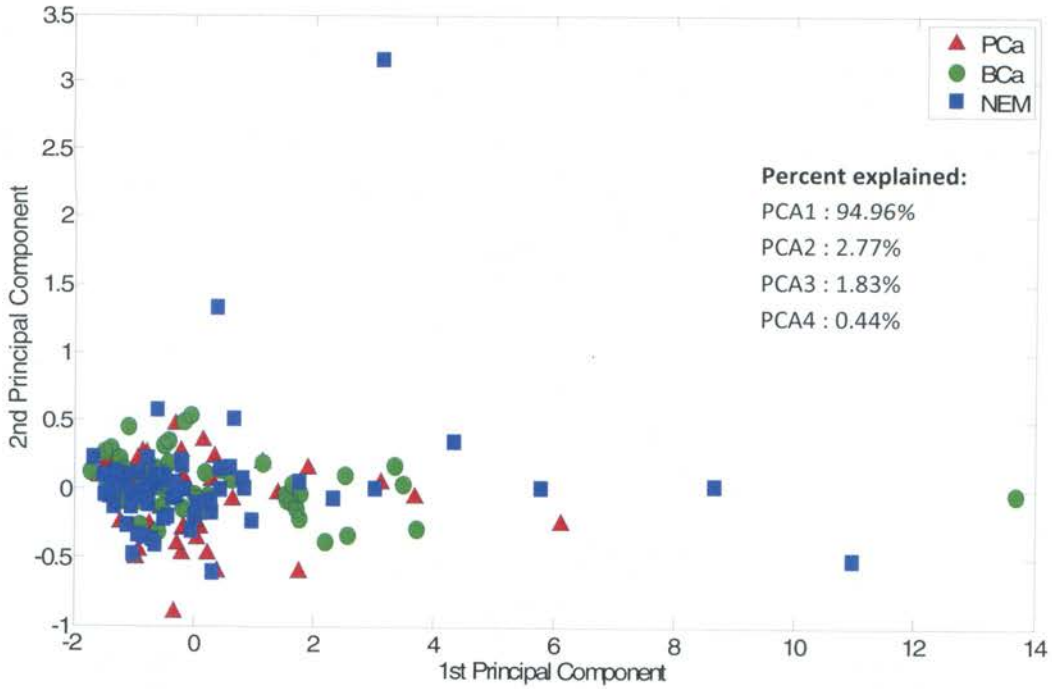

Figure 3. Principal component analysis plot between 1st principal component (PCA1) and 2nd principal component (PCA2) for PCa (n=63), BCa (n=63) and NEM (n=68).

From the *P* values obtained by the MANOVA test (Table 2), we can say that, for all metabolites, there is no significant difference between PCa, BCa and NEM ($P>0.05$). To further validate these observations a principal component analysis (Figure 3) was conducted, but we could not identify any clusters for different cancer types. Also, the 1st principal component was 94.96% and the 2nd principal component was 2.77 %. Since the first two principal components cover about 97.73%, we can say that these metabolites are highly correlated with each other. Due to this correlation, we cannot identify any clusters for cancer types in the principal component analysis graph (Figure 3), based on biomarker levels. Figure 4 shows a classification and regression tree (CART) for

classifying urine samples into PCa, BCa, and NEM, based on Pro, Kyn, Ura, and G3P levels in urine. The CART (Figure 4) shows how complex the relationship is between cancer types and biomarkers, and shows that this kind of model may be over-fitted due to the large number of branches. On the other hand, this classification model confirms the results from our previous statistical analyses, that we are unable to find a clearer and simpler relationship between cancer type and biomarkers. These urinary metabolomic profiles can fluctuate depending on the physiological and pathological states of the patients and extra caution is needed when they are pursued as biomarkers for cancer diagnosis.

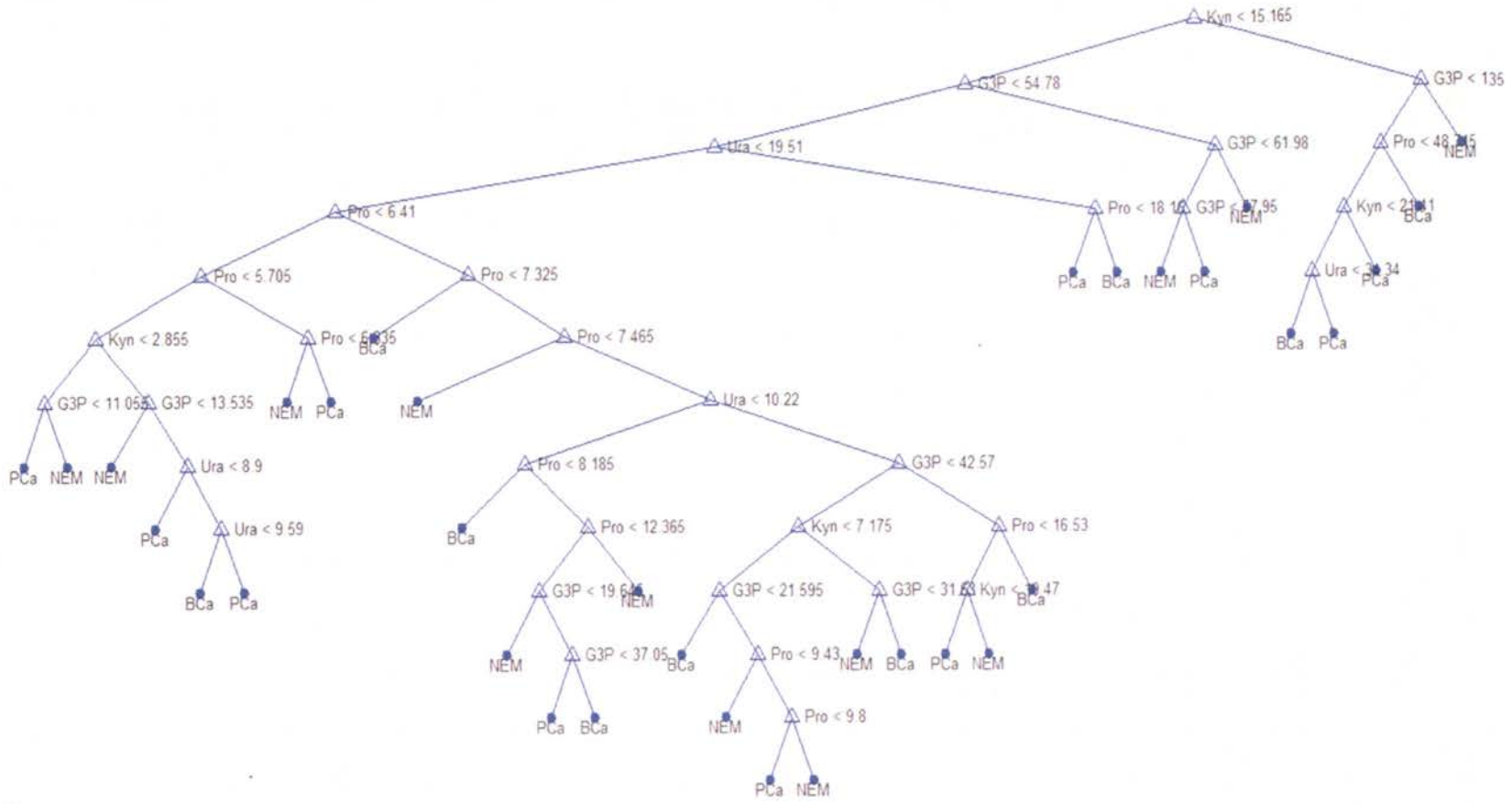

Figure 4. Classification and regression tree (CART) for classifying urine samples into PCa, BCa and NEM based on Pro, Kyn, Ura, G3P levels in urine.

4. CONCLUSIONS

The main conclusion of this study is Pro, Kyn, Ura and G3P are not reliable enough for PCa detection or for differentiating the aggressiveness of prostate cancer. In addition, they cannot differentiate prostate cancer from other related cancer types, such as BCa. Finally, these metabolites, as potential noninvasive urinary biomarkers for PCa, are questionable based the study results. These biomarkers need more independent validation studies before they contribute to clinical decision making. Accordingly, we believe that it is too early to use above metabolites with PSA as a viable method for diagnosing prostate cancer. Currently, PSA and DRE, though imperfect, provide the best methods for diagnosing PCa.

5. EXECUTIVE SUMMARY

- Urinary metabolomic profiles recently drew a lot of attention because a debate regarding their possible role as potential clinical markers for cancer diagnosis.
- Recently a subset of five metabolites including sarcosine, proline, kynurenine, uracil and glycerol-3-phosphate were found to be significantly increased in prostate cancer compared to the normal.
- In this study, levels of proline, kynurenine, uracil, and glycerol-3-phosphate in 126 patients with genitourinary malignancies were analyzed using a validated LC/MS/MS method and compared with no evidence of malignancy.
- Sarcosine was thoroughly studied compared to the other metabolites, but it was not included in this study due to the difficulties of analysis and its contradictions as a biomarker.

- The statistical results showed that these biomarkers cannot differentiate prostate cancer from no evidence of malignancy or from other related cancer types, such as bladder cancer.

- For prostate cancer, there is no significant difference in biomarker levels for T1, T2 stages and Gleason scores <7, ≥ 7.

- The correlation study, showed that age or serum PSA levels do not influence these metabolite concentrations in urine.

- The strong correlation between these metabolites and urinary creatinine concentrations implies that their occurrence is mainly due to renal excretion.

- We believe that it is too early to use above metabolites with PSA as a viable method for diagnosing prostate cancer more independent studies necessary to validate these metabolites as prostate cancer biomarkers.

FUTURE PERSPECTIVE

This study shows that these metabolites are not reliable enough for PCa detection or for differentiating the aggressiveness of prostate cancer. Although their role as potential noninvasive urinary biomarkers for PCa, are questionable based this study, more independent validation studies in large populations are necessary before we come to the final conclusions about their clinical decision making power. Normally contradictions happens in urinary metabolite biomarker studies (eg. sarcosine) becuase these metabolites are not specific to cancer. But their elevations give valuble informations about the status

of a disease. Overall this study provides a good insight of how urinary metabolomic analysis can utilize to investigate potential biomarkers.

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III. QUANTITATIVE DETERMINATION OF TAURINE AND RELATED BIOMARKERS IN URINE BY LIQUID CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY

ABSTRACT

Current urinary bladder cancer diagnosis is commonly based on a biopsy obtained during cystoscopy. This invasive method causes discomfort and pain in patients. Recently, taurine and several other compounds such as L-phenylalanine and hippuric acid in urine were found to be indicators of bladder cancer. However, because of a lack of sensitive and accurate analytical techniques, it is impossible to detect these compounds in urine at low levels. In this study, using liquid chromatography – tandem mass spectrometry (LC-MS/MS), a non-invasive method was developed to separate and detect these compounds in urine. $^{15}N_2$-L-glutamine was used as the internal standard, and creatinine acted as an indicator for urine dilution. A phenyl-hexyl column was used for the separation at an isocratic condition of 0.2% formic acid in water and 0.2% formic acid in methanol. Analytes were detected in multiple reaction monitoring (MRM) with positive ionization mode. The limit of detection range is 0.18-6 nM and the limit of quantitation ranges from 0.6 nM to 17.6 nM. The parameters affecting separation and quantification were also investigated and optimized. Proper clinical validation of these biomarkers can be done using this reliable, fast, and simple method. Furthermore, with simple modifications, this method could be applied to other physiological fluids and other types of diseases.

KEYWORDS

Taurine, Biomarkers, LC/MS/MS, Bladder Cancer, Urine

1. INTRODUCTION

Taurine plays a very important role in several essential biological processes. This sulfur- containing amino acid is not incorporated into protein, and it is the most abundant free amino acid in the heart, retina, skeletal muscle, leukocytes, and brain [1, 2]. Taurine, as well as inorganic sulfate, is a major end product of sulfur-containing amino acid metabolism in mammals [3]. The proteins in food give us sulfur-containing amino acids such as methionine and cysteine. Methionine is converted to cysteine via trans-sulfuration, and cysteine is then further metabolized through oxidation [4]. Cysteine is used in protein synthesis, and incorporated into glutathione. Other than that, it can also convert into taurine [4]. Two mechanisms can explain taurine accumulation in cells. It can be synthesized within cells, from cysteine, through a cysteine sulfonic acid decarboxylase or cysteine dioxygenase. Also, taurine can be taken up through a sodium-dependent transport, which is mediated by a specific taurine transporter [4]. Taurine accumulates in maternal tissue during pregnancy, and is released to the fetus via the placenta. Further, the newborn gets taurine via maternal milk [5]. Taurine levels in biological fluids help both in diagnosing diseases as well as in monitoring therapies [4]. Hence, taurine can act as a biomarker, and several studies have been conducted to investigate and determine if taurine is a biomarker for cancer. An *in vitro* nuclear magnetic resonance (NMR) study [6] reported that higher concentrations of taurine in breast cancer tissue samples were detected, as compared to the normal amount.

Furthermore, this study has postulated taurine as a potential indicator of tumor aggressiveness because the highest taurine levels were found in tumor grade 2 and 3 tissue samples [6]. Finally, another study reported that taurine levels in brain tumor tissues were significantly elevated, as compared to normal tissues [7].

A recent ^{1}H NMR study by Srivastava et al. [8] reported taurine, phenylalanine, and hippuric acid as possible fingerprint biomarkers for urinary bladder cancer. Using ^{1}H NMR spectra with reference to sodium-3-trimethylsilylpropionate [8], they have identified and quantified these biomarkers in urine samples of healthy controls, urinary tract infection patients, and bladder cancer patients. This motivated us to develop a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to simultaneously quantify taurine (Tau), L-phenylalanine (Phe), hippuric acid (Hip), and creatinine (Cre) in urine samples. This is a fast and more sensitive method compared to the ^{1}H NMR analysis.

Bladder cancer is the fourth and the ninth most common type of cancer among men and women in the United States, respectively. Each year, more than 50,000 men and 16,000 women are diagnosed with this disease[9]. The bladder, a hollow, muscular organ that stores urine, is located in the pelvis. About 90% of bladder cancers begin in the cell lining on the inside of the bladder, and are called transitional cell carcinoma [9]. Tobacco smoking and other carcinogens are the main causes of urinary bladder cancer. A biopsy obtained during cystoscopy is the most widely-used method for diagnosing bladder cancer. This invasive method causes discomfort and pain in patients and, also, its low sensitivity results in false positive diagnoses [8]. Thus, a reliable, non-invasive screening method is an urgent requirement in modern clinical settings. Although here we have

mainly focused on bladder cancer, this method could be applied to other cancer types and even to other types of diseases.

Creatinine, which Srivastava et al. did not include in their study [8], was included here as an analyte. Since a creatinine concentration corresponds closely to urine dilution, its levels must be monitored in any urinary biomarker analysis [10], with a biomarker-to-creatinine ratio representing the biomarker levels in urine. Phenylalanine is an essential, α-amino acid which is classified as non-polar because of the hydrophobicity of the benzyl side chain [9]. Phenylalanine is a precursor for many bio-molecules such as tyrosine, the monoamine signaling molecules dopamine, norepinephrine, and epinephrine, and the skin pigment melanin [9]. It is identified as a marker for oxidative damage in humans [11]. Another study demonstrated that phenylalanine levels are significantly correlated with Type 2 diabetes risk ($P < 0.0001$) [12]. Hippuric acid is a carboxylic acid found in urine, especially in herbivores [9]. Its excretion is associated with environmental-toxic exposures of aromatic compounds such as toluene, or dietary protein degradation and re-synthesis. Approximately 1-2 mM of hippuric acid is excreted daily in the urine, even in the absence of organic solvent exposure. This suggests that metabolic dietary sources of hippuric acid are also available [13]. In Srivastava's study [8], lower median levels of phenylalanine and hippuric acid were observed in urinary bladder cancer patients, compared to those in normal control urine samples, and taurine median levels were higher.

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) has been proven to be a useful tool for molecular structure identification and quantitative analysis in biological matrices due to its high selectivity and sensitivity. The technique has been

widely used in drug discovery [14], proteomics [15], pharmacokinetic [16] studies, and emerging containments in environmental sciences[17]. However, a LC-MS/MS method for quantitative determination of Tau and above related biomarkers is currently not available. A LC-MS/MS method for Tau analysis has been developed by Chaimbault et al. (2004) [18], but its limit of detection (LOD) was about 40nM, even when using alcohol as a ''sheath flow'' post-column addition. This was much higher than the Tau LOD value reported here. In this study, a fast, sensitive, and noninvasive LC-MS/MS technique has been developed to separate and quantitatively determine taurine and related biomarkers in urine matrices. Furthermore, this method eliminates laborious sample preparations such as solid phase extraction, liquid-liquid extraction, and also reduces the errors introduced by the derivatization procedures.

2. MATERIALS AND METHODS

2.1 Chemicals

Four standards, including taurine, L-phenylalanine, hippuric acid and creatinine, were purchased from Sigma–Aldrich (St Louis, MO, USA). L-Glutamine (Glu) isotope ($C_5H_{10}{}^{15}N_2O_3$), which was used as the internal standard (IS) for LC-MS/MS analysis, was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol (LC-MS grade) and Formic acid (99.9%), which were used to prepare the mobile phase, were purchased from Sigma-Aldrich (St Louis, MO, USA). Ultrapure water (MQ water) was obtained using a Milli-Q Advantage® A10 and Millipore Elix® water purification system (Millipore, MA, USA).

2.2 Urine Samples

Urinary bladder cancer (n=11) spontaneous urine samples were obtained from the Central Missouri Urology Clinic (Rolla, MO, USA). The "no-evidence-of-malignancy" (NEM) spontaneous urine samples (n=12) were collected from volunteers at Missouri University of Science and Technology (Rolla, MO, USA) and Central Missouri Urology Clinic (Rolla, MO, USA). After collection, the samples were immediately frozen and stored at -80 °C. The samples were thawed at room temperature and then diluted ten times using ultra-pure water and filtered with a 0.22 μm nylon membrane filter before analysis. A total of 20 μL of diluted urine was mixed with 20 μL of the internal standard solution (6 ppm) and 1460 μL of 0.2% formic acid in water. Then the sample was analyzed by LC/MS/MS.

2.3 High-Performance Liquid Chromatography (HPLC) Separation

A phenyl-hexyl, 3.0 μm, 3.0×150 mm column (Phenomenex, USA) and Agilent 1100 series LC system (Santa Clara, CA) were used for the separation. The sample injection volume was 10 μL. LC was performed at 25°C under a flow rate of 250 μL/min. Separation was achieved by an isocratic system with the mobile phase consisting of A: 0.2% formic acid in water and B: 0.2% formic acid in Methanol (100%). The isocratic program was 60% A and 40% B at a flow rate of 250 μl/min. Run-to-run time was 15 min.

2.4 Tandem Mass Spectrometry Detection

For the identification and quantification of the above metabolites in urine samples, an API 4000Q Trap MS/MS system (Applied Biosystems, Foster City, CA) was used. The mass spectrometer was operated in a multiple-reaction monitoring mode (MRM) with ESI-positive ionization. A turbo spray was used as the ion source and nitrogen was used as the curtain and cone gases. Optimal detection conditions were determined by direct infusion of each standard solution (100 ppb) in solvent A, using a syringe pump. MRM ion pairs for each compound were obtained as given in the Table 1.

Table 1. MRM parameters for all biomarkers, creatinine and internal standard in ESI positive mode.

Analyte	Q1 (amu)	Q3 (amu)	Dwell time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
L-Phenylalanine	166.1	120.2	150	31	10	21	6
Taurine	126	108	150	46	10	17	20
Hippuric acid	180.1	105.1	150	36	10	19	18
Creatinine	114	44.1	150	51	10	31	6
L-Glutamine (IS)	148	130	120	31	10	15	8

2.5 Method Validation

The LOD and limit of quantification (LOQ) were calculated at signal to noise ratios (S/N) of 3 and 10, respectively. Reproducibility and accuracy were determined by six consecutive analyses of analytes spiked in urine matrices of low, middle, and high concentrations. Urine -matrix based calibration curves were constructed and the lowest point in the calibration curve was chosen as the LOQ. Precision and accuracy were ensured at all levels during the analysis. In order to assure that these biomarker levels represented the physiological concentration, the amount is reported here as a ratio of biomarker concentration to creatinine concentration.

3. RESULTS

Method validation is extremely crucial for obtaining consistent, reliable, and accurate data for any analytical method. Therefore, great effort has been expended on method development and validation of these compounds. Each individual compound has been analyzed separately by different methods, such as LC-MS [18,19] and capillary electrophoresis [20], but not by one LC-MS/MS method in urinal matrices. In this study, 100 ppb standards were first prepared in different mobile phases such as 0.1% formic acid in MQ water, 5 mM ammonium formate in MQ water, and 5 mM ammonium formate plus 0.05% formic acid in MQ water, and injected directly into the mass spectrometer (**Table 2**). The first two mobile phases gave relatively high signal intensities required for further MRM study, in positive and negative modes, respectively.

Table 2. The effects of different mobile phase additives on the analytes.

Mobile Phase Additive	Ionization Mode	Observations
5 mM Ammonium Formate	Negative	High background was obtained for creatinine due to formate ions (m/z 45) present in the mobile phase.
5 mM Ammonium Formate + 0.05% Formic Acid	Negative	Phenylalanine peak intensity was very low.
10 mM Ammonium Acetate	Negative	Phenylalanine, hippuric acid and creatinine peak intensities were very low.
0.1 % Formic Acid	Positive	Relatively high peak intensities were observed for all analytes.
0.2 % Formic Acid	Positive	Higher peak intensities (1.5~2.0 times), better peak shapes, and lower backgrounds were observed for all analytes compared to 0.1% formic acid.

A 10 mM ammonium acetate buffer (pH 9.3) was tried, as given in the method of Chaimbault et al. [18], but it did not give required intensity for Phe, Hip and Cre peaks (Table 2). Although different concentrations of ammonium formate, such as 2.5 mM, 5mM, 10mM in MQ water, were used, they did not significantly affect the signal intensities. Both phenyl-hexyl and C18 columns were tried for the separation in different mobile phase compositions in both isocratic and gradient programs. The C18 column did not provide a good separation for the compounds of interest, and all compounds were eluted quickly with the matrix peak. This may have been due to its inability to retain and separate high polar analytes. On the other hand, a phenyl-hexyl column gave a good

separation of the analytes. Hence, further testing was done with 0.1% formic acid (for ESI positive mode) and 5 mM ammonium formate (for ESI negative mode) dissolved in MQ water and acetonitrile. The parent ions and daughter ions for each compound in both positive and negative modes were determined using multiple reactions monitoring (MRM). Because ammonium formate did not dissolve well in acetonitrile, acetonitrile was replaced with methanol. When 5 mM of ammonium formate were used as the mobile phase additive in the ESI negative mode analysis, creatinine showed a high background as compared to the others in the chromatogram (Table 2).

Based on our extensive experimental study, we found that an ESI negative mode could not be used for detection due to the high creatinine background. Therefore, further investigations of experimental conditions were conducted in an ESI positive mode using 0.1% formic acid as the mobile phase additive. Different concentrations of formic acid were tested as an additive, and 0.2% was found to be the optimum because it gave higher peak intensity, better peak shape, and a lower background (Table 2). Most intense ion pairs for each compound were determined by using direct infusion of 100 ppb standards dissolved in 0.2% formic acid in MQ water (as shown in Table 1). Compound dependent parameters, such as declustering potential (DP), entrance potential (EP), collision cell entrance potential (CE), and collision cell exit potential (CXP) are also listed in Table 1. Chromatographic separation was optimized after investigating different aqueous and organic phase combinations in both isocratic and gradient programs. An isocratic system, with the mobile phase consisting of 60% of 0.2% formic acid in MQ water and 40% of 0.2% formic acid in methanol, was selected for the separation of our compounds of interest.

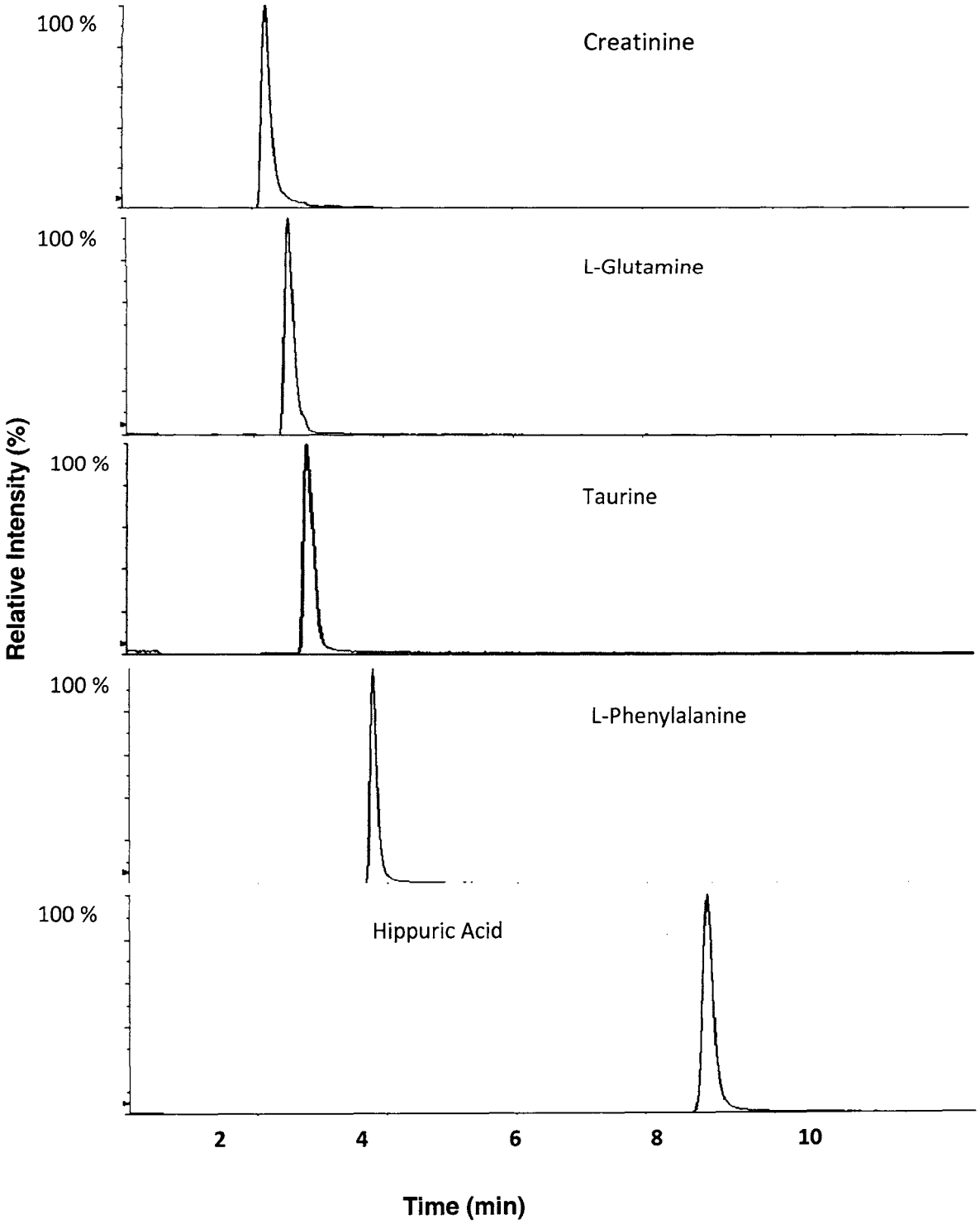

Figure 1. LC/MS/MS extracted ion chromatograms (XIC) of taurine (Tau), L-phenylalanine (Phe), hippuric acid (Hip), and creatinine (Cre) in urine samples. 15N2-L-glutamine was used as the internal standard, and creatinine acted as an indicator for urine dilution. The concentrations of all compounds were 50 ppb. Other experimental conditions were stated in the "Materials and Methods" section.

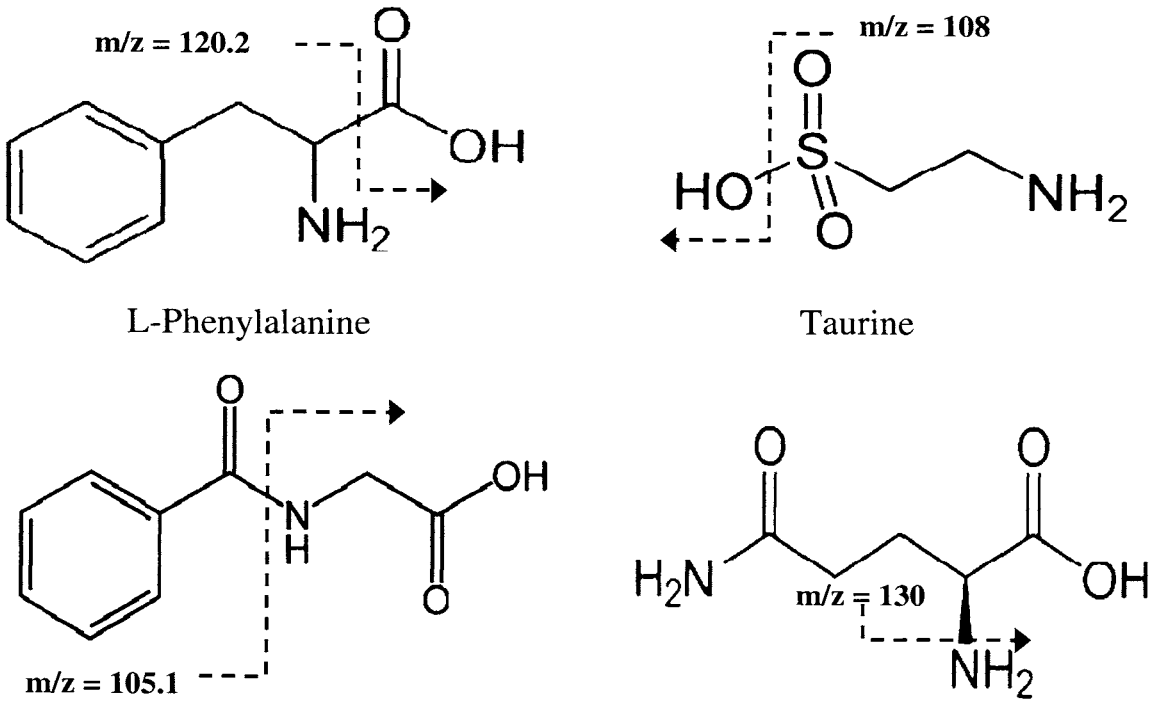

Figure 2. Structures and possible fragmentations of biomarkers, creatinine, and glutamine internal standard.

A MRM chromatogram of 50 ppb standards in a urine matrix is shown in Figure 1. The structures and possible fragmentations of all compounds in the MRM mode are shown in the Figure 2.

Table 3. Linearity, limit of detection, and limit of quantification of analytes in the urine matrix.

Analyte	Linear Range (μM)	Slope (peak area ratio/μM)	Intercept (peak area ratio)	R^2	LOD (nM)	LOQ (nM)
L-Phenylalanine	0.0006 - 1.513	7.07	0.147	0.999	0.18	0.6
Taurine	0.008 - 7.990	0.138	0.005	0.999	3.10	8
Hippuric acid	0.0006 - 3.907	1.828	0.044	0.999	0.20	0.6
Creatinine	0.018 - 17.680	0.498	0.455	0.999	6.00	17.6

The LOD and LOQ of each compound for this newly developed LC-MS/MS method were determined and are shown in Table 3. Phe and Hip showed very low detection and quantification limits in urine matrices, which is more than sufficient for quantitative analysis of these biomarkers in urine samples. Calibration curves were constructed by spiking standards on top of endogenous levels in a normal urine sample. Then the non-spiked urine sample was run to see the endogenous levels. Its peak areas were subtracted from the spiked urine to construct the calibration curves. Three samples were prepared for each concentration and an average was taken to make calibration curves. The same preparation steps were followed for the calibrants, as for the samples. LOQs were chosen as the lowest points in the calibration curves and concentrations of

standards were selected based on the expected concentration ranges in urine samples. The regression parameters, such as slope, intercept, linear range, and correlation coefficients (R^2), are also tabulated in Table 3. Good calibration linearities were obtained with R^2 values >0.999, for all compounds.

Table 4. Reproducibility and recovery of analytes in a urine matrix.

	Low Conc. (2 ppb)		Middle Conc. (50 ppb)		High Conc. (700 ppb)	
Analyte	RSD (%)	Recovery (%)	RSD (%)	Recovery(%)	RSD (%)	Recovery(%)
L-Phenylalanine	10	101	1.2	122	1.2	76
Taurine	8.8	85	1.7	108	1.4	113
Hippuric acid	11	84	3.8	95	2.3	109
Creatinine	*	*	6	80	7.4	125

* In urine creatinine concentration was normally at the high concentration levels. Therefore, the data at the low concentration level is not shown here.

The reproducibility and recovery of the method were systematically studied and the results are shown in Table 4. The reproducibility is represented as a percentage of the relative standard deviation (RSD) of each individual analysis of spiked standards. We can see clearly from Table 4 that more than 80% recovery was achieved for almost all of the compounds, except for Phe at high concentrations (700 ppb). To demonstrate the applicability of this method, 24 urine samples, including 11 BCa and 12 NEM, were analyzed.

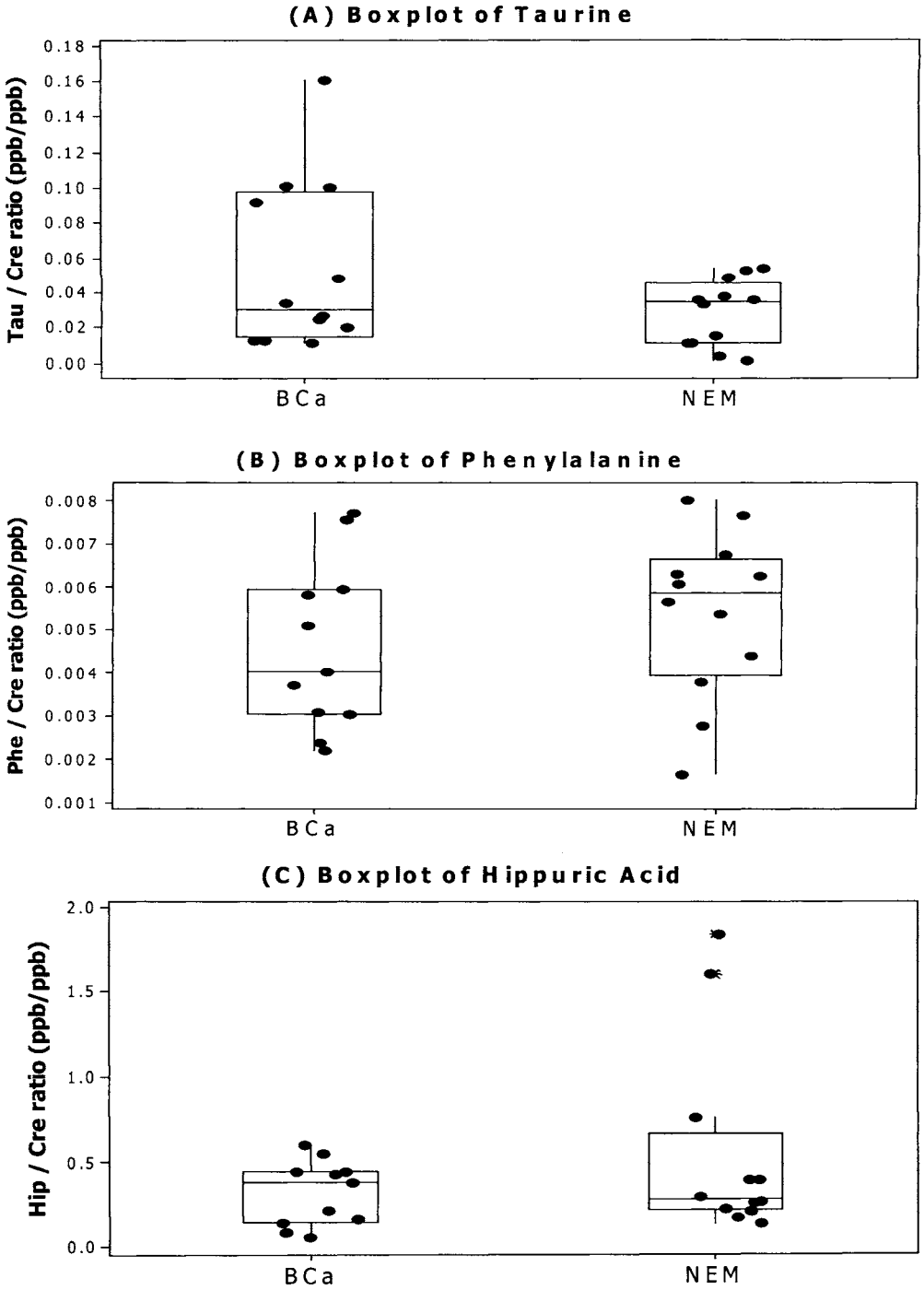

Figure 3. (A) Taurine to creatinine ratio in urine for BCa (n=11) and NEM (n=12); B) L-Phenylalanine to creatinine ratio in urine for BCa (n=11) and NEM (n=12); (C) Hippuric acid to creatinine ratio in urine for BCa (n=11) and NEM (n=12).

The concentrations of each biomarker were determined using the internal standard based calibration curves. Data are represented graphically in box-plots (Figure 3) as the ratio of analytes to creatinine for Tau, Phe, and Hip. Those biomarker levels in BCa and NEM can be compared using these data.

4. DISCUSSION

The discovery and quantitation of endogenous molecules that serve as biomarkers of cancer progression is a major challenge in modern clinical research. These indicators can dramatically improve early cancer detection and the efficiency of treatments. Several important attributes, such as sensitive and selective detection, multi-analyte analysis, and the ability to provide structural information, have made mass spectrometry a promising tool in biomarker discovery [21]. This study developed a new LC/MS/MS method for separating and quantifing taurine and related compounds in urine as potential biomarkers for non-invasive bladder cancer detection. A triple quadrupole operated in the MRM mode shows exceptional levels of sensitivity and selectivity. Here, a precursor ion is preselected and resolved in quadrupole (Q) 1, and then fragmented in Q2 by collision-induced dissociation. Finally, the resultant product ion is analyzed in Q3 [22]. Another advantage of this mode is that it results in extremely reproducible chromatographic peak shapes and intensity, because the precursor to product ion reaction is monitored many times per second [22].

In this experiment, negative mode detection was tried using 5 mM ammonium formate as an additive and positive mode detection was tried using 0.1% formic acid as an additive. The major problem with the negative mode was that creatinine showed a

high background signal compared to others. The creatinine ion pair in the negative mode was m/z 112.8/44.8 and formate ion m/z in the negative mode was 45. So, the creatinine signal can be seriously interfered by a formate ion signal in the negative mode. Thus, the positive mode was used for further analysis. Several studies have reported conducting urinary creatinine analyses by the ESI positive mode, using m/z 114/86 [23] and 114/44 [23]. The $^{2}H_3$ creatinine, which has ion transitions m/z 117/89 in the positive mode [23] , can be used as an internal standard for creatinine analysis. Most intense fragment ions were obtained for each compound in the MRM positive mode by direct infusion. Phe gave the most intense fragment of $C_8H_{10}N$ with m/z 120.2, and for Hip, it was C_7H_5O with m/z 105.1. Loss of a H_2O from Tau gave $C_2H_6NO_2S$ with m/z 108. The most intense ion pair for Cre was 114/44 and a $C_5H_8NO_3$ (m/z 130) fragment was obtained for Glu (IS). Optimal compound dependent parameters for each compound (Table 1) were obtained from direct infusion of standards into the mass spectrometer, because they do not depend on LC flow conditions. Sample preparation techniques are normally involved in bio-fluids analysis using LC/MS/MS, such as solid phase extraction (SPE), liquid-liquid extraction, filtrations, and derivatizations. Complex and time consuming sample preparations may significantly increase analysis cost and time. In this study, sample preparation was quick and simple. Ten-fold diluted urine samples were further diluted 75 times, using 0.2% formic acid in MQ water and injected into the LC/MS/MS for analysis. Because these analytes are present in relatively high concentrations in urine samples, extraction methods, like SPE, were not used in this study. Since the diluted sample was going through a HPLC column, most interference ions or compounds from the matrix were removed before the MS/MS analysis. Urine has become a popular medium for

biomarker discovery due to its non-invasive nature. Patients often prefer to give urine samples rather than blood or tissue samples. In most urinary biomarker analyses, creatinine has been included as an analyte, because the extent of creatinine concentration corresponds closely to urine dilution. It may be noted, however, that creatine can act as an interference for creatinine in LC/MS/MS, because creatine undergoes in-source loss of water and thereby mimics creatinine. This was investigated in this study, and there was a 0.3 min separation between the creatine and creatinine peaks. Even without a good separation, creatine contribution towards the creatinine peak (at 1 ppm) was only 0.35%, which is insignificant.

Even though MS/MS analysis does not depend on the chromatographic resolution, a good separation was obtained for all analytes. First peak, Cre, was eluted at nearly 2.3 minutes and it avoided the dead volume of the column. All other analytes were eluted within 10 minutes, with Hip eluted last at 8.9 minutes. The LOD and LOQ values demonstrated a good sensitivity of the method, which is adequate enough to detect very low concentrations of these analytes in urine. The urine-matrix based calibration curves gave good linearity and large concentration ranges. The reproducibility of the method was investigated for low, medium, and high concentration levels. The results showed a great reproducibility for all of these concentration levels. The recovery was consistent, precise, and reproducible, and was above 80% for most of the compounds, except for Phe at the high concentration level. This optimized method was utilized to analyze BCa and NEM urine samples from 23 donors; data are represented in Figure 3 as box plots. Also Table 5 shows the analytes concentrations in each urine sample. Using these data,

however, we did not expect to validate the accuracy of the results from Srivastava et al.[8].

Table 5. Analytes concentrations in urine samples.

Urine Sample	Hippuric Acid (mM)	Creatinine (mM)	Phenylalanine (mM)	Taurine (mM)
BCa 01	22.721	81.010	0.323	0.956
BCa 02	6.308	45.445	0.095	0.216
BCa 03	1.796	32.815	0.054	0.107
BCa 04	11.654	48.266	0.197	1.500
BCa 05	6.759	23.907	0.124	0.546
BCa 06	19.165	54.645	0.116	2.402
BCa 07	2.795	30.552	0.084	0.736
BCa 08	10.778	28.218	0.149	0.337
BCa 09	1.749	47.130	0.165	0.442
BCa 10	6.409	23.374	0.060	0.243
BCa 11	0.785	7.551	0.012	0.628
NEM 01	10.762	75.330	0.350	0.791
NEM 02	4.325	25.874	0.112	2.116
NEM 03	38.339	37.845	0.163	2.109
NEM 04	13.301	71.432	0.216	0.774
NEM 05	3.786	15.201	0.029	0.674
NEM 06	6.633	49.437	0.261	8.898
NEM 07	8.212	50.216	0.277	2.429
NEM 08	2.755	31.459	0.082	1.079
NEM 09	18.611	15.982	0.067	0.520
NEM 10	4.930	44.388	0.164	0.642
NEM 11	14.446	57.974	0.225	1.767
NEM 12	5.941	12.324	0.014	0.047

BCa : urinary bladder cancer urine samples; NEM : no-evidence-of-malignancy urine samples

The reason was that we did not have control over the diet, and other factors such as vitamin supplementary intake, of the cancer patients and healthy controls. These biomarker levels, especially taurine, can be elevated according to diet and treatment options of the cancer patients. The main goal of this study was to develop a fast, reliable, and simple method for quantifying these compounds in urine, so that a detailed clinical study can be done to validate these markers using a controlled diet for cancer patients and for healthy subjects. Taurine levels was found to be elevated not only in BCa, but in other diseases as well, such as polymytosis [24], dermatomyositis [24], Alzheimer [25], aging [26], and ischemia/reperfusion injury [27]. Hence, this method has considerable potential for applications other than BCa detection. However, it is still unclear how taurine and related compounds can be used as reliable prognostic or diagnostic biomarkers. Therefore, more detailed clinical studies have to be conducted in order to validate these markers. In any case, this LC-MS/MS method provides a valuable tool for urinary analysis of taurine and related biomarkers.

5. CONCLUSIONS

A reliable, fast, and simple HPLC-MS/MS method was developed to simultaneously separate and detect four biomarkers (Tau, Phe, Hip, Cre) in urine samples. These markers can be used as potential candidates for BCa detection. Without additional time-consuming sample preparation techniques, this method can completely separate the above compounds (using a phenyl-hexyl column) within 10 minutes. Parameters affecting LC separation and MS/MS detection were systematically investigated and optimized. A complete method validation was performed in the urine matrix.

Proper clinical studies have yet to be done using this method to validate these compounds as fingerprint biomarkers for BCa detection. Not only can this method be used for BCa, but it may be applied to detect other diseases and to monitor treatments. Furthermore, with simple modifications, this method could be used for detection of these biomarkers in other physiological fluids.

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IV. BIOMARKER ANALYSIS IN PROSTATE CANCER USING LC-MS AND CE-MS (REVIEW ARTICLE)

SUMMARY

Prostate cancer is one of the most common cancer types in men. In addition, it is the second leading cause of cancer death in the United States and Canada. Prostate cancer diagnosis is not a precise science yet. Discovery of potential biomarkers for early prostate cancer diagnosis and monitoring is crucially important. Liquid chromatography – mass spectrometry (LC-MS) and capillary electrophoresis – mass spectrometry (CE-MS) have been widely used analytical techniques in the biomarker discovery. This review will describe the applications of LC-MS with different ionization techniques such as ESI, APPI, APCI and CE-MS techniques used in prostate cancer biomarker analysis.

KEYWORDS

01. Biomarker - Any biological, chemical, or biophysical indicator of an underlying biological process.

02. Prostate Cancer - A hormonally mediated tumor that develops in the prostate gland of the male reproductive system.

03. LC-MS - An analytical tool which combines the separation of analytes by liquid chromatography and detection by mass spectrometry.

04. CE-MS - An analytical tool which combines the separation of analytes by capillary electrophoresis and detection by mass spectrometry.

05. ESI - An ionization technique used in mass spectrometry which a sample solution is sprayed across a high potential difference from a needle into an orifice and produced ionized species in the gas phase.

06. APCI - A chemical ionization process due to ionization of O_2 and N_2, which subsequently ionizes the solvent that then, ionizes analytes in the atmospheric pressure region.

07. APPI - Atmospheric pressure photo ionization is an ionization technique in which samples are ionized by ultraviolet light.

1. INTRODUCTION

1.1 Prostate Cancer and Current Diagnosis Methods

Adenocarcinoma of the prostate and transitional cell carcinoma of the urinary tract categorized as a genitourinary malignancy or urological cancer. Adenocarcinoma is a cancer of an epithelium that originates in glandular tissue. The majority of genitourinary malignancies are from adenocarcinoma of the prostate and transitional cell carcinoma of the bladder, ureters, and renal collecting system.

A healthy prostrate in a male reproductive system is a small soft gland which is located under the bladder in the front of the rectum. Its main function is to help produce and store seminal fluid. About twenty percent of the fluid containing semen is made up of small glands within the prostate [101]. The process of cell mutation in the prostrate glands results prostate cancer. Unfortunately, this has become the most common cancer

among American men. According to studies, one out of every ten men will develop this cancer during their life time, most often after the age of fifty [102].

Several factors have been identified that increase the risk of developing prostrate cancer. The hereditary factor, or genetic background, plays a prominent role. Apart from that, dietary patterns [1], medication exposure [2], and viral exposure [3] have also been found to have repercussions that can lead to prostrate cancer. Unfortunately, in its early stages prostrate cancer does not show any symptoms, however, it can cause elevated prostate specific antigen (PSA) levels that can be detected in routine medical examinations. Patients diagnosed with prostrate cancer have symptoms like frequent urination, increased urination at night, difficulty starting and maintaining a steady stream of urine, blood in the urine, and painful urination. Advanced prostate cancer can spread to other parts of the body, causing a variety of other symptoms. The most common symptom is bone pain, often in the spine, pelvis, or ribs. Leg weakness and urinary and fecal incontinence [4] can occur when cancer spreads to the spinal cord, which then becomes compressed as a result. Evaluation of prostrate cancer involves gauging its stage or the extent to which it has grown. This determination informs the prognosis and treatments. The most common system used to evaluate prostate cancer is the four-stage tumor, nodes, metastases, (TNM) system.

This TNM staging system determines whether or not the cancer is still confined to the prostate. In TNM system, clinical T1 and T2 cancers are found only in the prostate; T3 and T4 cancers have spread elsewhere. The Gleason score is another grading system for prostate cancer based on its microscopic appearance. A pathologist assigns a grade to the most common tumor pattern, and a second grade to the next most common tumor

pattern. These two grades are then added together to get a Gleason Score. Dr. Donald Gleason, a pathologist at the Minneapolis Veterans Affairs Hospital, developed this scoring system in the 1960s. These staging and grading systems determine the aggressiveness of prostate cancer and its treatment options.

PSA is a serine protease that liquefies semen coagulum. It is widely used in prostate cancer diagnosis and monitoring. PSA is a normal constituent of prostatic fluid and is therefore neither specific nor sensitive for prostate cancer. The major drawback of PSA is that it leads to false-negative or false-positive test results. Doctors consider a PSA level below 4.0 ng/mL normal, but one large study diagnosed prostate cancer in 15.2% of men with a PSA level at or below 4.0 ng/mL. Also false positives lead to over diagnosis followed by unnecessary treatment and monitoring of patients over long periods of time [5]. Although serum PSA levels can be elevated in prostate cancer, the only test that can fully confirm the diagnosis of prostate cancer is a biopsy. Before a biopsy is performed, however, several other tools may be used to gather more information about the prostate and the urinary tract. A digital rectal examination (DRE) may allow a doctor to detect prostate abnormalities. Trans-rectal ultrasound uses inaudible sound waves produced by a probe inserted into the rectum to create an image of the prostate. If the biopsy indicates cancer, the doctor will order other tests such as bone scans, x-rays, computed tomography (CT) or magnetic resonance imaging (MRI) to determine whether or how far the cancer has spread.

Normally, patients prefer not to damage their organs and tissues to give samples during the disease diagnosis process. They also prefer not to give blood for diagnostic tests. Noninvasive diagnosis procedures do not penetrate the body mechanically, nor

break the skin or enter a body cavity; therefore, they do not require an incision into the body or the removal of biological tissue. Biomarkers are good potential candidates in this non-invasive diagnosis.

2. APPLICATIONS OF LIQUID CHROMATOGRAPHY MASS SPECTROMETRY IN PROSTATE CANCER BIOMARKER ANALYSIS

Diagnosis of cancer at an early stage is a major challenge to modern cancer research. Biomarkers may be able to meet this challenge. A biomarker is any biological, chemical, or biophysical indicator of an underlying biological process. Biomarkers are used in many fields, such as biology, medicine, geology, astrobiology, and genetics. In medicine, a biomarker can be a substance that indicates a particular disease state. A cancer biomarker is defined specifically as *"a molecular, cellular, tissue, or process-based alteration that provides indication of current, or more importantly, future behavior of cancer"*[6] (Hayes et al., 1996).

Liquid chromatography-mass spectrometry (LC-MS) is a powerful, widely used tool for quantitative and qualitative biomarker analysis. LC/MS has proved useful due to its high sensitivity, and chromatographically co-eluting peaks can be identified by mass selectivity and is not depending on chromatographic resolution. The mass spectrum generates a chemical fingerprint of a compound, ensuring accurate peak assignment, even in the presence of a complex matrix. LC/MS can also determine or confirm known and unknown compounds using molecular mass. Furthermore, controlled fragmentation permits structural elucidation of unknown compounds. LC/MS permits rapid method development because it easily identifies eluted analytes without the need to validate retention time. Further, its sample matrix adaptability decreases sample preparation time.

Using LC/MS, quantitative and qualitative data can be collected easily with limited instrument optimization. These characteristics make LC/MS a promising tool for the identification of biomarkers.

2.1 Sample preparation for LC-MS

Biomarker analysis of human body fluid samples is restrained by three major factors. The large sample variation in biofluids requires numerous patient samples. The complexity and wide dynamic range of proteins in biological samples demand arduous biochemical analysis to identify protein markers. Sometimes identified biomarkers are not produced by the diseased cells, but by secondary body defense mechanisms[7] . LC/MS has been used to analyze many biomarkers directly or indirectly related to prostate cancer. Some studies entail simple sample preparation techniques, whereas others rely on complex, time consuming preparations. Urine is a popular medium for biomarker discovery because its collection is not invasive. Patients often prefer to give urine samples rather than blood or tissue samples. Creatinine levels in urine act as an internal standard. Since creatinine concentration corresponds closely to urine dilution its levels must be monitored. For animal studies, mice are often kept in metabolism cages under food deprivation [8]. Since urine samples have a very complex matrix, it should dilute before the analysis. In most cases, preparation of urine samples for analysis requires solid phase extraction (SPE). Oasis HLB, Strata-X[9], and C18 [10] cartridges are among the most widely used SPE columns for urine analysis. After samples are collected from a SPE cartridge, they must be dried using a vacuum evaporator and re-dissolved in high performance liquid chromatography (HPLC) grade solvents. . The analytes can also be extracted into the organic liquid phases[11]. Tissue samples must be

stored at -20°C. Higashi et al. (2006) developed a LC/MS assay to quantify androstenediol in prostatic tissue [9]. Before analysis, prostate tissue was minced and crushed before addition of the [7,7,16,16,17α-$^{2}H_5$]-Adiol internal standard and further homogenized [9]. It was then centrifuged several times and run through a SPE cartridge. Also incubation at an optimum temperature [11] is important in cell sample studies.Biomarker analysis of human plasma also requires that samples be cleaned using a procedure such as SPE [12]. Chen et al. reported on the application of in-gel iso-electric focusing LC-MS/MS to the mapping of the phosphoproteome in human prostate cancer cell lines [13].

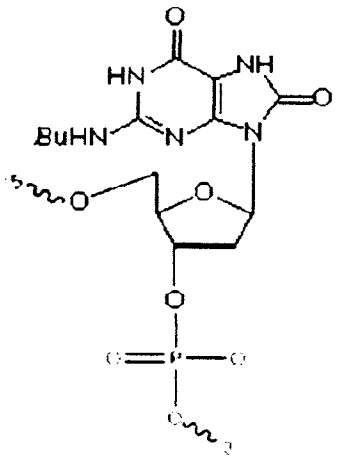
In protein biomarker analysis, high-abundance proteins always mask low-abundance proteins, therefore, removal of high abundance proteins is an important step in sample preparation. In a serum sample, after dilution and filtration, the three most abundant serum proteins (albumin, immunoglobulins, and transferrin) can be removed using multiple affinity removal spin cartridges [7] and cutoff ultracentrifugation columns. The tryptic diestion is a widely used technique[14-15] to study the protein primary structure and identification of proteins by analyzing the resultant peptides using mass sepectrometry. Trypsin digests protein by cleaving the peptide chain at specific sites such as after Lysine(K) or Arginine(R) residues except when followed by a Proline(P) residue. This techninique is eminently suitable for positive ionisation mass spectrometric analysis. The protein digestions can also be performed on a computer through a computer simulation. Those simulation experiments are called *in silico* digestions and they can be useful aids for planning proteomics experiments. Programs that perform *in silico* digestion include PeptideMass and PeptideCutter at ExPASy, MS-Digest at Protein

Prospector and Protein Digest at ISB. These tool generate a list of peptide sequences and provides descriptive and statistical analysis, such as molecular weight histograms and amino acid residue distributions.

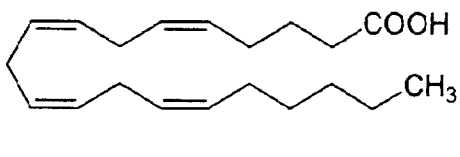
2.2 Electrospray Ionization - Liquid Chromatography Tandem Mass Spectrometric (LC-ESI-MS) Studies

Tandem mass spectrometry uses two or more quadrupoles to separate ions based on a sample's mass-to-charge ratio. Quadrupole-quadrupole, magnetic sector-quadrupole, quadrupole ion trap and the quadrupole-time-of-flight geometries are the most common tandem mass spectrometers in use today. In electrospray ionization (ESI), a sample solution is sprayed across a high potential difference from a capillary into an orifice in the interface. Heat and gas flows are then used to de-solvate the ions in the sample solution [103]. The droplets that detach from a tip of the capillary contain an excess of positive or negative charge as a result of the applied high voltage. The electrical field gradient attracts charged droplets towards the entrance of the mass spectrometer and charged analyte molecules are generated from the small charged droplets either by the charged residue model or by the ion evaporation model. Ion formation from the droplets is promoted by a flow of drying gas such as nitrogen. Multiple-charged ions may be produced by ESI, and number of charges increase with molecular weight. ESI has many advantages: It is useful for charged, polar or basic compounds, and it is the best method for analyzing multiple-charged compounds. It also permits the detection of high-mass compounds. ESI shows excellent detection limits due to a very low chemical background. It is compatible with MS/MS methods and can control the presence or absence of fragmentation by changing the interface lens potentials [8]. Two studies have analyzed 8-

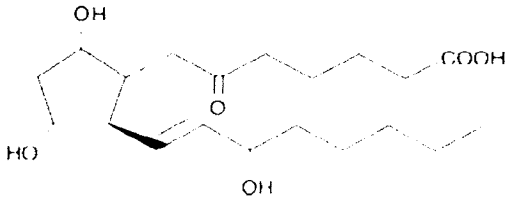
oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) (Figure 1) in the urine of human and rodents as a biomarker for oxidative DNA damage. In urine biomarker analysis, creatinine levels [16] are significant. Several studies have reported that urinary creatinine can be analyzed by tandem mass spectrometry, ESI positive mode, using selected reaction monitoring (SRM) (m/z 114 to 86)[8] , (m/z 114 to 44)[17] . Both studies used 0.1% formic acid in water/acetonitrile as the mobile phase. It is also possible to use $^{2}H_3$ creatinine, which has ion transitions m/z 117 to 89 in the positive mode [8] , as the internal standard for creatinine analysis. Figure 2 shows SRM chromatograms for the different ion pairs for creatinine analysis. The enzymatic activities of 15-Lipoxygenase 2 (15-LOX2, KDa) in normal and cancerous prostate cells have been analyzed in tandem MS and ESI negative mode[12] . This recently cloned human lipoxygenase shows tissue-restricted expression in prostate, lung, skin, and cornea. The protein level and enzymatic activity of 15-Lipoxygenase 2 has been found to be down-regulated in prostate cancers to a greater degree than in normal and benign prostate tissues [12] . Sardana et al (2007) used a cell- culture-based proteomic approach to find novel prostate cancer markers in the proteins secreted into the conditioned medium of the prostate cancer cell line PC3 $(AR)_6$ [18] . They have used a C-18 reversed-phase chromatography column with multiple reaction monitoring to analyze the peptides. They have found that serum-based concentrations of Mac-2BP candidate marker were higher than normal in prostate cancer.



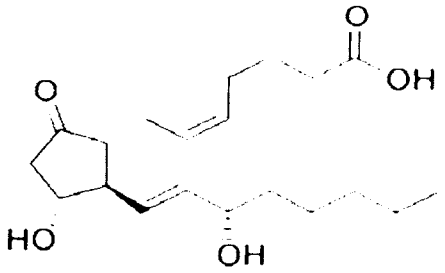
8-oxodG



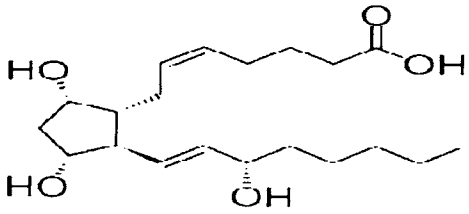
Arachidonic Acid



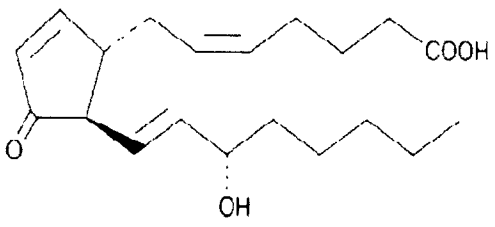
6-keto-$PGF_{1\alpha}$



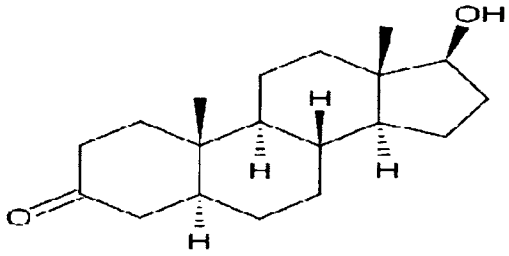
PGE_2



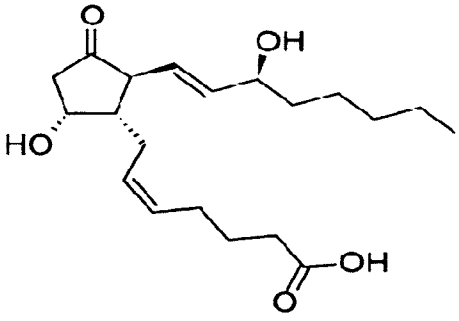
$PGF_{2\alpha}$



PGJ_2



Dihydrotestosterone (DHT)



PGD_2

Figure 1. Structures of some of the prostate cancer biomarkers

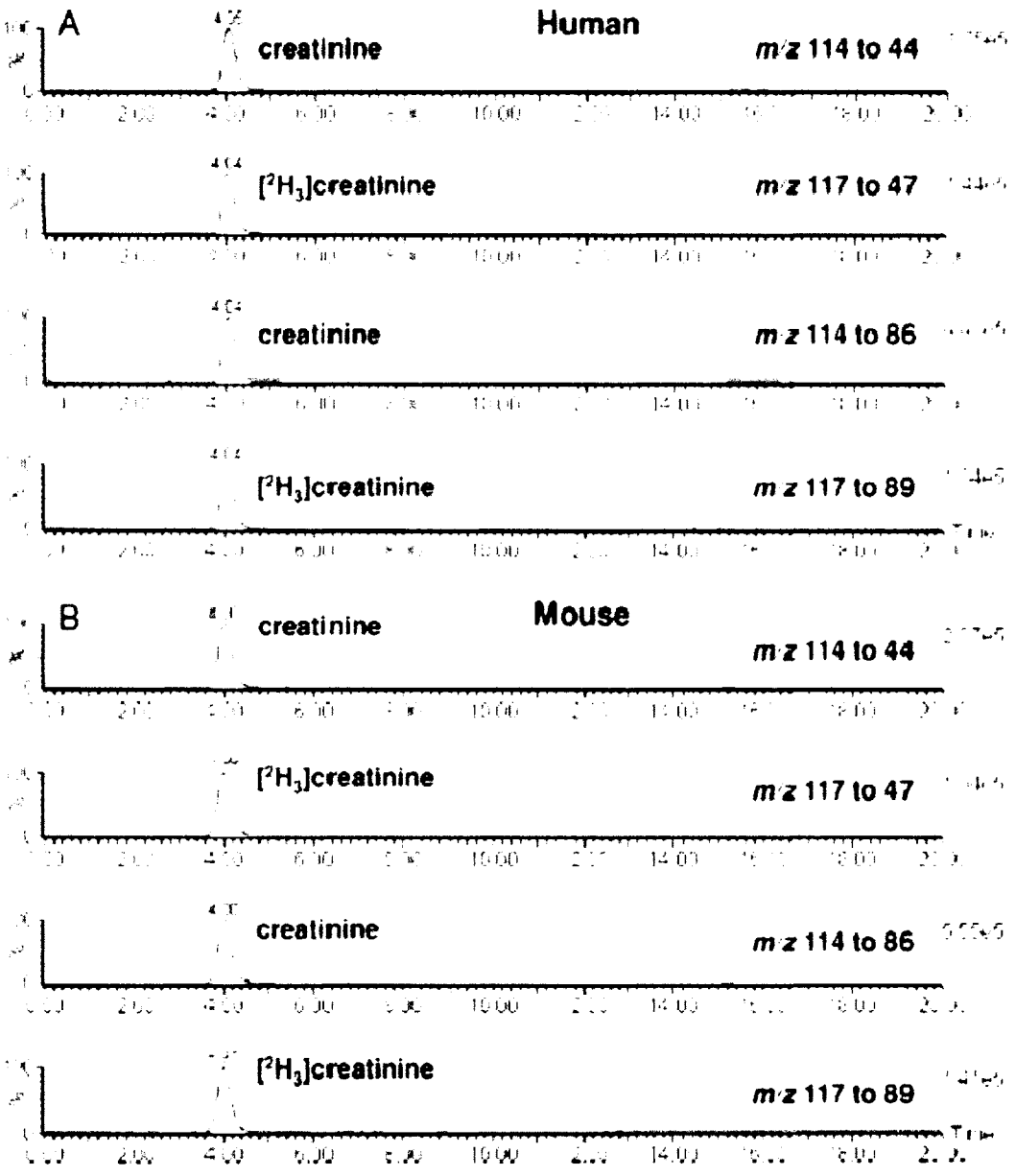


Figure 2. LC/MS/MS determination of creatinine in (A) human and (B) mouse urine. The typical LC/MS/MS SRM ion chromatogram is shown for the transitions m/z 114 to 44 and m/z 114 to 86 for creatinine plus m/z 117 to 47 and m/z 117 to 89 for [2H3]creatinine followi following gradient elution using solvent A, 0.1% formic acid in 10mM ammonium acetate (v/v), and solvent B, 0.1% formic acid in acetonitrile (v/v), at a flow rate of 120mL/min. Adapted from [8]. Reprinted with permission.

Another study by Nithipatikom et al.(2003) [10] used an LC-ESI-MS technique to detect the cyclooxygenase metabolites of arachidonic acid (6-keto-$PGF_{1\alpha}$, PGD_2 , PGE_2 , $PGF_{2\alpha}$, and PGJ_2) (Figure 1) produced by cultured cells. These metabolites were separated on a C18 column with water-acetonitrile mobile phase and detected in the ESI positive mode. They have used selected ion monitoring (SIM) of *m/z* 353, 335, 335, 319 and 317 to quantify 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2 respectively. Glycans are important cofactors in several biological processes and diseases, including immune system function and cancer. Nithipatikom's group analyzed the structures of glycans on immunoprecipitated proteins from small amounts of cell or tissue lysates by LC-ESI-MS [19]. Their analysis incorporated both capillary LC-MS/MS and nano-LC-MS/MS.

Most published studies of proteins in biological fluids describe limits of quantitation in the low μg/ml range when no immunoenrichment of the target protein is applied. However, the concentrations of known clinical biomarkers are usually in the ng/ml range. Fortin et al. (2009) proved that mass spectrometry permits protein quantification in a concentration range of clinical interest without immunoenrichment using prostate specific antigen (PSA) as a model[20]. They investigated the metabolism of limonin 17-*β*-D-glucopyranoside (LG) by noncancerous (RWPE-1) and cancerous (PC-3) human prostate epithelial cells using LC-ESI-MS in SRM mode [21]. In positive mode ESI-MS, LG showed an abundant sodiated species $[M+Na]^+$, whereas the protonated molecule was barely observable. That work showed that $[M+Na]^+$ further fragmented into the less abundant $[LARL+H]^+$ (limonin A-ring lactone) and a predominantly protonated aglycone molecule (limonin) due to in-source fragmentation. It acquired structurally informative fragment ions indicating loss of water, carbon

monoxide, and carbon dioxide. One metabolite peak in addition to LG was observed during HPLC analysis of the medium of PC-3 cells, while no such peak was present in the medium of RWPE-1 cells or control samples. This suggests that metabolism of LG is cell dependent. The differences in proliferation between the cancerous and non-cancerous prostate cells can be explained by the ability of PC-3 cells to metabolize LG to LARL. The above experimental process can be used to identify functional groups in the structure, and it may prove to be a useful tool in the investigation of the metabolism of limonoids [21]. Another group demonstrated the applicability of the LC-ESI-MS/MS method combined with derivatization to the determination of 5α-dihydrotestosterone and testosterone in the human prostate. This derivatization was very effective, and it increases the detectability of positive-ESI-MS [22] .

Sreekumar et al. (2009) [23] achived a breakthrough when they quantified 1,126 metabolites and discovered a subset of five metabolites, including sarcosine (Sar), proline (Pro), kynurenine (Kyn), uracil (Ura), and glycerol-3-phosphate (G3P), that increased significantly with disease progression from benign to PCA to metastatic prostate cancer [23]. Based on this metabolomic study, a new LC/MS/MS method with ESI positive mode ionization was developed by our group to quantify these five metabolites and creatinine in urine samples[17]. This method uses a phenyl hexyl column for separation, with 0.1% formic acid in water and 0.1% formic acid acetonitrile as the mobile phases [17]. Figure 3 shows the separation comparison of six metabolite standards plus creatinine between C18 and phenyl-hexyl columns.

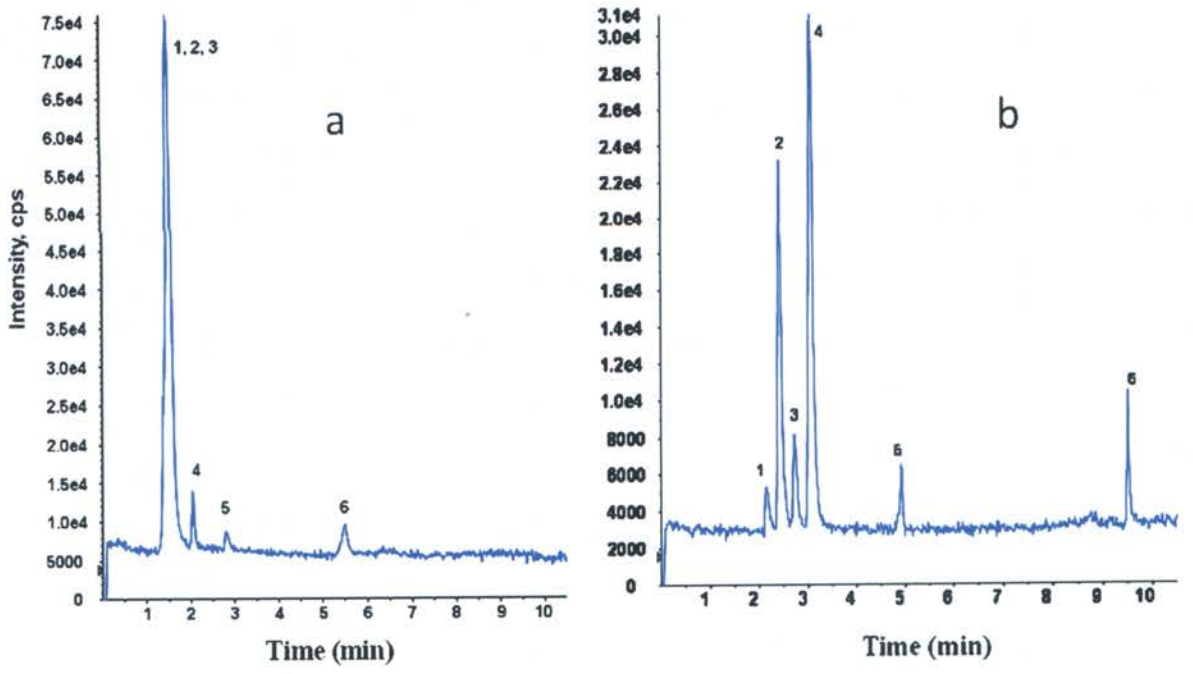


Figure 3. Separation comparison of six metabolite standards between C18 (3a) and phenyl-hexyl (3b) columns (for both C18 and phenyl-hexyl columns; dimentions, 3.0 μm, 3.0 × 150 mm ; temperature, 25 °C; flow rate, 250 μL/min; injected volume, 10 μL; mobile phase A, 0.1% formic acid in water; mobile phase B, 0.1% formic acid in acetonitrile (100%); gradient program, initial 98% A and 2% B, linear gradient to 60% A and 40% B in 5 min, return to initial conditions in 0.1 min at a flow rate of 250 μL/min, followed by equilibration for 10 min; run-to-run time, 15 min;). Peak identification: 1, glycerol 3-phosphate (G3P); 2, creatinine (CRE); 3, sarcosine (SAR) + internal standard (G); 4, proline (PRO); 5, uracil (URA); 6, kynurenine (KYN). Adapted from [17]. Reprinted with permission.

This method was used to analyze levels of sarcosine and related metabolites in 126 patients with genitourinary malignancies (63 prostate cancers and 63 bladder cancers) and compare them with normal (n=68) urine samples. The results showed that these biomarkers cannot differentiate prostate cancer from normal or from other related cancers, such as bladder cancer. Nor can they predict the aggressiveness of a tumor because no significant difference was apparent in biomarker levels for T1 and T2 stages and Gleason scores of <7and ≥7. Correlations results showed that age or serum PSA levels do not influence these metabolite concentrations in urine. Their strong correlation with urinary creatinine concentrations implies that their occurrence is mainly due to renal excretion[24]. Jentzmik et al.(2010) analyzed only sarcosine levels in urine and reported [25] that, after a digital rectal examination, sarcosine in urine fails as a marker of prostate cancer or aggressive tumors.

Although ESI tandem mass spectrometry is widely used in biomarker analysis, it has limitations. Multiple-charge species analysis can be difficult because it requires interpretation and mathematical transformation. ESI is not good for uncharged, nonbasic, and low-polarity compounds such as steroids. It can be very sensitive to contaminants such as alkali metals or basic compounds. Finally, it gives relatively lower ion currents and requires more complex hardware than other ion sources [8].

2.3 Atmospheric Pressure Chemical and Photo Ionization Liquid Chromatography Tandem Mass Spectrometric (LC-APCI/APPI-MS) Studies

In atmospheric pressure chemical ionization (APCI), a corona discharge is used to ionize the analytes in the atmospheric pressure region. This gas-phase ionization in APCI is more effective than ESI for analyzing less-polar species. High flow rates typical of standard-bore HPLC can be used directly in APCI without diverting the larger fraction of volume to waste [8]. This approach is a soft ionization technique and excellent LC-MS interface compatible with MS/MS methods. In atmospheric pressure photo ionization (APPI), samples are ionized using ultraviolet light. This process yields highly sensitive ionization for low polarity compounds. Adding a compound called a dopant can increase APPI sensitivity because dopants have lower ionization energy than the analytical target [104].

Androgen deprivation therapy is a widely used in the treatment of advanced prostate cancer; Therefore, quantitative determination of tissue androgen levels is important to understand the mechanism of prostate cancer recurrence during therapy [26]. Although LC/ESI/MS/MS is quite sensitive, it cannot accurately measure tissue androgens in small samples (e.g., prostate biopsies and micro-dissected radical prostectomy specimens). APPI [27] has been reported to increase the sensitivity of unsaturated keto-steroids. Several groups have used APPI to identify and quantify steroids [28], but none have included dihydrotestosterone (DHT) (Figure 1) as an analyte, perhaps due to its poor yield of DHT $[M + H]^+$ ions from the APPI [26]. The exclusion of analytes limits APPI as a tool for comprehensive steroid quantification [26]. Lih et al. reported a method to quantify seven intracellular androgens including DHT [26]. They obtained the required sensitivity for DHT and 5α-androstanedione (5α-ASD)

by selecting a novel $[M + 15]^+$ parent ion. They have hypothesized that the source for this $[M + 15]^+$ ion is the reaction of the keto-sterane in the APPI source with methanol from the mobile phase [26].

Table 1. Instrument parameters and limits of detection (S/N > 3) for ESI and APPI of targeted androgens. Adapted from [26]. Reprinted with permission.

Compound	rT (min)	Ionization	LOD (pg)	Parent	Fragment	DP (V)[a]	FP (V)[b]	CE (V)[c]
ASD	7.4	ESI	1	287.2	97	28	135	32.5
		APPI	0.5	287.2	97	28	115	32.5
T	8.3	ESI	1	289.2	97	28	135	33
		APPI	0.5	289.2	97	28	115	33
5α-diol	8.3	ESI	20	308.2	273.2	28	160	11.5
		APPI	5	273.2	255.2	20	100	21
DHEA	9.1	ESI	40	289.2	271.2	31	155	14
		APPI	10	253.2[d]	197.1[d]	30	100	30
5α-ASD	9.8	ESI	10	289.2	271.2	34	160	17
		APPI	20	303.2	253.2	26	120	25
DHT	10.3	ESI	3	291.2	255.2	42	175	22
		APPI	5	305.2	255.2	29	135	24
AND	11.6	ESI	4	291.2	255.2	22	110	20
		APPI	2	273.2	255.2	21	85	20

[a] DP = declustering potential; [b] FP = focusing potential; [c] CE = collision energy; [d] Although $[M+H-H_2O]^+$ was the most abundant ion formed by DHEA, $[M+H-2(H_2O)]^+$ was found to be a preferable parent ion for SRM quantitation due to the high yield of the m/z 197 fragment.

According to their theory, a nucleophilic attack of the keto oxygen by methanol would result in a hemiketal. The loss of H_2O from this hemiketal leads to a protonated unsaturated enol ether. The enol ether undergoes a retro-Diels-Alder reaction to form; fragment m/z 85 $(M+H)^+$ in methanol; or m/z 88 $(M+H)^+$ in trideuterated [26]. The shifting of this m/z 85 fragment ion with the change in mobile phase, indicates that it contains the modified portion of the parent molecule. Table 1 compares detection limits for APPI and ESI sources at a signal-to-noise ratio greater than 3. Figure 4 shows comparison of selected reaction monitoring analyses of T and DHT in ESI and APPI ionization.

Another study investigated androgen deprivation therapy (ADT), and more specifically a liquid chromatography–electron capture atmospheric pressure chemical ionization-mass spectrometric (LC-ECAPCI-MS) method to determine the level of androstenediol in prostatic tissue [9]. Several cell lines studies have reported that adiol is a strong activator of the androgen receptor, and some anti-androgens fail to block completely its androgenic activity[29]. Even after ADT, adiol can cause the growth of prostate cancer, and the influence of ADT on adiol is poorly understood. Adiol shows lack of sensitivity in ESI or APCI analysis due to its low proton-affinitive properties[29].

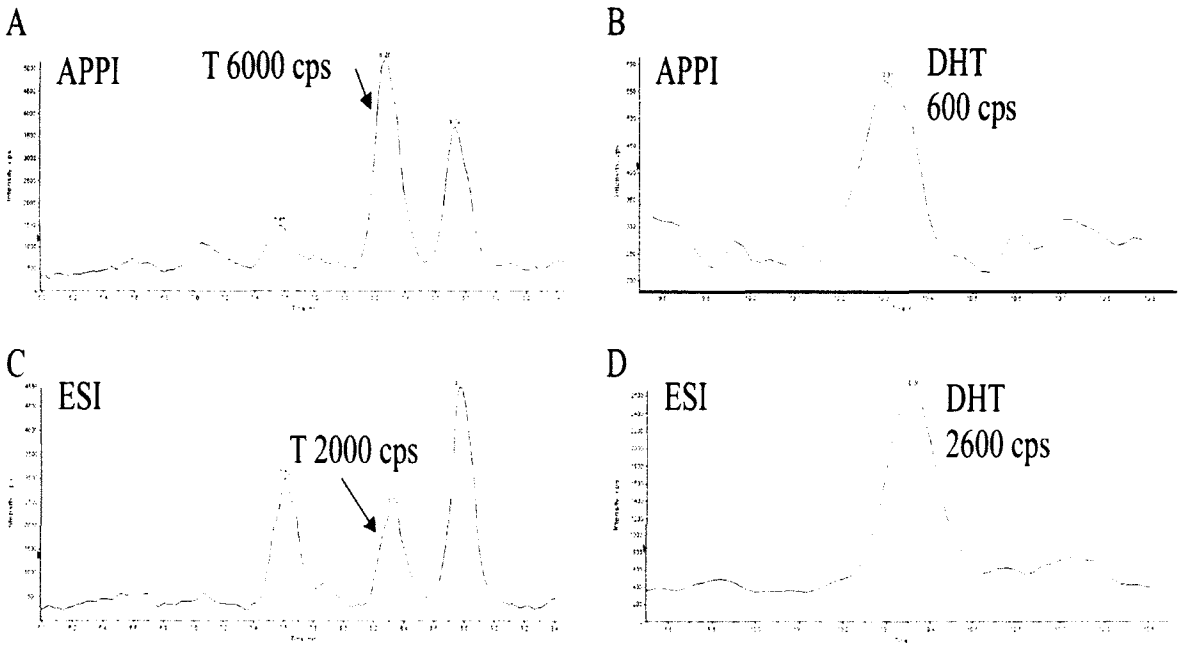


Figure 4. Selected reaction monitoring analyses of T and DHT using APPI (A and B) or ESI (C and D), respectively (cps) counts per second. Adapted from [26]. Reprinted with permission.

The electron capture APCI (ECAPCI)-MS [29] used is a highly sensitive technique for electron-affinitive compounds, and it uses a commercial APCI interface operating in the negative-ion mode. Here Adiol was derivatized with 4-nitrobenzoyl chloride and the detection response of the derivative was increased to a level 150 times greater than that of intact adiol[29]. This clinical study found that ADT cannot decrease the prostatic adiol level[29].

3. CAPILLARY ELECTROPHORESIS MASS SPECTROMETRY (CE-MS)

In capillary electrophoresis (CE) or capillary zone electrophoresis (CZE), species separate in the interior of a small capillary filled with an electrolyte, based on their size to charge ratio. Analyte migration is initiated by high voltage electric field that is applied between source and destination vials. All ions regardless of their charges are pulled through the capillary in the same direction by electroosmotic flow. Analytes are separated according to their electrophoretic mobility and detected near the outlet end [101]. Detection methods such as ultra violet (UV) , UV visible, fluoresce; mass spectrometry and surface enhanced raman spectroscopy widely use in capillary electrophoresis [101]. Compared to HPLC, CZE offers many advantages including stable constant flow, no gradient, fairly robust and inexpensive capillaries, compatibility with essentially all buffers and analytes, fast separation, and high resolution [30] .

In CE-MS, at the inlet one electrode can easily be interfaced with the capillary using the buffer. But the other electrode at the outlet is more difficult because it has to serve as the interface to the MS [30]. Several methods such as sheath-flow coupling, used to overcome this problem [30]. Several ionization techniques such as matrix assisted laser desorption ionization (MALDI), inductively coupled plasma (ICP) ionization, flow fast atom bombardment (FAB) have been used on CE-MS. CE-MS coupling became possible with the introduction of electrospray ionization-mass spectrometry [30] which is the most widely used ionization technique. In contrast to ESI, multiply charged ions are seldom observed in MALDI and FAB techniques. So the macromolecules with a molecular mass of a few thousand Dalton are usually detected as singly charged even-electron ions. Unlike in the other detectors, in CE-MS effluents from CE must physically transport to

the MS without sacrificing separation efficiency. Liquid-liquid junction interfaces, sheath-liquid interfaces, and sheathless interfaces are used for this purpose[24]. Electrospray optimum flow rate normally higher than the CE flow rate. Additional fluid or make-up-flow is added coaxially to overcome this problem[24]. Nonvolatile buffers such as phosphate and borate used in CE, cannot use in CE-MS because they adversely affect the MS performance. Hence, volatile buffers such as formic acid, ammonium formate, ammonium acetate used in CE-MS[24]. Interaction of the analytes with inner wall of the capillary is a major problem in CE. To overcome this problem coating which can covalently bonded to the capillary wall that can bear positive, neutral, or negative charges is done[24].

3.1 Prostate Cancer Biomarkers detection by CE-MS

Although the publications in prostate cancer detection by CE-MS are very limited, there are few articles published in this area during the past several years. Theodorescu et al. (2005) described a CE-MS method to analyse of several hundred urinary polypeptides within approximately 60 min in a small volume of urine with a high sensitivity [31] . This method has been used to identify new potential biomarkers of Prostate Cancer. First high abundance proteins were removed from urine and filtrate was applied on to RP-C2 column to remove urea, electrolytes, and salts, decrease matrix effects, and to enrich the polypeptides present [31]. The MS analysis was performed in ESI-TOF positive mode. The sheath liquid, containing 30% v/v iso-propanol and 0.4% v/v formic acid in HPLC-grade water, was applied coaxially. Here MS/MS analysis was performed by an Ultraflex MALDI TOF/TOF instrument (Bruker) [31]. The entire CE run was spotted onto a MALDI target plate using a Probot microfraction collector (Dionex) by depositing one

spot every 15 s, to retain migration time information. Importance of sample cleanup procedures was described earlier in this paper. In this specific case, albumin found in high concentrations in samples from patients with severe renal disease and elderly people [31]. This analysis resulted a prominent albumin peak (Figure 5A) and almost no additional polypeptides. Also this high albumin resulted co-elution of polypeptides and clogging of capillaries. So albumin was removed by ultracentrifugation (Figure 5B). The migration time was normalized because of its variability due to different amounts of analytes and ions in the samples. The time normalization was done by using an array of 200 frequently found urinary "anchor" polypeptides [31]. This study reveals that several polypeptides enable a correct classification of the prostate cancer with 92% sensitivity and 96% specificity [31].

Based on their pilot study, Theodorescu et al.(2008) have conducted another CE-MS study which was performed on a P/ACE MDQ CE (Beckman) system coupled on-line to a Micro-TOF MS [32]to validate the findings in a blinded prospective way. They have identified 12 new peptide biomarkers for prostate cancer. But, the defined biomarkers failed to distinguish between malign and benign samples with sufficient accuracy[32]. They hypothesized that absence of prostatic fluid in several of the collected midstream urine samples caused this false negative results.

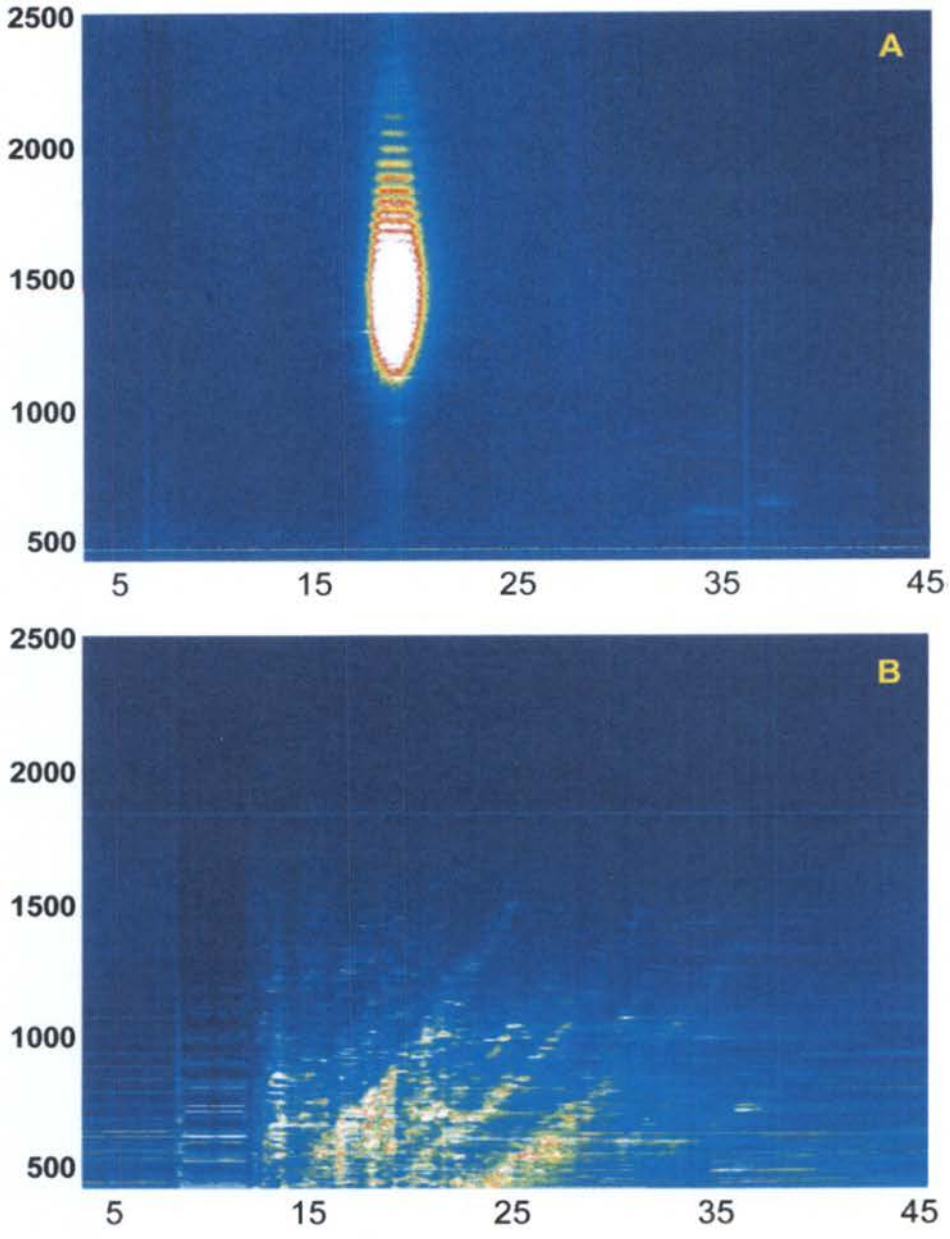


Figure 5. Raw data plot of CE-MS data from human urine obtained from a patient suffering from membraneous glomerulonephritis (MGN). Data obtained with the original sample preparation protocol are shown in A. Massive signal from albumin can be observed and only ca . 150 additional polypeptides are detectable. In B the same sample prepared using ultrafiltration is shown. Here approximately 1400 polypeptides could be detected. Evidently, the elimination of albumin results in the detection of .1000 additional polypeptides in this sample. Adapted from [31]. Reprinted with permission.

Void urine samples (n=86) and midstream urine control samples (n=138) were compared to define polypeptides that were present in the first void urine samples, but not in the midstream. Also 46 female urine samples were analyzed to exclude any non-seminal or prostatic fluid specific polypeptides[32]. In conclusion, they have discovered a panel of 12 urinary peptides present in initial voided urine, in combination with age and PSA acts as a predictor for the presence of prostate cancer. This may give guidance to patients and clinicians to perform additional evaluation[32].

Another study conducted by Coon et al. (2008) compared CE-ESI-TOF-MS electropherograms of urine samples from healthy volunteers to those from patients with several diseases, including prostate cancer. It showed statistically significant variation of peptides in prostate cancer, compared to the normal [33]. PSA in blood can bind to different protein inhibitors such as serine proteases, and can be found in serum as free or complexed PSA forms. This will increase the PSA heterogeneity, and Donohue et al. [34] (2005) studied PSA heterogeneity by CE in amine-modified electrolytes. Wittke et al. (2007) also discovered new proteomic biomarkers in urine, by CE-MS [35]. This study revealed that prostate and urothelial carcinoma can be detected by using disease-specific polypeptide patterns [35].

Table 2. Application of LC-MS and CE-MS techniques on prostate cancer biomarker investigation.

Separation Method	Ionization	Detection	Sample	Biomarker	Observation	Reference
LC	ESI	MS/MS	Urine	8-oxodG	Levels of 8-oxodG were not altered by tea polyphenols. In TRAMP mice, urinary 8-oxodG levels increased.	[8]
LC	MALDI	MS	Serum	Alpha-1-glycoprotein	–	[36]
LC	APPI	MS/MS	Tissue/Cells	Androgen	_	[26]
LC	Electron Capture APCI	MS/MS	Tissue	Adiol	Prostatic adiol level was not changed by androgen deprivation therapy.	[9]
LC	ESI	MS	Serum	Adiol-3S DHEA-S	_	[37]
LC	ESI	MS/MS	Cells	15-HETE	_	[11]
LC	ESI	MS/MS	Cells	Proteins	Concentrations of a novel candidate biomarker Mac-2BP were increased.	[18]
LC	ESI	MS/MS	Urine	Endogenous corticosteroids	_	[38]
LC	ESI	MS	Cells	Cyclooxygenase metabolites of arachidonic acid	–	[10]
LC	ESI	MS/MS	Plasma/Serum	ITIH4	_	[39]
LC	ESI	MS	Cells	limonin17-*b*-D-glucopyranoside	_	[21]
LC	ESI	MS/MS	Cells	Phosphoproteome	_	[13]
LC	ESI	MS	Cells	Glycans	Sensitive and specific method was developed to analyze *O*-glycosylation of MUC1	[19]
LC	ESI	MS/MS	Urine	Sarcosine/Metabolites	Method development	[17]
CE	ESI	MS	Urine	Peptides	Several peptides identified in cancer.	[31-32]
CE	ESI	MS	Urine	Peptides	Significant variation of peptides in prostate cancer.	[33]
CE	ESI	MS	Urine	Peptides	Found PCa specific polypeptide patterns	[35]

4. CONCLUSIONS AND FUTURE PERSPECTIVE

Mass spectrometry is a powerful tool in modern bioanalysis. Its high sensitivity and selectivity helps to determine or confirm known and unknown compounds based on the molecular weight. Mass spectrometry can be easily coupled with liquid chromatography compared to the capillary electrophoresis. Modern LC-MS does not depend on chromatographic resolution, thus co-eluting peaks can be easily identify by mass selectivity. LC/MS permits rapid method development and its sample matrix adaptability decreases the sample preparation time. Thus it is a promising tool in biomarker discovery.

In this review article we have focused on prostate cancer, which is a hormonally mediated tumor that develops in the prostate gland of the male reproductive system. It is one of the most common cancer types in men and is the second leading cause of cancer death in the United States and Canada. When we consider the limitations associated with current prostate cancer diagnosis methods, discovering new biomarkers will help a lot in early diagnosis and monitoring. Hence we have focused on applications of LC-MS and CE-MS techniques in prostate cancer biomarker detection. Table 2 summarizes the applications of LC-MS and CE-MS techniques.

Either LC/MS or CE/MS, sample preparation is an important step in biomarker discovery. Most cases SPE or liquid-liquid extraction is used to extract the biomarkers present in minute amounts. In protein biomarker analysis, high-abundance proteins always mask the low-abundance proteins, thus, removal of high abundance proteins is needed. Affinity removal spin cartridges or molecular weight cutoff ultracentrifugation are used for this. In MS various methods are used to ionize the analytes. This results in

the formation of charged particles or ions. This paper discussed the applications of ionization techniques such as ESI, APCI and APPI in the prostate cancer biomarker discovery. Relative to the other methods ESI is widely used because it is good for charged, polar or basic compounds. Using this m/z less than 3000 can be easily detected and give low chemical background. Complementary to ESI, APCI is a good method for uncharged, non-basic, low polar compounds such as steroids. APPI used UV light for ionization, and it is also a high sensitive ionization technique for low polarity compounds.

Publications about prostate cancer biomarker detection by CE-MS are very limited compared to LC-MS. Most of published papers based on polypeptide biomarkers for prostate cancer. In CE-MS, ESI is the most widely used ionization technique. Coupling of CE with MS is a challenge, and many techniques have been tried to achieve this. Sensitivity improvement can be obtained by using sheath-less interfaces, because they do not dilute the samples. Low sample, buffer consumption, and high resolution are the main advantages of CE-MS.

Altogether, proteomic biomarkers have been studied extensively compared to metabolomic or genomic studies. In future scientist will focus more on the metabolomic studies. Also they should explore more on CE-MS applications for prostate cancer biomarker discovery, because there are limited number of studies have been done.

EXECUTIVE SUMMARY

- A validated set of biomarkers is an urgent requirement for diagnose prostate cancer at an early stage.
- MS coupled with LC and CE widely is used for biomarker analysis.
- LC-MS is a powerful tool because of its high sensitivity, selectivity and it does not depend on chromatographic separation.
- In sample preparation SPE is widely used to extract biomarkers and molecular weight cutoff filters used to remove high abundance proteins which will mask the low abundance ones.
- ESI, APCI and APPI are widely used ionization methods in MS, ESI is good for charged polar compounds and APCI, APPI used for uncharged polar compounds.
- Publications in prostate cancer biomarker detection by CE-MS are very limited.

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SECTION

2. CONCLUSIONS

The studies included in this dissertation utilize different analytical techniques such as CE-LIF and LC/MS/MS to investigate and validate chosen biomarkers for non-invasive diagnosis of cancer. In the study, given in the first paper, eight pteridine molecules in urine were analyzed, and among those eight, five pteridines were elevated in cancer urine samples compared to the cancer-free urine samples. In the study given in the second paper, the levels of proline, kynurenine, uracil, and glycerol-3-phosphate in urine were analyzed using LC/MS/MS. The statistical analyses showed that these biomarkers were not reliable enough for prostate cancer detection or for differentiating the aggressiveness of prostate cancer. In the study given in the third paper, a validated, reliable, fast, and simple HPLC-MS/MS method was developed to simultaneously separate and detect potential urinary bladder cancer biomarkers such as taurine, L-phenylalanine, hippuric acid and creatinine in urine samples. The final paper is a review article that gives a detailed discussion about the applications of different LC-MS/MS and CE-MS techniques used in prostate biomarker discovery. In conclusion, the elevated concentrations of certain metabolites in human urine can indicate the current or future behavior of cancer but detailed clinical studies are necessary to validate them as reliable biomarkers.

VITA

Sanjeewa B. Gamagedara was born in Kandy, Sri Lanka. In 2002, he joined the Faculty of Science, University of Peradeniya, Sri Lanka. In 2006, he graduated with a Bachelor of Science (Honors) degree in Chemistry. From 2006 to 2007 he worked as a temporary assistant lecturer at the Department of Chemistry, University of Peradeniya. Also, he has completed an advanced diploma in Information Technology at Sri Lanka Institute of Information Technology, Colombo.

In 2007, Sanjeewa joined Dr. Yinfa Ma's research group at Missouri University of Science and Technology, Rolla, Missouri for his PhD in Analytical Chemistry. During his time as a graduate student, he worked both as a graduate research assistant and a graduate teaching assistant at the chemistry department. His research was mainly focused on investigating biomarkers for noninvasive early cancer diagnosis. During his graduate studies, he won several awards including John W. Claypool Award for Biomedical Research, and Marilyn and Richard Vitek Graduate Fellowship for Analytical Chemistry. His PhD in Chemistry was awarded in May, 2012.