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Quantitative analysis of co-enzyme a in a plant tissues and cancer biomarkers in urine samples by using capillary electrophoresis and liquid chromatography-mass spectrometry

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QUANTITATIVE ANALYSIS OF CO-ENZYME A IN PLANT TISSUES AND CANCER BIOMARKERS IN URINE SAMPLES BY USING CAPILLARY ELECTROPHORESIS AND LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

by

YONGQING JIANG

A DISSERTATION

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In Partial Fulfillment of the Requirements for the Degree

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Approved by

Dr. Yinf a Ma, Advisor
Dr. Philip Whitefield
Dr. Nuran Ercal
Dr. Paul Nam
Dr. Roger Brown
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ABSTRACT

Despite continuous effort and progress in cancer detection and therapy, cancer remains one of our greatest health concerns because of its low survival rate and rapid incidence increase. One reason for this is the late detection and therefore pre-cancer diagnosis is crucial. In pre-cancer studies, cancer biomarkers become significant because of the useful information it contains such as possible cancer type and stage. Separation and detection technique plays an important role in cancer biomarker identification.

Coenzyme A (CoA) facilitates more than 100 chemical reactions in cells. Because of its low abundance accumulated in cells, it is critical to develop a sensitive method to detect CoA compounds in biological samples in order to study it as a cofactor.

The valuable characteristics of capillary electrophoresis (CE), such as rapid analysis, high separation efficiency and minimal consumption of sample and buffer solutions, make it a unique analytical technique. The combination of the high specificity and sensitivity of tandem mass spectrometry (MS/MS) with the high separation ability of high performance liquid chromatography (HPLC) facilitates the sensitive and specific analysis in complex matrices like urine and blood. In this body of work, new methods were developed using CE-UV, CE-LIF and HPLC-MS/MS to determine modified nucleosides, CoAs, and sarcosine, proline, kynurenine, uracil and glycerol-3-phosphate. These methods were applied to measure the above mentioned analytes in either urine samples or cell extracts and proved to be simple, fast, reliable and powerful. Based on these newly developed methods, cancer biomarker screening is undertaken and new biomarkers will be identified.
ACKNOWLEDGMENTS

The work presented in this doctoral dissertation would never have been possible without the continual support and guidance of my advisor, Dr. Yinfa Ma. His direction in all aspects of my graduate work was invaluable and will be forever appreciated.

I would also like to thank my doctoral committee members, Dr. Philip Whitefield, Dr. Nuran Ercal, Dr. Paul Nam and Dr. Roger Brown, for the various ways in which they assisted me in my work and study.

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I would like to thank Joseph Council for his great help in capillary electrophoresis instrument maintenance and troubleshooting. I would also like to give my thank to chemistry department secretaries, Carol Rodman, Donna Riggs and Kathy Eudaly, who were constantly to help and provide timely information. I would like to thank my group members for their help.

Finally, I would like to thank my family, especially my parents and my husband, for their untiring support and encouragement, which took shape in so many ways. I thank my three-year old son for the vitality he brought to my life. Loves from family were and will always be strength to me.
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1. INTRODUCTION

1.1. EARLY CANCER DETECTION

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems and put high risk for human life. Figure 1.1 illustrates what happens when normal cells become cancer cells\(^1\). The body is made up of many types of cells. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. When cells become old or damaged, they die and are replaced with new cells. However, sometimes this orderly process goes wrong. The genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division. When this happens, cells do not die when they should and new cells form when the body does not need them. The extra cells may form a mass of tissue called tumor and malignant tumor is cancer. Cancer is a serious health problem because cancer cells can spread to distant parts of the body. For example, a melanoma (a cancer of pigmented cells) arising in the skin can have cells that enter the bloodstream and spread to distant organs such as the liver or brain\(^1\). Melanoma cells growing in the brain or liver can disrupt the functions of these vital organs and so is potentially life threatening.

Despite continuous effort and progress in cancer detection and therapy, cancer remains a significant health problem with a very low 5-year survival rate and a rapid increase in its incidence. For example, there are around 11 million new cancer cases and about 397,700 cancer-related deaths reported in United States in 2009\(^1,2\). Cancer is the
second leading cause of death in USA. One reason for the high incidence and low survival is the easier and more frequent exposure to carcinogens (cancer-causing agents) which is responsible for triggering most human cancers. Although scientists don't know all the reasons yet, many of the causes of cancer have already been identified. Besides intrinsic factors such as heredity, diet, and hormones, scientific studies point to key extrinsic factors that contribute to the cancer's development: chemicals (e.g., smoking, alcohol, and industry pollution), radiation, and viruses or bacteria. Failing to take appropriate steps to avoid these agents increases the cancer risk. Another reason for the poor survival is that many cancers are detected late, often after they have metastasized to distant sites. Once a cancer has spread, it is more difficult to eradicate. For many cancers, there is no any symptom at its early stage. Patients visit the doctor only when they feel pain or when they notice changes such as a lump in the breast or unusual bleeding or discharge. Sometimes with these very apparent symptoms, the cancer is already in its late stage and survival rate is very low. Therefore, early cancer screening even though without any symptom is crucial.

For many cases, successful prevention depends on the accurate evaluation of risk, and successful treatment depends on early detection. For example, the 5-year survival for colorectal cancer is greater than 90% if it is detected while it is still localized, 30-50% if detected with local lymph node involvement and only 10% if it has metastasized to distant sites. Finding cancer early may decrease a person's risk of dying from the cancer. Consequently, many oncologists and cancer biologists are working to develop methods that detect cancers at their early stages of development. Developing and improving methods for early cancer detection is currently a high priority for cancer researchers.
1.2. BIOMARKERS

For many decades, the microscopy of biopsied samples has continued to be the mainstay of definitive cancer diagnostic technique. However, this technique suffers from intra-observational subjectivity. Also, detection of many cancers at the microscopic level is often too late for successful intervention. Therefore, despite numerous technical innovations in the quality of microscopic imagery, we are still limited in our ability to detect cancer in their earliest stage of formation. After decades of basic research in attempting to unravel the underlying cellular and molecular mechanisms of cancer, the scientific community has uncovered novel candidate targets for the early cancer detection. By the time a tumor is detected, several biological changes have already
occurred. By reading these changes accurately, early detection and diagnosis of individual cancers can be improved. Therefore, diagnostic assays to detect the changes using biomarkers have considerable potential for early detection.

Normally, biomarkers are defined as cellular, biochemical and molecular alterations by which normal, abnormal or simply biological processes can be recognized or monitored. These alterations should objectively measure and evaluate normal biological process, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In cancer research and detection, a biomarker refers to a substance or process that is indicative of the presence of cancer in the body. It may be a molecule secreted by a malignancy itself, or it can be a specific response of the body to the presence of cancer. Gene mutations, alterations in gene transcription and translation, and alterations in their protein products can all potentially serve as specific biomarkers for disease. The characteristics of an ideal biomarker have been described as follows: (i) specific for the malignant process; (ii) tumor type specific; (iii) readily detectable in body fluids and tissue extracts; (iv) detectable early in the course of disease before the disease is clinically evident; (v) indicative of the overall tumor cell burden; (vi) indicative of the presence of micrometastases; and (vii) predictive of relapse. Biomarkers serve as hallmarks for the physiological status of a cell at a given time and change during the disease process. They are important tools for cancer detection and monitoring.

Cancer biomarker discovery has been and continues to be an active and productive area of research and its practitioners are using ever-more sophisticated and innovative technologies. Methods used for discovery frequently cast a wide net to allow for the identification of new biomarkers. In the discovery phase, investigators typically
identify genetic mutations in cancerous cells or compare gene or protein expression levels in cancer and normal tissues to identify biomarkers that are either elevated or depressed in cancerous tissues. Alternatively, differences in biological samples such as cell extracts, sera, urine or sputum from control and cancer patients are investigated.

1.3. URINE SAMPLES

“There is perhaps no excretion of the human body which possesses more interest to the medical practitioner, and probably none which throws so strong a light on the organic processes of the diseased as well as the healthy body, as the urine.”

Examination of urine to determine a patient’s health status has a long history, being recorded as first practiced in 4000 B.C. by Sumerian and Babylonian physicians. Ancient clinicians detected glucose in the urine by tasting it or observing whether it attracted ants. The presence of albumin in the urine has been measured as an indicator of renal disease for centuries and in early times could be detected via the so-called “foam test” to determine whether albumin was present in the urine in large amounts. In the modern clinical laboratory, routine urinalysis is frequently used to observe the status of a patient’s kidney function, bacterial infection, glucose levels, and for a variety of other diagnostic reasons. Moreover, urine sample collecting is relatively cheap, easy and requires minimal amount of training. Unlike blood and tissue collecting, urine collecting is also noninvasive, which is important sometimes. But, it still gives vital objective information about the patients internal functioning. Although there is room to dispute whether urine, blood, cerebrospinal fluid, or another bodily fluid has the greatest value in
the diagnosis of diseases, there is no doubt that urine is an important biological matrix for
the determination of a patient’s physical state\textsuperscript{10}.

With the aid of modern analytical instrumentation and a solid foundation in
biochemistry, interest in the components of human urine as disease biomarker has greatly
expanded. Recently, more and more studies have been reported in the area of
determination and screening of cancer biomarkers using compounds in urine sample,
including nucleosides, ribonucleic acid (RNA), 8-hydroxydeoxyguanosine, DNA
mutation, DNA-adduct, glycans, proteins, glycoproteins, and small biomolecules\textsuperscript{11}. The
latest developments and advances were focused on instrumental, methodical and data
analysis of different cancer biomarkers.

1.4. TECHNIQUES USED TO DETECT CANCER BIOMARKERS

Many different methods have been developed for screening cancer biomarkers
from saliva, sputum, serum, urine and tissues, such as high-performance liquid
chromatography- mass spectrometry (HPLC-MS) and gas chromatography- mass
spectrometry (GC-MS)\textsuperscript{12,13}, matrix assisted laser desorption ionization-mass
spectrometry (MALDI-MS)\textsuperscript{14-16}, two-dimensional polyacrylamide gel electrophoresis (2-
D PAGE)\textsuperscript{17-21}, surface-enhanced laser desorption ionization time of flight-mass
spectrometry (SELDI-TOF-MS)\textsuperscript{22-24}, and so on. These sensitive techniques have been
extensively used to identify disease biomarkers in human biofluid, and great amount of
information has been obtained. For example, Petricoin et al.\textsuperscript{25} have used the proteomic
patterns in serum samples from the SELDI-MS spectrum to identify ovarian cancers.
After studying of 50 ovarian cancer cases and 66 nonmalignant cases, their study results yielded a sensitivity of 100%, specificity of 95%, and positive predictive value of 94%. This technique could be effectively used for early-stage ovarian cancer screening. The applications of SELDI-TOF-MS for diagnostic proteomics have been recently reviewed by Issaq et al.\textsuperscript{23}. This review has briefly covered the fundamental principles of SELDI-TOF-MS, sample preparation, protein identifications, diagnostic applications for diseases including cancers, and other biological applications.

Capillary electrophoresis (CE) is a highly efficient analytical technique that has had a great impact in biomedical research and clinical and forensic practices in the last decade\textsuperscript{26-28}. CE has been coupled to different detection methods based on the nature of analytes\textsuperscript{29}, including ultraviolet-visible (UV) absorption, conductimetry, MS, patch clamp, electrochemical (EC) detection, and laser-induced fluorescence (LIF). With these versatile methods of detection, CE has been quite capable in studying a variety of analytes from smaller molecules (inorganic ions and organic molecules) to larger biomolecules (DNA and proteins). Compared to other sensitive analytical techniques such as HPLC and GC, which have been extensively used for analysis of biologically active substances in clinical routines, CE holds a number of distinct advantages: (i) A very small sample volume is needed for a single run (nL), which makes CE an ideal analytical technique for applications that require analysis of low nanoliter samples and subfemtomole quantities. Researchers have downscaled the sample volume for CE analysis to less than picoliters, but the mass detection limits remain in the zeptomole range\textsuperscript{30,31}. Recently, CE has been reportedly used in single cell analysis\textsuperscript{32,33} and even subcellular-level analysis\textsuperscript{34}. (ii) CE’s waste is safer for the environment than that from
HPLC, which produces large amounts of organic waste, or than that from GC, which produces volatile air pollutants\textsuperscript{27}. Other advantages are rapid analysis, great resolution, low cost, etc. All of these advantages designate CE as almost ideal for the analysis of numerous endogenous and exogenous substances present in biological fluids\textsuperscript{35,36}.

During the past ten years, high performance liquid chromatography-mass spectrometry-mass spectrometry (HPLC-MS/MS) has become one of the fastest growing analytical techniques for the determination of trace level compounds. This technique involves using two mass spectrometers, in tandem, as the detector for an HPLC. The ion fragments from the first mass spectrometer (parent ions) are accelerated into the second mass spectrometer, where secondary ionization occurs (daughter ions). This results in a highly sensitive and highly selective detection. Tandem mass combined with the separation power of HPLC minimizes interferences in the complex matrix and has revolutionized the way we do chemical analysis today. HPLC-MS/MS offers a revolution in the ability to identify compounds which are undetectable by other technologies. HPLC coupled with tandem mass has been applied in a wide range of research areas, including food safety, environmental protection, and pharmaceutical development.

1.5. THIS DISSERTATION

Different biomarkers have different functions in biological systems, but they each have their own special characteristics. Monitoring the concentrations of biomarkers in urine or cell extract is the easiest way to observe the clinical significance of a cancer patient’s status at regular intervals, and still be capable of predicting tumor formation and
relapse. In this dissertation, modified nucleosides, sarcosine and related metabolites were studied as the potential cancer biomarkers.

Nucleosides are primary constituents of ribonucleic acids (RNAs). When RNAs are biotransformed, the normal nucleosides can either be metabolized or reutilized to synthesize nucleic acid. However, in particular cases, some RNAs are transformed into modified nucleosides which can neither be further degraded nor reutilized, but are excreted intact in urine as end products due to a lack of specific phosphorylases. In cancer disease where cell proliferation takes place, RNA metabolism increases dramatically and higher concentrations of excreted modified nucleosides will be observed. Consequently, the levels of modified nucleosides in urine can reflect RNA degradation in the organism and so they can be used as potential cancer biomarkers. One example of the possible pathway from normal nucleoside (A) to modified nucleosides (m1A, I, m6A, m1I, m6,6 A) is shown in Figure 1.

Mapping the differential metabolomic profiles to their respective biochemical pathways as outlined in the Kyoto Encyclopedia of Genes and Genomes (KEGG, release 41.1, http://www.genome.jp/kegg) revealed an increase in amino acid metabolism and nitrogen breakdown pathways during cancer progression to metastatic disease. A similar enrichment network of amino acid metabolism was also identified by the bioinformatics tool Oncomine Concept Map4, 5(OCM, http://www.oncomine.org, P56310213), which is shown in Figure 1. Additionally, OCM found strong enrichment for increased ‘methyltransferase activity’ among metabolites upregulated in metastatic samples. Because amino acid metabolism and methylation were enriched during prostate cancer
progression, we focused on five metabolites (sarcosine, proline, kynurine, uracil and glycerol-3-phosphate) as potential cancer biomarkers.

For the determination of different biomarkers, CE and HPLC-MS/MS will be a highly efficient analytical technique with great potential in biomarker researches due to
their high qualities mentioned above. Because these techniques’ history is relatively short compared to many other analytical techniques, much work needs to be done to make CE and HPLC-MS/MS be widely used in routine tests in various clinic laboratories. In this dissertation, new methods were developed based on modified nucleosides, sarcosine and related metabolites as cancer biomarkers using CE-UV/LIF and HPLC-MS/MS. These methods were proved to be simple, fast, reliable and powerful. They are very crucial in cancer biomarker identification and confirmation. Through large amount of biological samples analysis using these newly developed assays, new biomarkers can be identified and confirmed and more lives will be saved.

Figure 1.3. Oncomine concept analysis of metabolomic profiles of prostate cancer progression⁵⁹. Network view of the molecular concept analysis for the metabolomic profiles of “over-expressed in PCA signature” (blue node). Each node represents a molecular concept or a set of biologically related genes. The node size is proportional to the number of genes in the concept. Each edge represents a statistically significant enrichment (P < 1 X 10⁻⁴). (Enrichments with interconnected’amino acid metabolism concepts’, indicating increased amino acid metabolism in PCA versus benign, are indicated by red edges.
1. A Fast Capillary Electrophoresis Method for Separation and Quantification of Modified Nucleosides in Urinary Samples

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ABSTRACT

Modified nucleosides are formed at the post-transcriptional stage by chemical modification of normal nucleosides within the RNA. These modified nucleosides cannot be reutilized or further degraded, but they are excreted in the urine as intact molecules. The elevated levels of modified nucleosides in the urine samples have been served as potential cancer biomarkers in many studies. Even though different analytical techniques have been reported for determining nucleosides levels, they are practically difficult to be used as a routine tool for early cancer screening. In this paper, a novel method was developed to separate and quantify 10 nucleosides (adenosine, cytidine, guanosine, uridine, inosine, xanthosine, pseudouridine, N2-methylguanosine, 1- methyladenosine, N2, N2-dimethylguanosine) in urine samples by using capillary electrophoresis with UV detector at 254 nm. A 50 µm (i.d.) ×38cm (effective length) fused silica capillary was used for the separation and a borate-phosphate buffer containing 25 mM CTAB at pH 9.50 was used as a background electrolyte. The separation was carried out at 15 kV under reverse polarity and completed in less than 10 min. The linear range of the analytes was from 5.0 to 500 µmol/L and the detection of limit was lower than 2.0 µmol/L. The effects
of pH, buffer concentrations, CTAB concentration and the operation voltages on the separation and quantification of the modified nucleosides were also investigated. The technique, developed in this study, is much simpler and faster compared to previous studies and can be used for quantifying modified nucleosides in urine samples.

KEYWORDS

Modified nucleosides; cancer biomarker; capillary electrophoresis

INTRODUCTION

Nucleosides are primary constituents of ribonucleic acids (RNAs). When RNAs are biotransformed, the normal nucleosides can either be metabolized or reutilized to synthesize nucleic acid. However, in particular cases, some RNAs are transformed into modified nucleosides which can neither be further degraded nor reutilized, but are excreted intact in urine as end products due to a lack of specific phosphorylases. In cancer disease where cell proliferation takes place, RNA metabolism increases dramatically and higher concentrations of excreted modified nucleosides will be observed. Consequently, the levels of modified nucleosides in urine can reflect RNA degradation in the organism and so they can be used as potential cancer biomarkers.

Modified nucleosides in human urine as possible cancer biomarkers have been of interest since 1970s. Some studies reported that elevated levels of some nucleosides in
the urine samples can be served as potential cancer biomarkers and the urinary profile of modified and normal nucleosides have been widely studied as markers of leukemia\textsuperscript{40}, breast cancer\textsuperscript{41-43}, thyroid cancer\textsuperscript{11}, uterine cervical cancer\textsuperscript{44}, liver cancer\textsuperscript{45}, lung cancer\textsuperscript{46}, bladder cancer and colorectal cancer\textsuperscript{47,48}, head and neck cancer\textsuperscript{49}, and rheumatoid arthritis process\textsuperscript{50}. Profiles of urinary nucleosides could also be used in monitoring progress of the cancer disease and the response of individuals to an applied therapy\textsuperscript{51}. Nevertheless, no specific pattern has been discovered up to date. Searching for the specific biomarker for specific cancer is very crucial for early cancer diagnosis. In many cases, an efficient separation and determination technique is required to assess the levels of these biomarkers.

Different analytical techniques have been reported for separating and determining normal and modified nucleosides in urine samples, including immunoassays\textsuperscript{52}, high performance liquid chromatography (HPLC) and capillary electrophoresis conjugated with UV detection\textsuperscript{53-58}, photodiode-array detection\textsuperscript{55}, and mass spectrometry (MS)\textsuperscript{49,59-62}. However, there is no routine cancer diagnostic tool based on levels of urinary nucleosides in clinical laboratories. One reason is that there is no specific nucleoside as biomarker to a specific type of cancer. Another major reason is due to the lack of practicable analytical techniques to conveniently obtain levels of urinary nucleosides. Even though both Immunoassay and HPLC have been demonstrated for analysis of nucleosides, the procedures are tedious and the methods are time-consuming. In addition, HPLC consumes a large amount of organic solvents and the organic is not environmental friendly.
High-performance capillary electrophoresis (HPCE) has proven to be a rapid and simple technique for separating charged biomolecules with very high resolution. Its unique advantages, such as a relatively short time of analysis, high separation efficiency with consumption of minimal amount of sample and buffer solutions, make HPCE a valuable technique for determination of urinary nucleosides. Micellar electrokinetic chromatographic (MEKC) methods with sodium dodecyl sulfate (SDS)–borate–phosphate buffer have been applied for determination of urinary nucleosides by many researchers \(^{11,44,56,63}\). However, it was found that the method had a long separation time and the SDS in the background electrolyte (BGE) caused a poor reproducibility due to the frequent generation of air bubbles in the separation column. In this paper, we have developed a simple and fast HPCE method for the determination of urinary nucleosides using \(\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{NaH}_2\text{PO}_4\) as BGE and cetyltrimethylammonium bromide (CTAB) as BGE additive. The reproducibility and migration time were greatly improved. The optimized method has been used for analysis of 12 important urinary nucleosides from both normal and breast cancer-carrying subjects. The main goal of this study was to develop a fast and reproducible HPCE method to study modified nucleosides that present in urine samples from both cancer-carrying patients and healthy controls, so that it can be used to provide nucleoside profile information for potential early cancer screening.
MATERIALS AND METHODS

Chemicals

12 nucleoside standards, including adenosine, cytidine, guanosine, uridine, inosine, xanthosine, pseudouridine, $N^2$-methylguanosine, 1'-methyladenosine, $N^2,N^2$-dimethylguanosine, 8-hydroxy-2'-deoxyguanosine, 5-hydroxymethyl-2'-deoxyuridine, and creatinine (for normalization purpose) were purchased from Sigma–Aldrich (St Louis, MO, USA). All other chemicals used for the background electrolyte preparation and capillary rinsing, such as sodium phosphate, sodium tetraborate, CTAB, methanol, sodium hydroxide and hydrochloric acid, were also obtained from Sigma–Aldrich (St. Louis, MO, USA). Deionized water (18.2 MΩ) from Millipore Simplicity 185 - system (Millipore, MA, USA) was used to prepare standard solutions, BGE and other solutions.

Preparation of BGE

The BGE solution containing 25 mM Na$_2$B$_4$O$_7$, 25 mM NaH$_2$PO$_4$ and 25 mM CTAB was prepared with deionized water and the pH was adjusted to 9.50 by adding 1.0 M NaOH in the buffer before diluting it to the final volume. The buffer solution was filtered through the 0.45μm membrane filter before use.

Preparation of Standard Nucleoside Solutions

The 10 mM stock solutions of adenosine and xanthosine were prepared in 1.0 M NaOH and guanosine was in 50% formic acid. The 10 mM stock solutions of other nucleosides were prepared in deionized water. All stock solutions were kept at –20 ºC. The working nucleoside solutions were prepared by diluting the stock solutions with
deionized water to the concentration range of 1–5000 µM (1, 5, 10, 50, 100, 500, 1000 and 5000).

**Preparation of Urine Samples**

Spontaneous urine samples from one healthy adult and one breast cancer patient were collected from the Ellis Fischel cancer center (Columbia, MO). After collection, the samples were frozen and stored at −80 °C immediately. Before the analysis, the samples were thawed at room temperature. Each urine sample was prepared for both nucleosides and creatinine analysis.

Nucleosides were extracted from urine samples through affinity chromatography gel as described in the literature\textsuperscript{44}. Basically, each of 3 mL extraction (SPE) tube purchased from Supelco (Bellefonte, PA, USA) was packed with Affi-gel 601 (200 mg). The gel was conditioned by washing sequentially with 15 mL 0.1 M formic acid in 50% methanol and 0.25 M ammonium acetate (pH 8.6) prior to be used as phenylboronic acid (PBA) columns for SPE in affinity mode. 25% ammonia was added to urine samples to adjust pH to the range of 8.2 to 8.6 and then centrifuged. For the analysis of nucleosides, 1 mL supernatant was loaded to a preconditioned PBA column, followed by the addition of 0.5 mL 0.25 M ammonium acetate (pH 8.6) and standing for 5–10 min. The PBA column was then successively rinsed with 4.0 mL 0.25 M ammonium acetate (pH 8.6), 0.3 mL 50% methanol twice and 0.5 mL 0.1M formic acid in 50% methanol. The rinsed column was eluted with 3 mL 0.1M formic acid in 50% methanol. The eluent was evaporated to dryness in the Turbovap LV evaporator (Zymark, Hopkinton, MA, USA) at 50 °C, and then the residue was dissolved in 200 µL water for HPCE analysis. For the creatinine analysis, a method developed by Yufang Zheng etc. was used with minor
modification. Briefly, the urine supernatant was diluted 8-fold in deionized water and then injected to CE column for direct analysis.

**Instrumentation**

All CE experiments were carried out on a Beckman Coulter P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA, USA) equipped with a UV-absorbance detector. Electrophoretic data were acquired and analyzed by 32 Karat software version 4. Separations were performed in fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with 50µm (i.d.) × 38cm (effective length). New capillaries were conditioned by rinsing with methanol for 15 min, deionized water for 5 min, 1.0 M HCl for 5 min, followed by deionized water for 5 min again, then 1.0 M NaOH for 20 min and deionized water for 5 min. The capillary was rinsed with deionized water for 5 min and then pre-run with BGE for 20 min under -15 kV every morning to obtain the best reproducibility. Samples were injected into the capillary at 0.5 psi for 10 s. After each analysis, the capillary was rinsed successively with 0.1 M NaOH for 1.0 min, deionized water, and BGE for 2.0 min respectively. Nucleosides separation was carried out at -15 kV at 25 °C and the wavelength of the UV detector was set at 254 nm. For the analysis of creatinine, 30 mM phosphate at pH 6.0 was used as BGE and the separation was completed under 15KV with the detection wavelength at 214 nm. Other conditions were the same as those of nucleosides.
RESULTS AND DISCUSSION

Nucleosides are suitable to be analyzed by capillary electrophoresis, due to their negative charges in a wide pH range, diverse molecular weight, and hydrophilic property. The quality of the electrophoretic separation and the time required for this separation depend on a number of analytical parameters, such as running buffer composition and pH, buffer concentration and additives, the applied voltage, the length and diameter of the capillary, and the sample size introduced, and so on. All these conditions were investigated systematically in this study in order to get the optimum separation conditions. Figure 1 showed the separation of 12 nucleoside standards under optimal conditions by using HPCE. The running buffer was composed of 25 mM Na$_2$B$_4$O$_7$ + 25 mM NaH$_2$PO$_4$+ 25 mM CTAB (pH=9.50) and the separation voltage was -15 kV with UV detection at 254 nm.

Effect of Buffer Concentration

Tetraborate, which can form complex network with hydroxyl group, has been used to improve the separation of catechols, carbohydrates and nucleosides. After preliminary experiments, an electrolyte containing tetraborate, phosphate and CTAB was selected as the running buffer. Five different tetraborate concentrations (15, 25, 50, 75 and 100 mM) were examined to compare the separation efficiency and peak-to-peak resolutions of 12 nucleosides. With the increase of the tetraborate concentration above 25mM, the migration time became longer and longer without any improvement of the nucleoside separations. When the concentration was decreased to 15mM, the resolution was getting worse and several peaks merged together and cannot be separated.
Figure 1. Electropherogram of 12 nucleoside standards at optimized conditions by HPCE with UV detection at 254nm. Capillary: 50µm (i.d.) × 48cm (38 cm to detection window); Applied voltage: -15KV; Temperature: 25°C; Sample injection: 0.5psi for 10s; Running buffer: 25 mM Na2B4O7 + 25 mM NaH2PO4+ 25 mM CTAB, pH=9.50; Peak identification: 1, adenosine (A), 2, cytidine (C), 3, guanosine (G), 4, uridine (U), 5, inosine (I), 6, xanthosine (X), 7, pseudouridine (Pseu), 8, N2-methylguanosine (m2G), 9, N1-methyladenosine (m1A), 10, 8-hydroxy-2’-deoxyguanosine, 11, 5-hydroxymethyl-2’-deoxyuridine, 12, N2,N2-dimethylguanosine (m22G).

Meanwhile, the current went up significantly at higher buffer concentrations and the Joule heating generated affected the separation. Therefore, the optimized tetraborate concentration was maintained at 25 mM. Phosphate was used as co-ion because of its UV transparency at 254 nm. After a series of experimental studies, 25mM was chosen as the optimal concentration. Under this condition, the current was about 60 µA at 25°C, which did not produce significant amount of Joule heating. The running buffers were replaced every 12 runs to keep the ionic strength balanced on both sides of the running buffer, so that reproducible data can be obtained.
Effect of Buffer pHs

The most crucial parameter for the nucleoside separation by using HPCE is the pH of the running buffer. It not only affects the solute charge, but also influences the capillary wall surface and will cause a concomitant change in electroosmotic flow (EOF). Most nucleosides, with cis-diol structures, will bind with boric acid at high pHs and form negatively charged complex. Therefore, four different pH values (9.02, 9.32, 9.50 and 9.78) were examined for the separation of 12 nucleosides. The results were shown in Figure 2. It was clearly shown that the HPCE separation of nucleosides was very sensitive to pHs. Under pH 9.02, pseu and U, X and C, and G and A coeluted with each other, respectively. In addition, m2^G coeluted with system peak. As pH increased to 9.32 or above, m2^G eluted after the system peak. With the increase of the pH to 9.50,

![Figure 2. The pH effect on the separation of 12 nucleoside standards. The experimental conditions were the same as those of Figure 1, except for the pHs of the running buffer. Peak identifications were the same as those in Figure 1.](image-url)
the migration time became longer but the resolution also increased and all 12 nucleosides can be well separated. When the running buffer pH increased to 9.78, X and C couldn’t be well separated. For the examined pHs, these 12 nucleosides can only be separated at 9.50. It was clear to see that the nucleosides have different mobilities at about pH 9.50 under the present separation conditions. Therefore, the optimal pH for the separation of these 12 nucleosides was set at 9.50.

**Effect of Additive Concentration**

Additives are widely used in HPCE separations acting as solubilizing agents for hydrophobic solutes or as wall modifiers. For the separation of nucleosides, SDS has been used as surfactant at 300 mM in most of the studies to help improving the separations. However, several problems have been encountered by adding SDS in the running buffer: (1) bubbles were easily generated at such a high surfactant concentration, (2) relatively long separation time (normally 30–40 min) and pH shifting were observed, and (3) poor reproducibility after 3 runs and noisy baseline were often obtained. Without additives, these nucleosides couldn’t be well separated because of their close PIs. Therefore, CTAB was used in our study to help the separation. CTAB, which is a cationic surfactant, was used to cover the silanol groups and make the capillary wall positively charged in order to prevent positively charged particles from adsorbing on the wall. In addition, the use of CTAB in this method also reversed the direction of the EOF and form micelle simultaneously at above CMC (1.3 mM), which helped in the nucleoside resolution. Moreover, the presence of CTAB as an additive enhanced the interaction between BGE and nucleosides, predominantly anionic species, improving the nucleoside
separation. Total of seven CTAB concentrations (5, 10, 15, 25, 50, 75, and 100 mM) were examined in our study and the results are shown in Figure 3. Under the CTAB concentrations below 25 mM, only 11 peaks were observed and m$_2^3$G and m$_1^1$A merged together, although the migration time was shorter. At 50 and 75 mM CTAB concentrations, m$_2^2$G co-eluted with the system peak. When CTAB concentration reached to above 100 mM, crystals were found in the running buffer after one day. Therefore, 25 mM CTAB was chosen as the buffer additive in this study and the separation of 12 nucleoside standards can be completed in 7 min.

Figure 3. Effect of CTAB concentration on the separation of 12 nucleoside standards. The experimental conditions were the same as those of Figure 1, except for the CTAB concentrations. Peak identifications were the same as those in Figure 1.
**Effect of Applied Voltage**

Three voltages (20, 18 and 15 kV) were examined in our study. Under 20 and 18 kV, X and C cannot be separated and merged as a single peak and m1A also was hidden in I. Although the migration time was longer at 15 kV, the peak resolution was greatly improved within the reasonable time. Therefore, 15 kV was selected as the optimized separation voltage in our study.

**Linearity, Detection Limit, Reproducibility, and Recovery study**

Completely study of linearity, detection limit, reproducibility, and recovery of modified nucleosides of this HPCE method was conducted and the data were summarized in Table 1 and Table 2. Since this method can also be used to determine the nucleoside levels in other biological samples, CE and SPE methods were validated separately. In the linearity study, nucleoside concentrations selected were based on the expected concentration range in the urine samples. The following concentrations in DI H2O were used to construct the calibration curves: 0, 5, 10, 25, 50, 100, 250, 400, 500 µM for A, C, G, U, X, I, m22G, m1A; 0, 10, 25, 50, 100, 250, 500, 1000 µM for m2G, 8-hydroxy-2’-deoxyguanosine and 5-hydroxymethyl-2’-deoxyuridine; 0, 50, 100, 200, 400, 1000, 2000, 5000 µM for pseu; and 5, 20, 100, 1000, 2000, 5000 µM for creatinine. The regression parameters such as linearity range, slope, intercept and correlation coefficients are presented in Table 1. The averages of the correlation coefficients for six injections were between 0.9843 to 1.0000, which indicates a good linearity. The limit of detection (LOD) of this method was defined as the concentration where the ratio of signal to noise was 3. As listed in Table 1, the limit of detection of this method is from 0.56 to 1.67 µM, which is sensitive enough for nucleoside detection in urine samples.
Table 1. Linearity and detection limit of nucleosides and creatinine. The experimental conditions were the same as those of Figure 1.

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>Linear range (µM)</th>
<th>Slope (peak area/µM)</th>
<th>Limit of confidence for slope (p=95%)</th>
<th>Intercept (peak area)</th>
<th>Limit of confidence for intercept (p=95%)</th>
<th>( R^2 )</th>
<th>LOD (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseu</td>
<td>50 -5000</td>
<td>30.3</td>
<td>26.9 -33.7</td>
<td>11.5</td>
<td>-83.6 -107</td>
<td>0.994</td>
<td>0.67</td>
</tr>
<tr>
<td>U</td>
<td>10 -400</td>
<td>65.8</td>
<td>60.8 -70.8</td>
<td>-46.5</td>
<td>-187 -93.7</td>
<td>0.997</td>
<td>1.06</td>
</tr>
<tr>
<td>X</td>
<td>5 - 500</td>
<td>82.4</td>
<td>80.9 -83.8</td>
<td>1.64</td>
<td>-38.7 -42.0</td>
<td>0.999</td>
<td>0.78</td>
</tr>
<tr>
<td>C</td>
<td>5 - 400</td>
<td>70.7</td>
<td>65.9 -75.5</td>
<td>-74.3</td>
<td>-209 -60.4</td>
<td>0.998</td>
<td>1.67</td>
</tr>
<tr>
<td>G</td>
<td>5 - 250</td>
<td>88.4</td>
<td>84.8 -91.9</td>
<td>-43.8</td>
<td>-144 -56.3</td>
<td>0.999</td>
<td>0.77</td>
</tr>
<tr>
<td>A</td>
<td>5 - 500</td>
<td>94.7</td>
<td>88.0 -101</td>
<td>-66.4</td>
<td>-254 -121</td>
<td>0.998</td>
<td>1.09</td>
</tr>
<tr>
<td>I</td>
<td>10 -400</td>
<td>128</td>
<td>117 -138</td>
<td>-52.8</td>
<td>-345 -239</td>
<td>0.997</td>
<td>0.56</td>
</tr>
<tr>
<td>m^1A</td>
<td>10 -500</td>
<td>88.8</td>
<td>88.6 -88.9</td>
<td>-59.5</td>
<td>-65.0 -54.1</td>
<td>1.00</td>
<td>0.76</td>
</tr>
<tr>
<td>11*</td>
<td>10 -500</td>
<td>75.3</td>
<td>74.4 -76.1</td>
<td>-20.4</td>
<td>-44.2 -3.31</td>
<td>1.00</td>
<td>0.98</td>
</tr>
<tr>
<td>10*</td>
<td>10 -500</td>
<td>116.7</td>
<td>95.9 -137</td>
<td>118</td>
<td>-467 -703</td>
<td>0.996</td>
<td>0.55</td>
</tr>
<tr>
<td>m^2G</td>
<td>10 -1000</td>
<td>74.0</td>
<td>67.3 -80.6</td>
<td>-7.69</td>
<td>-196 -180</td>
<td>0.997</td>
<td>1.26</td>
</tr>
<tr>
<td>m^2G</td>
<td>5 - 500</td>
<td>79.0</td>
<td>75.7 - 82.2</td>
<td>-8.38</td>
<td>-88.6 -71.9</td>
<td>0.984</td>
<td>0.74</td>
</tr>
<tr>
<td>Creatinine</td>
<td>5 -5000</td>
<td>0.068</td>
<td>0.065 -0.071</td>
<td>-0.99</td>
<td>-7.0 -5.1</td>
<td>0.999</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*10, 8-hydroxy-2'-deoxyguanosine; 11, 5-hydroxymethyl-2'-deoxyuridine.
Table 2. Reproducibility and Recovery for nucleosides and creatinine*

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>RSD (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseu</td>
<td>3.6</td>
<td>108</td>
</tr>
<tr>
<td>U</td>
<td>7.2</td>
<td>104</td>
</tr>
<tr>
<td>X</td>
<td>7.9</td>
<td>82.6</td>
</tr>
<tr>
<td>C</td>
<td>6.4</td>
<td>113</td>
</tr>
<tr>
<td>G</td>
<td>6.8</td>
<td>83.8</td>
</tr>
<tr>
<td>A</td>
<td>5.7</td>
<td>92.9</td>
</tr>
<tr>
<td>I</td>
<td>8.3</td>
<td>81.2</td>
</tr>
<tr>
<td>m1A</td>
<td>7.2</td>
<td>89.4</td>
</tr>
<tr>
<td>5-hydroxymethyl-2'-deoxyuridine</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>8-hydroxy-2'-deoxyguanosine</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>m2G</td>
<td>5.1</td>
<td>105</td>
</tr>
<tr>
<td>m2'G</td>
<td>6.7</td>
<td>108</td>
</tr>
<tr>
<td>Creatinine</td>
<td>4.1</td>
<td>95.6</td>
</tr>
</tbody>
</table>

*The experimental conditions were the same as those of Figure 1.

N/A- not applicable

The reproducibility of relative peak areas, which is expressed as a percentage of relative standard deviation (RSD), was determined by six consecutive analyses of the normal urine sample. As showing in Table 2, the developed method was reproducible and these nucleosides were very stable during the analysis. The good reproducibility is not only due to the usage of CTAB as additive, but also due to the capillary rinsing procedure at the beginning of each day and the one between runs.
The recoveries of the nucleosides were determined by spiking the standards into a 1.0 mL urine sample. From the data in Table 2, we can see that the recoveries for both 8-hydroxy-2’-deoxyguanosine and 5-hydroxymethyl-2’-deoxyuridine were 0, which means they were not extracted by the affinity gel used in this study. While phenylboronate affinity chromatography selectively binds the cis-diol groups available on the nucleoside ribose sugar, the missing hydroxyl functional groups on the 2’ position for 8-hydroxy-2’-deoxyguanosine and 5-hydroxymethyl-2’-deoxyuridine makes these two nucleosides not have cis-diol structures any more. Therefore, the present nucleoside extraction method from urine may not be applicable to the non-cis-diol nucleosides. Therefore, the sample preparation method becomes the limiting step of the whole nucleoside analysis by using this newly developed fast CE separation method. For the rest of 10 nucleosides, a range of 81.2% to 113% recovery were obtained, which is acceptable.

**Analyses of Urine Samples**

In order to demonstrate the applicability of the developed method, it was applied to two urine sample analyses collected from a healthy person and a breast cancer patient. A capillary electropherogram of nucleosides in the urine extract from the healthy person is shown in Figure 4. The average nucleoside levels excreted in urine samples from a non-cancer carrying person and a cancer patient were shown in Table 3. The peaks were identified by comparing migration times of the unknown peaks with those of the standard nucleosides eluted under the same condition, and by spiking the urine sample with pure single nucleoside standards. The levels of the urinary nucleosides were calculated by using standard calibration curves, and then were transformed into nM nucleoside/µM creatinine. Creatinine has been used in many clinical studies as internal standard since its
concentration strictly corresponds to urine dilution. Many studies have shown that urinary nucleosides excretion from human beings is little affected by diet, and when normalized to urinary creatinine, the daily excretion rate is remarkably constant in an individual\textsuperscript{57,68}. The results obtained through our newly developed HPCE-UV method were comparable to those of previous CE-UV method and HPLC method. More urine samples will be analyzed by using this newly developed method for potential early cancer screening using nucleosides as biomarkers.

Figure 4. A representative electropherogram of nucleosides in a urine extract of a healthy subject. The experimental conditions and peak identifications were the same as those of Figure 1.
Table 3. Average nucleoside levels excreted in urine samples from a normal subject and a breast cancer-carrying patient (nM nucleoside/µM creatinine)

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>Normal subject mean ± SD</th>
<th>Cancer patient mean ± SD</th>
<th>Literature data for normal subject&lt;sup&gt;43, 44, 50, 63, 69-71&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseu</td>
<td>24.0 ± 3.2</td>
<td>48.2 ± 2.4</td>
<td>13.0~42.0</td>
</tr>
<tr>
<td>U</td>
<td>1.01 ± 0.10</td>
<td>1.22 ± 0.08</td>
<td>0.21~0.99</td>
</tr>
<tr>
<td>X</td>
<td>0.76 ± 0.04</td>
<td>1.30 ± 0.09</td>
<td>0.24~0.86</td>
</tr>
<tr>
<td>C</td>
<td>0.57 ± 0.29</td>
<td>7.55 ± 0.62</td>
<td>0.01~0.78</td>
</tr>
<tr>
<td>G</td>
<td>10.0 ± 0.1</td>
<td>11.7 ± 1.0</td>
<td>0.01~10.70</td>
</tr>
<tr>
<td>A</td>
<td>3.51 ± 0.21</td>
<td>6.09 ± 0.18</td>
<td>0.18~4.70</td>
</tr>
<tr>
<td>I</td>
<td>7.38 ± 0.59</td>
<td>12.3 ± 0.2</td>
<td>0.09~8.80</td>
</tr>
<tr>
<td>m&lt;sup&gt;1&lt;/sup&gt;A</td>
<td>3.01 ± 0.23</td>
<td>3.34 ± 0.31</td>
<td>2.02~2.90</td>
</tr>
<tr>
<td>m&lt;sup&gt;2&lt;/sup&gt;G</td>
<td>0.59 ± 0.01</td>
<td>2.46 ± 0.14</td>
<td>0.26~2.00</td>
</tr>
<tr>
<td>m&lt;sup&gt;2&lt;/sup&gt;2G</td>
<td>0.32 ± 0.01</td>
<td>2.03 ± 0.32</td>
<td>0.36~1.74</td>
</tr>
</tbody>
</table>

**CONCLUSION**

A simple and fast HPCE method was developed for separation and quantitation of 10 modified nucleosides in urine samples. The separation time was reduced dramatically and the reproducibility was significantly improved, compared to previous CE and HPLC methods, which is very valuable for clinic diagnosis. The factors that affect separation efficiency, such as pH, voltages, buffer composition and concentrations, have been systematically investigated and optimized. This method can be used for urinary nucleoside determination for early cancer screening in which nucleosides are used as biomarkers.
ACKNOWLEDGEMENT

This research was supported by Environmental Research Center and startup funds awarded to Yinfa Ma from Missouri University of Science and Technology. The authors sincerely thank Ellis Fischel Cancer Center (Columbia, MO) for providing urine samples from breast cancer-carrying patients.
2. Quantitative Determination of Sarcosine and Related Compounds in Urinary Samples by Liquid Chromatography with Tandem Mass Spectrometry

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ABSTRACT

Current prostate cancer (PCa) diagnosis, based on the triad of digital rectal examination (DRE), blood prostate-specific antigen (PSA) level measurement and trans-ultrasound guided prostate biopsy, is not a precise science. The widely used PCa biomarker PSA has poor sensitivity and specificity, leading to false-negative and false-positive test results. Recently, sarcosine, proline, kynurenine, uracil and glycerol-3-phosphate were found in large concentrations in metastatic prostate cancer urine samples. By measuring all these five metabolites, doctors may be better able to diagnose prostate cancer with high accuracy. However, there is no method reported to detect these compounds in urine sample simultaneously. In this paper, a novel method was developed to separate and quantify 6 metabolites including creatinine in urine samples by using liquid chromatography with tandem mass spectrometry. Chromatographic characteristics of the analytes were determined using a phenyl-hexyl column with 0.1% formic acid in water and acetonitrile respectively under a gradient program. The six metabolites were detected in the multiple reaction monitoring (MRM) modes with ESI-positive mode. The
linear range of the analytes was from 0.0026 to 44.6 µmol/L and the detection of limit was lower than 2.61 nmol/L. The effects of stationary phase, mobile phase and mass spectrometry on the separation and quantification of the six metabolites were also investigated. The technique developed in this study is simple, fast, sensitive and selective and will be used for quantifying these six metabolites in urine samples for potential early cancer screening.

**KEYWORDS**

Sarcosine; prostate cancer biomarker; LC-MS/MS

**INTRODUCTION**

Prostate cancer (PCa) ranks as the third most common cancer in men in the world\textsuperscript{72,73}. In US and Europe, PCa is the most common cancer diagnosis and the second most common cause of cancer-related deaths in men\textsuperscript{74}. Furthermore, the disease incidence is increasing steadily over the years, although its etiology remains unknown. However, prostate cancer diagnosis is not a precise science yet. Normally, 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\textsuperscript{75,76}. The typical exam combines a digital probe of the prostate to check for swelling or lumps and a blood test to reveal PSA levels. If an abnormal DRE and/or PSA levels are found, a
prostate biopsy is often recommended. The definitive diagnosis of cancer is based on histological assessment of the needle biopsy tissue material\textsuperscript{77}. Although, DRE allows the clinician to “feel” the prostate, it has a poor sensitivity and typically only allows the detection of relatively large tumors which usually represent locally advanced disease. Even when a biopsy reveals cancer, it sometimes remains unclear whether the cancer is aggressive and at risk of spreading, or indolent. Biopsy doesn’t always reveal the true disease condition. Therefore, biomarkers are needed for the prostate cancer diagnosis.

Currently, PSA is the only biomarker widely used in the diagnosis and management of patients with PCa. However, it has poor sensitivity and specificity for cancer detection, leading to false-negative and false-positive test results. Also, PSA is unable to distinguish indolent from aggressive tumors. The results is nearly 30,000 men die of prostate cancer in the United States each year, but millions of others who have the disease are not even aware of it\textsuperscript{78}. Many men with indolent disease are overtreated and at the same time aggressive cases are not caught. Thus, more and more researchers began to question PSA as PCa biomarker\textsuperscript{79}. There is an urgent need for clinically validated biomarkers which will improve the diagnosis and management of PCa.

In 2009, sarcosine, an N-methyl derivative of the amino acid glycine, was revealed to highly increase during prostate cancer progression to metastasis and can be detected non-invasively in urine\textsuperscript{39}. Four other metabolites, including proline, kynurenine, uracil and glycerol-3-phosphate, were also found in large concentrations in metastatic prostate cancer by the same research team. However, only sarcosine was currently studied for the potential role in prostate cancer progression. In our work, we proposed that five metabolites, including sarcosine, proline, kynurenine, uracil and glycerol-3-
phosphate, can be used as a biomarker panel and doctors might be better able to diagnose prostate cancer and its progression by using this panel. During the biomarker confirmation and early cancer screening, a simple but powerful separation and quantification technique is crucial.

The structures of the five metabolites and creatinine (for normalization purpose) are shown in Figure 1. From Figure 1 we can see that five of the molecules are amines. The determination of molecules carrying amine groups is usually performed by ion-exchange chromatography followed by post column derivatization and then detected by mass spectrometer or UV. Some studies use pre-column derivertization and then analyze the analytes by MS. Isotope dilution method coupled with LC-MS or GC-MS has also been employed by some researchers and samples need to be modified to their t-butyl dimethylsilyl derivatives before analyzed by mass detector. However, the above mentioned analytical techniques are complicated, time-consuming and have high requirement to the operating person. These problems will prevent the findings from being applied to the clinic and new method needs to be developed.

Tandem mass spectrometry (MS/MS) is a powerful technique for the quantitative analysis of small molecules. The combination of the specific parent mass and the unique fragment ion is used to selectively monitor for the compound to be quantified. When it is conjugated with HPLC, most ions and other components from the sample matrix suppressing the signal output are removed and potential isobars are separated. HPLC-MS/MS facilitates the sensitive and specific analysis, especially in complex matrices like urine and blood. In recent years, the use of HPLC-MS/MS has become more popular in
hospital laboratories and is applied to the quantitative analysis of small molecules such as metabolites and pharmaceuticals.\textsuperscript{86}

![Structures of six molecules](image)

Sarcosine  
Creatinine  
Kynurenine  
Proline (Pro or P)  
Uracil  
Glycerol 3-phosphate

Figure 1. Structures of six molecules

In this paper, a robust HPLC-MS-MS technique was developed to separate and quantify six molecules simultaneously in a single run. Simplicity of sample preparation, short analysis time and super specificity and sensitivity of this method make it suitable for biomarker screening using urine samples. Healthy subjects, prostate cancer and other cancer patients’ urine samples were analyzed using the newly developed method. The main goal of this study was to establish an efficient analytical technique to simultaneously measure the six compounds that are present in urine samples from both cancer-carrying patients and healthy controls, and therefore provide a key tool for new prostate cancer biomarker identification and confirmation. More urine sample analyzing
is ongoing by using the established method, new cancer biomarkers will be confirmed and potential early cancer screening can be conducted based on these findings.

**MATERIALS AND EXPERIMENTS**

**Chemicals**

Six metabolite standards, including sarcosine, proline, kynurenine, uracil, glycerol-3-phosphate, and creatinine (for normalization purpose) were purchased from Sigma–Aldrich (St Louis, MO, USA). L-Glutamine isotope which was used as internal standard for LC-MS/MS analysis was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Formic acid (99%) and LC-MS grade acetonitrile which were used to prepare mobile phase were purchased from Sigma-Aldrich (St Louis, MO, USA). LC-MS grade water (18.2 MΩ) from ultra-high purity water system from Millipore Inc. (Millipore, MA, USA) was used to prepare standard solutions, mobile phase and other solutions.

**Urine Sample Collection and Preparation**

Spontaneous urine samples from 10 healthy adults, 7 prostate cancer patients and 3 other cancer patients were collected from Missouri University of Science and Technology (Rolla, MO, USA), the Ellis Fischel cancer center (Columbia, MO, USA), and Central Missouri Urology Clinic (Rolla, MO, USA). After collection, the samples were frozen and stored at -80°C immediately. Before analysis, samples were thawed at room temperature and then diluted 3 times using water. 10 μL of diluted urine was mixed
with 10 μL of the internal standard solution and 1480 μL of 0.1% formic acid in water. The sample was then ready for LC-MS-MS analysis.

**High-Performance Liquid Chromatography (HPLC)**

Agilent 1100 series LC system (Santa Clara, CA) and a phenyl-hexyl, 3.0 μm, 3.0×150 mm column (Phenomenex, USA) were used to conduct the metabolite separation. 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 injected volume was 10 μL.

**Tandem Mass Spectrometry (MS/MS)**

API 4000Q trap MS/MS system (Applied Biosystems, Foster City, CA) was used to detect the molecules. Mass spectrometer was operated in multiple-reaction monitoring mode (MRM) under the ESI-positive mode. Turbo Spray was used as the ion source. The capillary voltage was set at 5.5 kV. 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 solutions (20 ppb) in solvent A using a syringe pump. Parent-ion and daughter-ion scans were performed using argon as the collision gas at a pressure of 3.8×103 mbar and a flow of 0.2 mL/min.
Data Normalization

Human biological samples, such as urine, exhibit a large amount of variation caused by physiological factors such as sex, state of health, age, diet, stress, or diurnal cycles among others. The experiment data thus require normalization to account for the variation and to give each sample equal importance in analysis by multivariate statistics. All the metabolite experiment data was normalized according to creatinine concentration in this study.

Method Validation

Because of the number of analytes and complexity of the sample matrixes, linearity and limit of quantification (LOQ) were determined by spiking 3 independent urine samples with known concentrations of the analytes. LOQ was chosen as the lowest standard on the matrix-based calibration curve. Reproducibility and accuracy were determined by 5 consecutive analyses of spiked urine sample at low, middle and high analytes concentrations. This ensured that the precision and accuracy can be assessed for all studied metabolites at all levels.

RESULTS AND DISCUSSION

A quantitative assay should be specific, accurate and sensitive. The method development time should be as short as possible without sacrificing the above attributes. Assays based on mass spectrometry quantitation combined with HPLC separation can satisfy these criteria if the conditions are optimized. It is realized that parameters related to sample pretreatment, chromatography, analyte ionization, and mass
spectrometric analysis are all strongly interrelated and will significantly affect the quality of separation and quantification of the developed technique. These important issues are discussed below.

**Sample Preparation**

A wide variety of sample pretreatment methods have been applied in quantitative bioanalysis using LC-MS, including liquid-liquid extraction, solid phase extraction (SPE), or protein precipitation. Compared to the fast MS analysis, some sample pretreatment methods are tedious and time-consuming and therefore limit the analysis efficiency. In this study, less time need be spent on sample preparation because of the combination of high selectivity and sensitivity of tandem mass and high separation performance of HPLC. The samples only need to be diluted 450 times and then ready for LC-MS/MS analysis. Because of the use of HPLC, most interference from the matrix was removed before MS/MS quantification. The high sensitivity of tandem MS enables us to detect the interest analytes after dilution with aqueous phase and the high selectivity ensures that without extraction and other pretreatment method, the analytes can still be detected without interference. This eliminating the need for extensive sample preparation and hence reducing the effort spent on method development and routine analysis will save a lot of time and money in bioanalysis.

**Optimization of Liquid Chromatography Conditions**

Although tandem MS can surmount some chromatographic problems, good chromatography will give better quantification and calibration curves. So it is better to separate component peaks with LC than with the MS. If components are separated well
on the column, more time per component can be used to scan resulting in better signal to noise ratios. Better signal to noise ratios are easier to integrate, give better reproducibilities and also an increase in sensitivity. Also, co-elution might cause competition effects during the ionization. In our study, the optimized liquid chromatography conditions were chosen based on the interested analytes and all six metabolites including creatinine are completely separated, as shown in Figure 2.

Figure 2. A representative chromatogram of 6 metabolites standards at optimized conditions by HPLC-MS/MS. Column: 3.0 μm, 3.0×150 mm (phenyl-hexyl); Temperature: 25°C; Flow rate: 250 μL/min; Injected volume: 10 μL; Mobile phase A: 0.1% formic acid in water and 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 was 15 min; MS/MS conditions are the same as that in Table 1. Peak identification: 1, Glycerol 3-phosphate (G3P), 2, Creatinine (CRE), 3, Sarcosine (SAR), (G), 4, Proline (PRO), 5, Uracil (URA), 6, Kynurenine (KYN).
From the analytes structures which are shown in figure 1, we know that four of them are polar and at the same time five of the metabolites are amines except glycerol phosphate. Currently, metabonomics studies with LC-MS predominantly use reversed-phase (RP) chromatography, in which C18 column conducts the separation most of the time\textsuperscript{88,89}. Although C18 column is very common, it is not suitable to retain and therefore separate highly polar analytes. A commonly used strategy to separate polar compounds by C18 column is to add ion-pair reagent in the mobile phase\textsuperscript{90}. Tridecafluoroheptanoic acid (TDFHA) as additive was tested in our study. However, the small hydrophilic molecules, sarcosine, proline, glycerol-3-phosphate and creatinine, had no retention on column and couldn’t be separated. They were eluted out the column quickly with matrix. Also, ion-pair reagent bleeding, column block and bad reproducibility problems were also observed. Phenyl-hexyl column, which can provide excellent retention for aromatic, polar and amines under pH=1.5 ~ 10, was therefore used in our study. Compared with C18 column, phenyl-hexyl column revealed good separation for our analytes, which is shown in Figure 3.

In HPLC, one of the eluent components is water and another component of any binary eluent is organic solvent. ACN is used as organic phase in this study because of its higher solvent strength and lower viscosity than MeOH in mixtures with water. 0.1% formic acid was added to both aqueous and organic phase for better peak shape and high ionization efficiency purpose.
Gradient program was optimized using the so-called 60/60. That is, near 0% B to 60% B was run first in 60 minutes and the chromatographic character of the studied compounds on the column was learned. Under 60% B, all analytes were eluted out the column. Then both B% and time were decreased subsequently until they reached 40% B at 5 minutes where complete separation could still be obtained. Decreasing the elution time and B% further couldn’t get good separation any more. In order to let the compounds stack on the front of the column, give sharp peaks and get better separation through long column path, 98% aqueous phase at the beginning of gradient program was our choice. We didn’t start the program at 100% aqueous phase and 0% organic phase, because the alkyl chains of the stationary phase would get away from the high aqueous environment, mat down on the particle and not efficient at capturing the analytes if we
did so. Therefore, the gradient program was started with small percentage of organic phase - 2% in this study.

**Optimization of MS/MS Conditions**

HPLC coupled to mass spectrometry (MS) is increasingly being used for urinary metabonomic studies. Mass spectrometry is a highly sensitive but selective technique. Used in conjugation with HPLC, which provides separation of the components, mass spectrometry allows detection and quantification of low-level metabolites. In order to develop a MS/MS method, each parameter could have been optimized for each compound. However, for source/gas parameters, such as curtain gas, collision gas, ion spray voltage and ion sources, quite similar values were obtained for them. In all cases, their variation did not lead to a significant variation in the intensity of the signal (data not shown). Thus, same parameters were chosen for all compounds as shown in materials and experiments part in this study.

For other parameters, it was necessary to establish for each molecule their own ones. For example, DP applied to the interface is specific for each precursor ion, and CE is specific for each fragment ion. For this purpose, 20 ppb standard solutions of each compound were infused by syringe pump into the mass spectrometer under similar flow rate and mobile phase components to that in chromatography so that the actual sample analysis condition was simulated. The most abundant and specific transition was selected for use in the multiple reaction monitoring (MRM) mode with positive polarity. When choosing the precursor/daughter ion pair, caution was applied in order not to choose the too close pairs since the molecular weight are pretty close for some analytes. The parameters were obtained and shown in Table 1 with the precursor/daughter ion masses.
The specificity of the MS/MS method is linked to the combined analysis of the precursor ion and one of its fragments. This technique is much more sensitive than others. Other transitions characteristic of each molecule can also be used for confirmation of results, if necessary.

Table 1. MS/MS parameters for 6 molecules including creatinine.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Q1</th>
<th>Q3</th>
<th>Dwell(ms)</th>
<th>DP(V)</th>
<th>EP(V)</th>
<th>CE(V)</th>
<th>CXP(V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcosine</td>
<td>89.9</td>
<td>44</td>
<td>120</td>
<td>26</td>
<td>10</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>Proline</td>
<td>115.939</td>
<td>70.2</td>
<td>120</td>
<td>41</td>
<td>10</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>209.014</td>
<td>94.1</td>
<td>120</td>
<td>36</td>
<td>10</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Uracil</td>
<td>112.906</td>
<td>70</td>
<td>120</td>
<td>67</td>
<td>10</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Glycerol-3-phosphate</td>
<td>173</td>
<td>99</td>
<td>120</td>
<td>56</td>
<td>10</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Creatinine</td>
<td>113.949</td>
<td>44.1</td>
<td>120</td>
<td>1</td>
<td>10</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>Glutamine (IS)</td>
<td>148.027</td>
<td>130</td>
<td>120</td>
<td>31</td>
<td>10</td>
<td>15</td>
<td>8</td>
</tr>
</tbody>
</table>

In order to get good mass quantification, several strategies were also used, such as using fresh solvent weekly when analyzing samples, getting sharp peaks as best as we can, not using nonvolatile buffer, acid and base, additives such as TFA and ion-pair reagents, and using good IS (far enough from non-labeled analytes). In this study, L-Glutamine isotope was used as an internal standard. It not only controlled HPLC injection and ionization variability, also corrected the matrix effect of urine.
Method Validation

Complete study of linearity, limits of quantification, reproducibility, and accuracy of the six compounds using HPLC-MS/MS method was conducted in urine matrix and the data were summarized in Table 2 and 3. In the linearity study, calibration curves were prepared by spiking 3 urine matrixes with known concentrations of the analytes and the average was taken. Concentrations of standards were chosen on the basis of the expected concentration range in urine samples. The analyte concentrations in unknown samples were estimated by shooting unknown samples along with low, middle and high standards. The regression parameters such as linearity range, slope, intercept and correlation coefficients are presented in Table 2. The correlation coefficients were between 0.9986 and 0.9999 ($R^2 > 0.99$), which indicates a good linearity. The lowest standard on the matrix-based calibration curve was selected as the limit of quantification and also the analytes response at the LOQ was more than 5 times the response compared to blank response. As listed in Table 2, the limit of quantification of this method is from 2.61 to 17.7 nM, which is sensitive enough for these six molecules detection in urine samples.

The reproducibility of relative peak areas, which is expressed as a percentage of relative standard deviation (RSD), and the accuracy were determined by 5 consecutive analyses of urine sample at low, middle and high analytes concentrations. This ensured the possibility of precision and accuracy assessment for all studied metabolites at all levels. As showing in Table 3, the developed method was reproducible and these metabolites were very stable during the analysis. Also, the accuracy is $100 \pm 20\%$, which revealed that the method is valid for urine sample analysis. The good reproducibility and accuracy is not only due to the sample dilution, optimization of MS/MS condition and the
choosing of good internal standard, but also due to not using other additives in mobile phase except formic acid and the complete separation of analytes.

Table 2. Linearity and quantification limit of metabolites and creatinine in urine matrix.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Linear range (µM)</th>
<th>Slope (peak area ratio/µM)</th>
<th>Intercept (peak area ratio)</th>
<th>R²</th>
<th>LOQ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.0177-44.0</td>
<td>0.0761</td>
<td>10.6</td>
<td>0.9986</td>
<td>17.7</td>
</tr>
<tr>
<td>G3P</td>
<td>0.0029-17.4</td>
<td>0.0211</td>
<td>-0.107</td>
<td>0.9994</td>
<td>2.91</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>0.0048-9.61</td>
<td>0.205</td>
<td>0.523</td>
<td>0.9999</td>
<td>4.81</td>
</tr>
<tr>
<td>Proline</td>
<td>0.0026-8.69</td>
<td>0.24</td>
<td>0.814</td>
<td>0.9999</td>
<td>2.61</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>0.0056-56.1</td>
<td>0.0732</td>
<td>0.0942</td>
<td>0.9999</td>
<td>5.62</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.0045-44.6</td>
<td>0.048</td>
<td>0.375</td>
<td>0.9999</td>
<td>4.46</td>
</tr>
</tbody>
</table>

Analyses of Urine Samples

In order to demonstrate the applicability of the developed method, it was applied to 10 urine sample analyses collected from 5 healthy persons and 5 cancer patients. The average metabolites levels excreted in urine samples from them were shown in Table 4. The levels of the urinary metabolites were calculated by using matrix-based calibration
curves, and then were transformed into nM metabolites/µM creatinine. Creatinine has been used in many clinical studies as internal standard since its concentration strictly corresponds to urine dilution. Normally, the urinary excretion from human beings is little affected by diet, and when normalized to urinary creatinine, the daily excretion rate is remarkably constant in an individual \(^{57,68}\). More urine samples are currently being analyzed by using this newly developed HPLC-MS/MS method for prostate cancer biomarker identification and confirmation by measuring these metabolites.

Table 3. Reproducibility and Recovery for metabolites and creatinine in urine matrix

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Low conc.</th>
<th>Middle conc.</th>
<th>High conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RSD (%)</td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>20.1</td>
<td>129</td>
<td>15.3</td>
</tr>
<tr>
<td>G3P</td>
<td>13.2</td>
<td>117</td>
<td>9.1</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>7.27</td>
<td>111</td>
<td>3.56</td>
</tr>
<tr>
<td>Proline</td>
<td>7.11</td>
<td>112</td>
<td>6.95</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>5.89</td>
<td>90</td>
<td>6.31</td>
</tr>
<tr>
<td>Uracil</td>
<td>10.6</td>
<td>108</td>
<td>7.98</td>
</tr>
</tbody>
</table>
Table 4. Average metabolites levels excreted in urine samples from five normal subjects and five cancer-carrying patients (nM metabolites /µM creatinine)

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Normal subject mean ± SD</th>
<th>Cancer patient mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol-3-phosphate</td>
<td>28.7 ± 0.97</td>
<td>86.6 ± 1.78</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>4.16 ± 0.35</td>
<td>7.68 ± 1.13</td>
</tr>
<tr>
<td>Proline</td>
<td>2.64 ± 0.02</td>
<td>35.0 ±1.31</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>27.1 ± 1.08</td>
<td>118 ± 2.43</td>
</tr>
<tr>
<td>Uracil</td>
<td>2.78 ± 0.21</td>
<td>5.3 ± 1.14</td>
</tr>
</tbody>
</table>

CONCLUSION

A simple but powerful HPLC-MS-MS method was developed for simultaneous separation and quantification of 6 molecules (sarcosine, proline, kynurenine, uracil, glycerol-3-phosphate and creatinine) in urine samples for the first time. Without using other additives except formic acid, the six compounds were completely separated on phenyl-hexyl column within 10 min. The thorough separation directly resulted in good mass quantification. The factors that affect separation efficiency and quantification quality have been systematically investigated and optimized. This method is being used for urinary metabolites determination for early cancer screening in which these five metabolites are used as biomarkers. Simplicity of sample preparation, fast and complete separation, super specificity and sensitivity of LC-MS/MS and simultaneous analysis of these molecules in urine samples by LC-MS/MS in positive mode is certainly useful for
diagnosis and therapy assessment of prostate cancer. The developed method is promising and can also be used to detect the six metabolites in other biological matrix, such as blood.

ACKNOWLEDGEMENT

This research was supported by Environmental Research Center from Missouri University of Science and Technology. The authors sincerely thank Central Missouri Urology Clinic (Rolla, MO) and Ellis Fischel cancer center (Columbia, MO) and the volunteers from Missouri University of Science and Technology for providing urine samples.
3. Determination of Polyamines in Bacterial Cell Extracts by Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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ABSTRACT

Polyamines such as spermidine (SPD), spermine (SPM), cadaverine (CAD) and putrescine (PUT) are small, polycationic molecules that are required for optimal growth in all cells. In order to detect cell extracts polyamines accurately, a sensitive CE-LIF method was validated and successfully applied to determine biogenic PAs in bacterial cell extracts. FITC was used for the off-line derivatization and the FITC-PAs were separated in less than 8 min at a voltage of 20 kV. This CE-LIF method was proved to be sensitive, simple, fast, low cost and environment friendly and suitable to detect PAs in biological samples. Intracellular concentrations of polyamine molecules are maintained by de novo synthesis and transport pathways. The human pathogen possesses a putative polyamine transporter (pot) operon D (potD). Through the measurement of polyamine intracellular concentrations, the data suggested that potD is involved in polyamine transport and is important for pathogenesis within various infection models. Polyamine
transport was associated with the ability of a human pathogen to cause disease for the first time in this study.

KEYWORDS

Capillary electrophoresis; polyamines; laser-induced fluorescence; cell extract

INTRODUCTION

Polyamines (PAs) such as spermidine (SPD), spermine (SPM), cadaverine (CAD) and putrescine (PUT) (as shown in Figure 1) comprise a group of ubiquitous aliphatic, polycationic molecules which are found in central neurons system\textsuperscript{92}, tissue cells\textsuperscript{93, 94} and body fluids including serum\textsuperscript{95} and urine\textsuperscript{96}. They play an important role in cell’s function and growth, DNA replication, protein and nucleic acids synthesis, and gene expression\textsuperscript{97-99}. Intracellular polyamines are derived from both de novo synthesis from amino acids and intracellular uptake from the environment. The intracellular levels of polyamines are tightly regulated by multiple mechanisms involving both biosynthesis and transport processes. Although polyamines have been shown to have multiple effects on protein synthesis and cell proliferation for all cell types, polyamine uptake and synthesis in pathogenic bacteria has not been well studied. Also, several scientists reported that PA concentrations in the urine of some tumor patients were higher than those of normal people\textsuperscript{100, 101}. Despite of the limitations of PAs as biomarker for malignant tumors, PAs are now still considered as one group of the tumor markers in humans (although not as a
sole marker). Therefore, the ability to detect PAs concentrations in all type of cells would be of clinical significance in both pathogenesis studies and early cancer screening.

Methods for separation and determination of PAs have been established by using gas chromatography (GC)\textsuperscript{102,103}, high-performance liquid chromatography (HPLC)\textsuperscript{104-106}, and capillary electrophoresis (CE)\textsuperscript{107-109} with UV detection. Since PAs do not absorb at the UV/Vis range, they were detected either by indirect detection or through derivatization\textsuperscript{110,111}. These studies used 4-(1-pyrene) butyric acid \textit{N}-hydroxysuccinimide ester (PSE), dansyl chloride, and other derivatization reagents to derivatize PAs before separation by HPLC or CE. However, the detection sensitivity cannot meet the requirement of some analysis due to the variety of biological samples. In our laboratory, one PAs quantification method, capillary electrophoresis coupled with laser induced fluorescence (CE-LIF), was developed by labeling PAs with fluorescein-5-isothiocyanate (FITC) and then detected by LIF in order to improve detection sensitivity. Also, the developed method can separate and detect all PAs and catecholamines (CA\text{s}) simultaneously with high sensitivity, which is very crucial in understanding the mechanism of tumor cells’ malignancy, early cancer detection, and follow-up after anticancer drug treatment. In this work, we have applied for the first time the CE-LIF method to separate and quantify PAs in bacteria cell extracts and associated the polyamine transport with the ability of a human pathogen to cause disease.
EXPERIMENTS

Chemicals

All chemicals were of analytical reagent grade unless stated otherwise and used without further purification. CAD, PUT, SPD, SPM were purchased from Sigma (St. Louis, MO, USA).

A 0.1 mmol/L stock solution of each PA was prepared in 0.01 mol/L cold perchloric acid and stored at 4 °C before use. FITC isomer I was purchased from Molecular Probes (Eugene, OR, USA). A 0.45 mmol/L of FITC stock solution was prepared with acetonitrile containing 0.8% (v/v) methanol and 0.5% (v/v) pyridine. A 45 µmol/L of FITC working solution was prepared from the above stock solution with further dilution by using the same acetonitrile mixture solution. A 0.05 mol/L Na2HPO4 derivatization buffer solution was prepared by dissolution of 7.80 g/L of Na2HPO4 in deionized water, adjusted to pH 10.95 with 1.0 mol/L NaOH. A 40 mmol/L sodium tetraborate and 60 mmol/L boric acid mixture buffer solution was adjusted to pH 9.0 with
1.0 mol/L NaOH and was used as the background electrolyte (BGE). The working solutions of PA standards were diluted with this BGE solution. Deionized water (18.2 MO) was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

**Sample Extraction**

The pellets were washed three times with 15 mL 0.3 mol/L sucrose (pH 7.40) under 1400 rpm for 5min. A 500-600 µL aliquot of 15% iced trichloroacetic acid (TCA) was added to the cell pellets. The samples were stored at 4 ºC for 2 h. PAs and CAs that were complexed to DNA/RNA, proteins, and phospholipids were released under these treatment conditions. Proteins and other macromolecule precipitates were removed by centrifugation at 10,000×g for 15 min at 4 ºC. The TCA-soluble fraction was extracted six times with 6 times with 1.5mL diethyl ether to remove TCA. Then the solution was set to 1.0 mL. After the pH was increased to 1-2 through ether extraction, the sample extract were then used for derivatization to determine total PAs.

**Derivatization Procedure**

10 µL PAs working solution or extracts was mixed with 10 µL of 45 µmol/L FITC, 200 µL of 0.05 mol/L Na₂HPO₄ buffer, and vortexed and reacted in the dark at 25 ºC for 6 hrs 40min. The solution was then diluted twice with H2O before injection to the CE system. A blank solution was prepared at the same time to serve as a control.

**Instrumentation**

A Beckman P/ACE MDQ CE system with an LIF detector was used for all electrophoretic separations. Excitation was at 488 nm (argon ion laser) and the emission intensity was monitored at 520 nm (band-pass filter, bandwidth 10 nm). A 50 µm ID
fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA) of 58 cm length (48 cm from inlet to the detector window) was used and themostated at 25 °C. The capillary was first conditioned with 1.0 mol/L HCl, 1.0 mol/L NaOH and methanol for 2 min each as described in the P/ACE MDQ CE instrumental manual. Then the capillary was rinsed with deionized water for 2 min at a pressure of 60 psi and equilibrated with BGE buffer before sample analysis. Samples were injected by pressure at 0.5 psi for 15 s, and separations were performed under 20 kV for 15 min with a positive high voltage. The data were collected and processed by Beckman P/A CE 32 Karat software Version 4.0. The capillary was rinsed 2 min with 0.1 mol/L NaOH, water, and BGE after each run.

RESULTS AND DISCUSSION

A representative electropherogram of FITC-PA derivatives and unreacted labeling agent is shown in Figure 2, demonstrating the excellent separation of these analytes under the optimized conditions. Four PAs, which exist in biological cell extracts and are of interest to biological researchers, were completely separated in less than 8 min. The migration order was SPM, SPD, CAD, PUT, and FITC. This elution order can be explained on the basis of the molecular structure and mass-to-charge ratio. Because the negative charges on FITC-PAs are from FITC, the high mass-to-charge ratio results in a higher mobility. Due to the similar mobility of FITC-PAs, they moved in very short migration times to the detector side.
Figure 2. Electropherogram of standard mixtures of FITC-PAs under optimal derivatization and separation conditions with LIF detection. Capillary: 50 µm ID × 58 cm length (48 cm from inlet to the detector window); Temperature: 25 ºC; Sample injection: 0.5 psi for 15 s; Separation voltage: 20 kV; Separation time: 15 min; Detector: 488nm (excitation) and 520 nm (emission); BGE: 40 mmol/L sodium tetraborate and 60 mmol/L boric acid mixture buffer solution, pH = 9.0.

**Linearity, Reproducibility, Detection Limits and Recovery Study**

The linearity, reproducibility, detection limits, and recoveries for determination of FITC-PA derivatives were completely studied and are listed in Table 1. The reproducibility was expressed as relative standard deviation (RSD) values for both migration time and peak area and was calculated by using 0.50 mmol/L of PA standard solutions (n = 5). The RSD values of migration time were between 0.98% and 2.76% and the RSD for peak areas were between 1.86% and 3.15%, which means this technique is reproducible. The linearity of the method was determined by using standard mixtures at six concentrations from 0.05 to 1.0 mmol/L, correlation coefficients ranged from 0.9568 to 0.9975, and detection limits varied from 1.97 and 2.98 nmol/L with a signalto-noise
ratio (S/N) of 3, which is sensitive enough for PA analysis in cell extracts. The recoveries are between 87.4 and 92.5, which indicate that the method is reliable.

Table 1. The linearity, reproducibility and detection limits of FITC-PA derivatives by CE-LIF. Derivatization and separation conditions are the same as that in Figure 2.

<table>
<thead>
<tr>
<th>PA Standards</th>
<th>RSD (%)</th>
<th>Linearity ($R^2$)</th>
<th>Detection Limit (nM, S/N=3)</th>
<th>Recovery (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Migration Time</td>
<td>Peak Area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermidine</td>
<td>1.86</td>
<td>2.17</td>
<td>0.9896</td>
<td>2.05</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.98</td>
<td>1.86</td>
<td>0.9975</td>
<td>1.97</td>
</tr>
<tr>
<td>Spermine</td>
<td>2.14</td>
<td>3.12</td>
<td>0.9682</td>
<td>2.98</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>2.76</td>
<td>3.15</td>
<td>0.9568</td>
<td>2.76</td>
</tr>
</tbody>
</table>

**Analysis of polyamines in bacterial cell extracts.**

Bacterial cell samples were analyzed (n = 5) using the optimized CE-LIF method. PA peaks in the cell extracts were identified by retention time and standard addition. The results obtained from the analysis of bacterial cell extracts are shown in Table 2.

Polyamines have been shown to be involved in various steps of cell growth. Both WU2 and WU2ΔpotD were able to grow in the presence of methylglyoxal bis(guanylhydrazone) (MGBG) and α-difluoromethylornithine (DFMO), although WU2ΔpotD grew much more slowly, possessing an extended lag phase compared with WU2 results. To determine whether the final intracellular polyamine concentrations for
Table 2. Concentration of intracellular polyamines for strains WU2 and WU2ΔpotD

<table>
<thead>
<tr>
<th>Growth Medium and Strain</th>
<th>Concentration (µM)</th>
<th>Spermidine</th>
<th>Cadaverine</th>
<th>Putrescine</th>
</tr>
</thead>
<tbody>
<tr>
<td>THY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WU2</td>
<td>25.56 ± 0.64</td>
<td>0.289 ± 0.048</td>
<td>0.479 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>WU2ΔpotD</td>
<td>21.64 ± 0.455</td>
<td>3.194 ± 0.375</td>
<td>3.555 ± 0.351</td>
<td></td>
</tr>
<tr>
<td>THY+ inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WU2</td>
<td>102.61 ± 3.08</td>
<td>3.897 ± 0.329</td>
<td>7.161 ± 0.237</td>
<td></td>
</tr>
<tr>
<td>WU2ΔpotD</td>
<td>22.64 ± 1.025</td>
<td>2.181 ± 0.195</td>
<td>3.37 ± 0.201</td>
<td></td>
</tr>
<tr>
<td>CDM + choline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WU2</td>
<td>18.935 ± 1.35</td>
<td>0.668 ± 0.119</td>
<td>1.44 ± 0.084</td>
<td></td>
</tr>
<tr>
<td>WU2ΔpotD</td>
<td>21.875 ± 0.76</td>
<td>0.342 ± 0.02</td>
<td>0.519 ± 0.048</td>
<td></td>
</tr>
<tr>
<td>CDM + choline + inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WU2</td>
<td>59.27 ± 5.185</td>
<td>1.127 ± 0.412</td>
<td>1.199 ± 0.042</td>
<td></td>
</tr>
<tr>
<td>WU2ΔpotD</td>
<td>7.075 ± 0.48</td>
<td>0.116 ± 0.013</td>
<td>0.115 ± 0.023</td>
<td></td>
</tr>
</tbody>
</table>

Note: potD – polyamine transporter D; WU2- mouse-virulent serotype 3 strain; WU2ΔpotD – potD deficient mutant; THY – Todd-Hewitt yeast extract; CDM-chemically defined medium.

both WU2ΔpotD and WU2 were similar, the concentration of intracellular polyamines was compared for both WU2 and WU2ΔpotD. In Table 2, the intracellular concentrations of the polyamines cadaverine, spermidine, and putrescine are compared for WU2 and WU2ΔpotD grown in THY or CDM plus 1 mg/ml choline with or without MGBG and DFMO added to the medium. The intracellular levels of the all three polyamines for WU2ΔpotD grown in both THY and CDM plus 1 mg/ml choline were similar, and these
concentrations did not differ much after the addition of MGBG and DFMO. For WU2, the intracellular levels of spermidine and putrescine did not differ much for both THY and CDM plus 1 mg/ml choline with or without MGBG and DFMO. Interestingly, the addition of MGBG and DFMO to the both mediums did increase the intracellular concentrations of cadaverine within WU2 fivefold for cells grown in THY and threefold for cells grown in CDM plus choline. Alternative polyamine transport systems have been previously reported for E. coli. The unaffected growth kinetics along with the increased levels of intracellular cadaverine within WU2 in the presence of MGBG and DMFO supported the idea of the existence of alternative polyamine transport systems in the pneumococcal as well.

CONCLUSION

A sensitive CE-LIF method was successfully validated and applied to determine biogenic PAs in bacterial cell extracts. FITC was used for the off-line derivatization and the FITC-PAs were completely separated in less than 8 min at a voltage of 20 kV. This CE-LIF method was proved to be sensitive, simple, fast, low cost and environment friendly and suitable to detect PAs in biological samples. By quantifying PAs in bacterial cell extracts, potD was proved to be involved in polyamine transport and is important for pathogenesis within various infection models. Polyamine transport was associated with the ability of a human pathogen to cause disease for the first time.
4. Separation and Quantification of Short-Chain Coenzyme A in Plant Tissues by Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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ABSTRACT

Coenzyme A (CoA) is a group of active metabolic compounds that facilitate over 100 chemical reactions in animal and plant cells. It mainly serves as an acyl carrier in many metabolic reactions and initiates the tricarboxylic acid cycle that produces more than 90% of the energy required for life processes. Measurements of short-chain and long-chain CoA compounds in a variety of tissues by using HPLC and CE-UV detection have been reported, but these techniques do not allow one to simultaneously determine all the possible coexisting CoA’s and their derivatives in plant tissues with sufficient sensitivity. In this paper, a method of quantitative determination of 5 short-chain CoA’s in plant tissues by using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was developed. Under optimized derivatization and electrophoresis conditions, different CoA’s that were derivatized with FITC were separated and quantified at the pmole level. A fused silica capillary with a 75 \textmu m (i.d.) \times 57 cm was used for the separation and 150 mM borate buffer (pH 9.00) was used as a background
electrolyte. The separation was carried out at 25 kV and completed in less than 13 min. The effects of derivatization time, buffer concentrations, and pH value on derivatization efficiency were also investigated systematically. This newly developed can be used to detect CoA’s in both plant and animal tissues.

KEYWORDS

Coenzyme A; capillary electrophoresis; laser-induced fluorescence; plant tissue

INTRODUCTION

Coenzyme A (CoA) is one of the most active metabolic compounds. It facilitates more than 100 chemical reactions in cells, including the metabolism of amino acids, carbohydrates, and lipids\textsuperscript{112-114}. Most importantly, CoA initiates the tricarboxylic acid (TCA) cycle that produces more than 90% of the energy required for life processes\textsuperscript{115}. CoA serves primarily as an acyl carrier in many metabolic reactions due to its active thiol (SH) group, which covalently bonds to an acyl group to form thioesters\textsuperscript{116,117}. The acyl group can then be actively transferred to various acceptor molecules due to its high free energy released during hydrolysis, as in the case of fatty acid biosynthesis. The chemical structure of CoA, and the different portions of the molecule are illustrated in Figure 1. Research data have also shown that CoA and its derivatives have a site-specific and reversible interaction with certain enzyme systems\textsuperscript{118} and can act well below the critical
micelle level\textsuperscript{119}. They are considered to be important effectors in cell metabolism because of this unique function. Despite the importance of this molecule in many crucial reactions, it accumulates at low concentration in cells. In fact its low abundance has been a major hindrance in elucidating the metabolism of this molecule and elucidating metabolic processes in which it is required as a cofactor. To discover the detailed functions of CoA and its derivatives in tissues and subcellular organelles, it is critical to develop an efficient, simple, and sensitive method to detect CoA compounds in biological samples.

Different methods have been reported for determining short-chain and long-chain CoA compounds in a variety of tissues, including enzymatic assays\textsuperscript{120, 121}, high-performance liquid chromatography (HPLC) conjugated with either spectroscopic or fluorometric detection and capillary electrophoresis with UV detection\textsuperscript{122, 123}. Since enzymatic assays only allow the determination of one CoA compound at a time, they are not useful for simultaneous determination of all possible coexisting CoA's and their derivatives. Although the HPLC method has long been used as the main technique for measurement of acyl CoA esters, it has quite a few drawbacks, including (1) long running times (45-120 min) associated with generating a large amount of organic wastes, (2) inadequate separation of unknown compounds with short-chain CoA's leading to misidentification and inaccurate quantitation, (3) large shifts of baseline during gradient elution programming, which often requires further solvent purification, and (4) tedious procedures for sample preparation and sample analysis. High-performance capillary electrophoresis (HPCE) has proven to be a rapid, simple, and sensitive technique for separating charged biomolecules with very high resolution\textsuperscript{124-127}. However, the current
method using UV as detector has the low sensitivity problem and cannot meet the requirement of CoA measurement in some biological samples. In this paper, we have demonstrated a capillary electrophoresis-laser induced fluorescence (CE-LIF) method for the first time to separate and quantify CoA compounds in plant leaves. CoA compounds first were derivatized with fluorescein-5-isothiocyanate (FITC), separated by CE and then detected using by LIF in order to improve detection sensitivity. This fast, sensitive and reliable method can be applied to determine CoA’s in both plant and animal tissues.

Figure 1. Chemical structure of Coenzyme A

MATERIALS AND METHODS

Chemicals

CoA standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). FITC was obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals that were used for buffer preparation and capillary rinsing, such as borate, acetone, methanol, sodium hydroxide and hydrochloric acid, were also from Sigma-Aldrich (St. Louis, MO, USA)
and of analytical reagent grade unless stated otherwise. Deionized water (18.2 MΩ) from Millipore Simplicity 185 - system (Millipore, MA, USA) was used to prepare standard solutions, BGE and other solutions.

**Preparation of BGE**

The derivertization buffer and BGE solution containing 170 mM and 150 mM borate respectively were prepared with deionized water and the pHs were adjusted to 9.00 by adding 1.0 M NaOH in the buffer before diluting it to the final volumes. These solutions were filtered through the 0.45µm membrane filter before use.

**Preparation of Standard CoA Solutions**

The 1.0 mM of CoAs and FITC stock solutions were prepared in DI water and acetone respectively. All stock solutions were kept at −20°C. The working solutions were prepared by diluting the stock solutions to the desire concentrations.

**Sample Extraction**

Plant leaves were harvested, immediately frozen in liquid N₂ and stored at −80°C until used. Before analysis, the frozen leaves (0.5–1.0 g fresh weight) was powdered using mortar and pestle, and then the powder was suspended in 2.0 mL of 5 % (w/v) iced trichloroacetic acid (TCA). The TCA suspensions were vortexed, centrifuged, and the supernatant was recovered. Repeated partitioning against diethyl ether removed TCA from the extracts, which were then dried under vacuum. The residues were dissolved in DI H2O and ready for derivatization.
Derivatization Procedure

12 µL CoA working solution or extracts was mixed with 30 µL of 1.0 mmol/L FITC, 258 µL of 0.2 mol/L borate buffer, vortexed and then reacted in the dark at 40°C in dry incubator for 10hrs. The solution was then diluted twice with H2O before injection to the CE system. A blank solution was prepared at the same time to serve as a control.

Instrumentation

All CE experiments were carried out on a Beckman Coulter P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA, USA) equipped with a LIF detector. Excitation was at 488 nm (argon ion laser) and the emission intensity was monitored at 520 nm (band-pass filter, bandwidth 10 nm). Electrophoretic data were acquired and analyzed by 32 Karat software versions 4. Separations were performed in fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with 75µm (i.d.) × 57cm (effective length). New capillaries were conditioned by rinsing with methanol for 15 min, deionized water for 5 min, 1.0 M HCl for 5 min, followed by deionized water for 5 min again, then 1.0 M NaOH for 20 min and deionized water for 5 min. The capillary was rinsed with deionized water for 5 min and then pre-run with BGE for 20 min under 25 kV every morning to obtain the best reproducibility. Samples were injected into the capillary at 0.5 psi for 10 s. After each analysis, the capillary was rinsed successively with 0.1 M NaOH for 1.0 min, deionized water, and BGE for 2.0 min respectively. CoA separation was carried out at 25 kV under 25 °C.
RESULTS AND DISCUSSION

CoAs are suitable to conjugate with fluorescence agent FITC and then be analyzed by capillary electrophoresis, due to the amine group in the molecule, the charges they carry, diverse molecular weight, and hydrophilic property of the derivatives. The quality of the CoA measurement in tissue depends on a number of derivatization and analytical parameters, such as derivatization and running buffer composition, pH, and concentration, the applied voltage on CE, the length and diameter of the capillary, and the sample size introduced, and so on. All these conditions were investigated systematically in this study in order to get the optimum derivatization and separation conditions. Figure 2 showed the separation of 5 CoA standards under optimal conditions by using CE-LIF. The derivatization was conducted in a 170 mM borate buffer at pH=9.00 under 40°C for 10hrs in dark. The running buffer was composed of 150 mM borate (pH=9.00) and the separation was conducted at 25 kV under with LIF detector 25 °C.

Optimization of Derivatization

FITC has been widely used in the derivatization of amines in CE\textsuperscript{109,129}, because it can react with primary and second amines and forms a derivative with an excitation maximum at 488 nm, which is coincident with the argon laser, and an emission maximum at 516 nm. The product information recommended condition for FITC derivatization is 0.2 mol/L carbonate buffer. In order to use the same buffer in derivatization and separation, and at the same time avoid the bubble problem of carbonate at the same time, borate was used as derivatization buffer. Eight different borate concentrations (30, 50, 100, 150, 170, 200, 250 and 300 mM) were examined to compare the derivatization
efficiency, which is shown in Figure 3. With the increase of the borate concentration, the peaks got more and more intensive until it reached 170 mM. Above 170 mM, no apparent derivatization efficiency improvement was observed with the increasing of derivatization buffer concentration. Since more concentrated buffer will adversely affect stability of derivatives\textsuperscript{109}, we chose 170 mM as the derivatization buffer concentration, even though high concentration buffer is of benefit to the derivatization.

Figure 2. Electropherogram of the separation of 5 CoA standards under optimal conditions by using CE-LIF. The derivatization was conducted in a 170 mM borate buffer at pH=9.00 under 40°C for 10hrs in dark. The running buffer was composed of 150 mM borate (pH=9.00) and the separation voltage was 25 kV with LIF detector. The separation was finished in a capillary with 75µm (i.d.) × 57cm (effective length) at 25°C. Sample was injected under 0.5psi for 10s. Peak identification: 1, Isovaleryl coenzyme A; 2, Acetyl coenzyme A; 3, n-Propionyl coenzyme A; 4, Isobutyryl coenzyme A; 5, Malonyl coenzyme A.
The most crucial parameter for the amine derivatization using FITC is the buffer pH. Slightly basic buffer would be beneficial for derivatization\textsuperscript{109}, since the derivatization involves the deprotonation of the amino-group, and basic solution will help shifting the dissociation equilibration to the deprotonation and therefore improve the derivatization efficiency. Buffer pH effects on derivatization efficiency of five CoAs were investigated and the results were shown in Figure 4. The results in figure 4 demonstrated that the derivatizing pH had remarkably effects on the derivatization efficiency and the efficiency increased continuously as pH increased until about pH 9.0, at which the fluorescence response achieved the highest value. Afterwards, the reaction efficiency decreased as the pH went up. Therefore, an optimized pH for derivatization in this study was maintained at 9.0 for the highest reaction efficiency. The pH of the derivatization buffer not only affected derivatization efficiency, but also affected separation result of capillary electrophoresis. A good separation will be obtained when the pH of derivatization buffer is the same as that of the running buffer\textsuperscript{109}. When the pH of derivatization buffer is lower than that of the running buffer, the acidity of sample band will result in the adsorption of FITC on the capillary wall and the tailing system peak will interfere with sample peaks. Therefore, the pH of the derivatization buffer was the same as that of the running buffer in this study, which will be discussed later.
Figure 3. Effect of derivatization buffer concentration on the separation of 5 CoA standards. The experimental conditions were the same as those of Figure 2, except for the borate concentrations.

Figure 4. The derivatization pH effect on the separation of 5 CoA standards. The experimental conditions were the same as those of Figure 2, except for the pHs of the derivatization buffer.
FITC to CoA ratio was also studied by using n-Propionyl CoA (due to the structural similarity of CoAs) and the result was shown in Figure 5. The efficiency of the derivatization reaction increased with the increasing of the FITC to CoA ratio. However, too much FITC will introduce interference and result in poor separation and quantification. For optimal detection sensitivity and accuracy, a FITC to CoA ratio of 5 was adopted in this study.

![Graph showing FITC to CoA ratio effect on peak area](graph.png)

Figure 5. FITC to CoA ratio on the separation of 5 CoA standards. The experimental conditions were the same as those of Figure 2, except for the FITC to CoA ratio. Peak identifications were the same as those in Figure 2.

The temperature and time of derivatization reaction were also found to affect the derivatization efficiency. After a series of experiments (data was not shown), 40ºC was considered the best temperature, since the relatively higher temperature speeded up the reaction; but higher than 40ºC, the derivatization product will decompose. The systematic study of the derivatization time effect on derivatization efficiency is shown in Figure 6.
We chose 10 hrs as derivatization time prior to injection to CE for analysis. Longer reaction time may induce product decomposition.

Figure 6. The derivatization time effect on the separation of 5 CoA standards. The experimental conditions were the same as those of Figure 2, except for the time of the derivatization. Peak identifications were the same as those in Figure 2.

Optimization of Separation

Running buffer is the key media for CE separation and so optimization running buffer is very important. Good buffer should have the following characteristics: good resolution; low current, low Joule heat; high fluorescence quantum yield and good buffer pH range and capacity. In this study, borate solution has been chosen as the running buffer, because of its buffer pH range, low current conductivity and good pH range for FITC derivatives. As mentioned above, the derivatization reaction was conducted in the alkaline solution. The running buffer pH should be basic in order to get good separation.
and sensitivity. The pH of the running buffer will influence the fluorescence intensities of FITC derivatives. According to the FITC product information\textsuperscript{130}, the fluorescence intensities are low and steady when pH is below 3.0. With the increasing of pH from 3.0 to 6.0, the fluorescence intensities rapidly increase. When pH is higher than 6.0, the fluorescence intensities reaches to a maximum and become steady. Therefore, good detection sensitivity and signal stability can be obtained under a basic condition; For the current study, four pHs (8.50, 9.00, 9.50 and 10.00) were tested and the result is shown in Figure 7. The optimal sensitivity and separation was obtained under pH 9.00. Separation efficiency was also greatly affected by the BGE concentration. Since FITC derivatized products were negatively charged and migrated against the EOF, a low molar concentration of BGE buffer, which decreased the EOF, decreased the separation efficiency. On the other hand, when the BGE concentration was too high, the EOF and Joule heating greatly increased, therefore causing a decrease in separation efficiency. Based on the above consideration, 150 mM borate was used.

The effect of applied voltages on the separation of five analytes was also investigated (data not shown). It was found that with the increases of the applied voltage, the analytes migration times were decreased and fast separation was obtained. However, when the applied voltage was higher than 25 kV, the analyte peaks were too close and it is hard to observe a baseline separation. In addition, due to the Joule heat caused by the increased electrophoretic current, the peak width expanded, which reduced the separation efficiency. Therefore, 25 kV was selected as the optimized applied voltage in our study.
Figure 7. The pH effect on the separation of 5 CoA standards. The experimental conditions were the same as those of Figure 2, except for the pHs of the running buffer. Peak identifications were the same as those in Figure 2.

**Linearity, Detection Limits, and Reproducibility**

A representative electropherogram of FITC-CoA derivatives and unreacted labeling agents is shown in Figure 2, demonstrating the excellent separation of these analytes under the optimized conditions. Five CoAs, which commonly exist in biological extracts and are of interest to biological researchers, were completely separated in less than 13 min. The migration order was 11, FITC, 1, 9, 8, 3. This elution order can be explained on the basis of the molecular structure and mass-to-charge ratio. For all 5 FITC-CoAs, the mass-to-charge ratio is high and a higher mobility is expected. Therefore, they moved in very short migration times to the detector side.

The linearity, reproducibility and detection limits for determination of FITC-CoA derivatives are listed in Table 1. The reproducibility was expressed as relative standard
deviation (RSD) values for both migration time and peak area and was calculated by using 0.1 mmol/L CoA standard solutions \((n = 5)\). The RSD values of migration time were between 1.97% and 3.89% and the RSD for peak areas were between 2.86% and 5.46%, which indicates a good reproducibility. The linearity of the method was determined by using standard mixtures at six concentrations from 0.5 to 500 nmol/L and correlation coefficients ranged from 0.9932 to 0.9988. Detection limits varied from 0.32 and 1.87 nmol/L with a signal to- noise ratio (S/N) of 3, which is sensitive enough to detect tissue extract CoA’s.

Table 1. The linearity, reproducibility and detection limits of FITC- CoA derivatives by CE-LIF. Derivatization and separation conditions are the same as that in Figure 2.

<table>
<thead>
<tr>
<th>CoA Standards</th>
<th>RSD (%)</th>
<th>Linearity ((R^2))</th>
<th>Detection Limit (nM, S/N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Migration Time</td>
<td>Peak Area</td>
<td></td>
</tr>
<tr>
<td>Acetyl coenzyme A</td>
<td>2.27</td>
<td>3.38</td>
<td>0.9986</td>
</tr>
<tr>
<td>Malonyl coenzyme A</td>
<td>1.97</td>
<td>2.86</td>
<td>0.9972</td>
</tr>
<tr>
<td>Isobutryl coenzyme A</td>
<td>2.06</td>
<td>3.05</td>
<td>0.9988</td>
</tr>
<tr>
<td>n-Propionyl coenzyme A</td>
<td>2.13</td>
<td>3.32</td>
<td>0.9968</td>
</tr>
<tr>
<td>Isovaleryl coenzyme A</td>
<td>3.89</td>
<td>5.46</td>
<td>0.9932</td>
</tr>
</tbody>
</table>
Analysis of Coenzyme A’s in Plant Leave Cell Extracts

Plant leaves were analyzed ($n = 5$) using the optimized CE-LIF method. CoA peaks in the cell extracts were identified by retention time and standard addition. The extraction recoveries for CoA’s present in leave extracts and results obtained from the analysis of cell extracts are shown in Table 2. The results were comparable to those of HPLC methods for CoA’s in plant tissue$^{131}$.

Table 2. CE-LIF results of Coenzyme A levels in plant tissue. The experimental conditions were the same as those of Figure 2. Amounts reported here were expressed as the mean ± standard deviation for three measurements.

<table>
<thead>
<tr>
<th>CoA’s</th>
<th>Concentration (nmol/g fresh weight)</th>
<th>Recovery (%)  ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl coenzyme A</td>
<td>$5.19 ± 0.2$</td>
<td>$87.6 ± 1.64$</td>
</tr>
<tr>
<td>Malonyl coenzyme A</td>
<td>$0.96 ± 0.1$</td>
<td>$83.5 ± 2.21$</td>
</tr>
<tr>
<td>Isobutyryl coenzyme A</td>
<td>$3.55 ± 0.05$</td>
<td>$90.8 ± 3.22$</td>
</tr>
<tr>
<td>n-Propionyl coenzyme A</td>
<td>$0.92 ± 0.01$</td>
<td>$88.7 ± 1.58$</td>
</tr>
<tr>
<td>Isovaleryl coenzyme A</td>
<td>$2.01 ± 0.05$</td>
<td>$91.2 ± 3.16$</td>
</tr>
</tbody>
</table>

CONCLUSION

A sensitive CE-LIF method was successfully developed for determination of CoA’s in plant cell extracts. FITC was used for the off-line derivatization and it was
found that the derivatization efficiency was the highest in 170 mM borate buffer at pH 9.00. Using 150 mM borate BGE at pH 9.0, FITC-CoA’s were separated in less than 13 min at a voltage of 25 kV. This CE-LIF method is sensitive, fast, at low cost and environment friendly. It can be applied to measure CoA’s in both plant and animal tissue and has the potential to be used for metabolite study in which CoA is required.

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