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Biomarker discovery using urinary metabolomics for noninvasive early cancer detection

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BIOMARKER DISCOVERY USING URINARY METABOLOMICS FOR NONINVASIVE EARLY CANCER DETECTION

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ABSTRACT

The development of new screening methods for the early detection of cancer remains one of the foremost challenges facing modern cancer research. The emergence of new analytical technologies and their application to “omics”-based approaches has provided researchers with powerful new tools for molecular biomarker discovery that may benefit early screening of cancer. This dissertation outlines several key advances made toward the application of urinary metabolomics to cancer biomarker discovery. The term urinary metabolomics here refers to the investigation of small metabolites in urine as potential disease biomarkers. The advantage of using this approach lies in its noninvasive sampling characteristics and robust analytical feasibility. The dissertation begins with the development of two analytical methods for the determination of sarcosine in urine for the early detection of prostate cancer. The next three papers discuss the analytical challenges facing the determination of pteridine derivatives in biological samples and present a new method to adjust their levels to patient hydration status and time since last urination. Briefly, pteridines and their derivatives function as intermediates in the metabolism of various vitamins and cofactors, where altered levels of pteridines have been reported in the urine of patients with several types of epithelial cancers, among other diseases. The following paper explores the possibility of using urinary metals as potential cancer biomarkers in a proof-of-concept study. The final two papers investigate the biological variation of urinary pteridines in order to better understand how urinary metabolites naturally fluctuate, and apply this information to a new method for the comprehensive determination of pteridine derivatives in urine. Taken together, this body of research presents new opportunities and challenges in the discovery of new cancer biomarkers.

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Paper VIII

LIST OF TABLES

SECTION

1. INTRODUCTION

1.1. CHALLENGES FACING EARLY CANCER DETECTION

Cancer is among the leading causes of death worldwide with over 8 million deaths reported in 2013.[1] Moreover, the global burden of cancer is expected to increase as risk factors for developing cancer, such as smoking, obesity, and sedentary lifestyles, become more prevalent.[2] Early cancer detection can reduce disease mortality by identifying premalignant and malignant tumors at an early stage of development when treatment may be more effective. However, conventional screening methods, such as medical imaging, mammograms, and colonoscopies, often involve invasive procedures that may be uncomfortable for patients or require advanced medical facilities that limit accessibility and affordability. False positives that result when a screening method cannot distinguish indolent and aggressive disease further contribute to the problem of overdetection and expose patients to unnecessary treatment. Overdetection resulting from insensitive mammograms and prostate specific antigen (PSA) screening has become especially problematic in the management of breast and prostate cancers, respectively. [3-5] For example, the extent of overdetection in breast cancer management is estimated to be as high as one in three breast cancers, prompting the American Cancer Society in 2016 to recommend new guidelines aimed at reducing the frequency of mammogram screening by raising the suggested age for women to begin screening and decreasing the frequency of periodic screening among women over 50.[6] In this way, new screening methods for detecting cancer earlier and more reliably are needed to better identify individuals who are at increased risk for developing cancer.

1.2. THE EMERGENCE OF URINARY METABOLOMICS

Modern research on early cancer detection has shifted focus onto the development of molecular biomarkers capable of reflecting underlying cancer biology. Whereas

conventional screening methods have limited spatial and temporal resolution that require the tumor to grow to a minimum size before detection is possible, molecular biomarkers are capable of providing details prior to disease onset and throughout the natural progression of the disease. Collectively, the applications of advanced high-throughput technologies to reveal the biochemistry of cancer and other diseases is referred to "omics" approaches, and can be classified by type of molecule studied. For example, genomics is concerned with the genetics of a given condition, while proteomics is concerned with the proteins involved in a given condition, and metabolomics involves the investigation of small metabolites in relation to a given condition, among other specialty subclasses. Previous efforts to characterize the genomes and proteomes of cancer have revealed remarkable complexity and heterogeneity, even among histologically similar tumors.[7] Nevertheless, the discovery of potential screening biomarkers from these genomic and proteomic studies has remained limited by difficulty in implementing such screening methods. Specifically, genomic analysis can readily reveal whether an individual is at increased risk for developing cancer, but analysis of genes alone cannot provide information on whether a malignant tumor already exists. Moreover, genomic analyses are generally limited to tissue samples for which an invasive biopsy from the suspected cancer site must be taken. Transcriptomics, concerned with the relationship between gene expression and disease, is an emerging "omics" approach that successfully bridges genomics with a responsive set of molecular biomarkers with better translatability to bodily fluids, such as serum.[8] Protein biomarkers have greater applicability to cancer screening methods than genetic markers while maintaining a high degree of disease specificity. Nevertheless, protein analysis in a clinical diagnostic setting requires specialized analytical techniques and invasive sampling techniques. In contrast, metabolites represent the final response of an organism to environmental, genetic, and disease factors [9, 10]. The responsiveness of metabolites to disease phenotype enables monitoring of subtle changes in the pathophysiology of multifactorial diseases, such as cancer[11]. Although the relationship between cancer development and cellular metabolism is complex and can be unique to individual tumors, cancer metabolism is often characterized by increased glycolysis and increased fatty acid synthesis, among other metabolic processes needed to support rapidly dividing cells, collectively referred to as the Warburg effect.[12-15]

Furthermore, metabolites may be sampled noninvasively in a myriad of biospecimen types, such as exhaled breath, saliva, and urine, with a greater variety of analytical techniques than is possible for genomic or proteomic biomarkers.[16, 17].

Among these biospecimen types, urine confers several advantages to cancer screening applications and is particularly well-suited for metabolite analysis[17, 18]. Namely, urine can be sampled noninvasively and economically in large volumes suitable for long-term storage. Urine further does not contain proteins, immunological factors, cells, and other matrix components common to serum and tissue lysates that require substantial pretreatment of samples prior to analysis. Nevertheless, the application of metabolomics-based approaches to cancer biomarker discovery in urine has only recently benefitted from the remarkable technological breakthroughs that have supported other "omics" strategies. Although urinary metabolites have been studied in relation to cancer for decades (e.g. [19, 20]), urinary metabolomics presents several challenges that comprise some of the major themes of this dissertation. For example, unlike genes or even proteins, metabolites can occur physiologically where their urinary levels become altered in the presence of cancer. This consideration requires the application of rigorous statistical models to determine whether a given metabolite concentration will be considered abnormal. The significance of this feature is highlighted by the shortcomings of the PSA test for prostate cancer in which PSA and other kallikrein proteins can become elevated due to benign prostatic hypertrophy and other conditions.[21] Moreover, urinary metabolites must be adjusted to patient hydration status and time since last urination. As this dissertation will later present, conventional strategies for rendering these corrections may be flawed or inappropriate for a given study population. Finally, the human urine metabolome is remarkably complex with over 4000 reported metabolites,[22] a feature that complicates the sensitive and specific determination of urinary metabolites due to the high potential of interfering compounds. This dissertation will outline the development of several analytical methods embedded with innovations to overcome these challenges. These new developments in the application of urinary metabolomics to cancer biomarker discovery have led to significant progress in a rapidly expanding and evolving field.

1.3. ANALYTICAL TECHNIQUES USED IN URINARY METABOLOMICS

Quantitative techniques for urinary metabolites can select from a rich variety of standalone and hyphenated analytical platforms for the optimal detection of selected metabolites. Mass spectrometry is a preferred detection method given its excellent sensitivity and multiplexing capacity. For quantitative analyses, tandem mass spectrometers in particular can provide fast acquisition times, a variety of possible scan modes, and wide dynamic ranges that allow these instruments to monitor trace level metabolites to primary metabolites such as amino acids within a single run. Tandem mass spectrometry may be further hyphenated with separation techniques, such as gas chromatography, liquid chromatography, or capillary electrophoresis for enhanced separation of urinary compounds and increased multiplexing capacity. Briefly, gas chromatography – tandem mass spectrometry (GC-MS/MS) is useful for the quantitative analysis of volatile compounds, and through the use of chemical derivatization, an additional subset of normally non-volatile compounds. However, the small number of volatile compounds present in urine and the potential selectivity issues and complicated procedures involved with chemical derivatization have generally limited its application for this purpose.[23]

Liquid chromatography continues to be the most widely used separation technique in the determination of urinary metabolites. The technique is best suited for the analysis of non-volatile and thermally labile compounds that comprise the overwhelming majority of urinary metabolites. The analytical capabilities of the technique have recently benefited from new column technologies including the development of novel stationary phase chemistries and reduced particle sizes that enable highly selective separation of metabolites in urine matrices. Liquid chromatography can further readily interface with a range of detector types, including mass spectrometry, UV absorbance, and fluorescence. However, liquid chromatography – tandem mass spectrometry (LC-MS/MS) continues to be the most common configuration given its superior sensitivity and selectivity and relative accessibility compared with other analytical techniques. Moreover, reference level accuracy can be obtained with this configuration through the use of stable isotope internal standards and isotope labelling strategies. For this reason, LC-MS/MS has been implemented in clinical diagnostic settings for the quantitative determination of various

metabolic biomarkers. This dissertation covers several advances made in the development of multiplexed LC-MS/MS methods for the simultaneous determination of pteridines and other compounds.

Finally, capillary electrophoresis presents another high-resolution separation technique for the analysis of highly polar or ionic compounds. Capillary electrophoresis has found limited applications in urinary metabolomics and cancer biomarker discovery given its poor reproducibility and challenges associated with its hyphenation to certain detector types, such as mass spectrometry. However, recent innovations in interface designs and advanced separation modes have overcome many of these earlier challenges.[24, 25] Taken together, these technological innovations have enabled extensive metabolic profiling in urine, resulting in the discovery of new putative biomarkers for the early detection of cancer.

1.4. URINARY PTERIDINES AS MODEL BIOMARKERS

Recent urinary metabolomics studies have revealed an array of putative biomarkers ranging from modified nucleosides[26] to kynurenine pathway intermediates[27] to pteridines[28] to many others. Among these, the clinical investigation of urinary pteridines has best exemplified progress made in the application of urinary metabolomics to cancer biomarker discovery through the translational challenges that have been overcome and is discussed in great detail later in this dissertation. Briefly, pteridines and their derivatives function as intermediates in the metabolism of several vitamins and cofactors. Pteridines are one of the most widely studied classes of metabolites in urine, owing to their original isolation in urine and the resulting analytical methods that were developed for their quantitation in urine.[29] Although many of these early studies examined the putative roles of pteridines in cancer given their relationships to the DNA nucleotide and folic acid metabolic pathways, and reported elevated levels of several such pteridines in certain epithelial cancers,[30-34] the clinical investigation of urinary pteridines and their biological function in cancer have until recently been frustrated by limited analytical capabilities. However, the biological function of some pteridines, such as 5,6,7,8-tetrahydrobiopterin (BH4) and neopterin, have been elaborately studied and provide important context for the probable roles of other pteridine

derivatives in cancer. Briefly, BH4 is derived from guanosine triphosphate and functions as an essential cofactor for several notable classes of enzymes, including amino acid hydroxylases involved with neurotransmitter biosynthesis, nitric oxide synthase which regulates vasodilation and neuroprotection, and alkylglyercol monooxygenase which mediates the biosynthesis of ether lipids involved with structural components, chemical signaling, and more. For this reason, BH4 deficiencies can present a spectrum of clinical disorders, and is consequently used as a screening biomarker for several diseases. Meanwhile, neopterin and its derivatives are produced selectively in monocytes, macrophages, dendritic cells, and endothelial cells following activation by the cytokines and interferon gamma (IFN-γ) released by T lymphocytes and natural killer cells in a similar manner to BH4.[35] As a result, neopterin has been studied extensively as a marker for activated cellular immune response in an array of medical conditions, ranging from bacterial and viral infections to autoimmune diseases to cancer. In addition to BH4 and neopterin derivatives are many other pteridines and related lumazines that have been reported in humans, but whose biological function and biosynthetic pathways remain to be established.[34]. This dissertation presents several analytical methods for the determination of these compounds in urine and cell lysates in order to better understand their roles in cancer as putative screening biomarkers.

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PAPER

I. A NOVEL ENZYMATIC TECHNIQUE FOR DETERMINATION OF SARCOSINE IN URINE SAMPLES

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ABSTRACT

Metabolites impart a significant importance to the understanding of biological reactions, and consequently, to the development of diagnostic and therapeutic techniques for specific diseases. Furthermore, there has been recent interest in metabolite concentrations present in urine for potential noninvasive disease diagnosis. The detection of specific metabolites, however, presents certain analytical difficulties such as low or ambiguous specificity of the techniques. This study developed a new technique, utilizing oxidative, enzymatic production of formaldehyde from the metabolite to produce a pH-induced change observed by fluorescein in acetone. This probe displays high sensitivity towards pH imbalances, and coupled with high enzymatic specificity, forms an accurate method to measure metabolite concentrations. Sarcosine was used as a model analyte in this study due to its potential for serving as a prostate cancer biomarker. Sarcosine was treated with sarcosine oxidase to generate formaldehyde, which was further oxidized to formic acid, and subsequently measured by the corresponding change in fluorescein. A good linearity was revealed with a correlation coefficient of 0.9961 and a detection limit of 20 $nmol \cdot L^{-1}$. This method was applied to sarcosine analysis in nine urine samples. The results suggest that this is a viable, cost-effective technique for determination of sarcosine in urine samples without interferences such as alanine.

KEY WORDS

Sarcosine, sarcosine oxidase, fluorescein, assay, urine analysis

1. INTRODUCTION

1.1. Background

Tests for prostate-specific antigen (PSA), a common marker for prostate cancer diagnostics, cannot accurately differentiate between benign prostate conditions and active prostate cancer.[1] This well-practiced technique, which relies upon an abnormally high presence of the antigen in serum, thought to be correlated with increased prostate gland size, is also well-known for being both false positive- and negative-prone.[2] It has further been shown that the mortality rate does not significantly improve with PSA screening.[3] This lack of sufficient diagnostic techniques for prostate cancer highlights the need for more effective screening methods.

Whereas many diagnostic techniques rely on the presence of antigens, like PSA, metabolites can serve and equally important diagnostic role. Indeed, metabolites, as the intermediates and products of metabolic exchanges, have an intimate relationship with systemic changes in an organism. An important implication of this is that of carcinogenesis; while cancer has been traditionally seen in terms of gene regulation, affecting control and signal processes, disjoint from metabolites, it is becoming increasingly apparent that metabolites operate as key functions in the development of several types of cancer through numerous mechanisms, such as modulating various signal transduction systems.[4]

Effective analysis and quantification of such metabolites in applicable media may then characterize the progression of certain cancers when practical difficulties would otherwise exist.[5,6] An example of this concept, applied to prostate cancer, was utilized in an attempted metabolomic profiling of biopsy-positive prostate cancer patients and biopsy-negative prostate control individuals that suggested a correlation between sarcosine levels and progression of the disease.[7] This finding brought about great interest in determining sarcosine in various media, and although the correlation between sarcosine and prostate cancer has since been refuted[8,9], it adds stress to the importance of metabolites, such as sarcosine, as novel markers for cancer diagnostics.

Analytical methods for direct determination of metabolites from cells, plasma, or urine often include a combination of liquid chromatography and gas chromatography with mass spectroscopy. However, the major disadvantages of these techniques are high instrumentation costs and the interferences from other chemicals; for example, in the case of sarcosine, alanine has the same exact mass as sarcosine and often co-elutes with it, traditionally complicating normal chromatographic techniques.[10] Although chromatographic techniques have been reported to fully separate alanine and sarcosine, [11,12] these techniques require instrumentation not generally available to clinics.

Substrate-specific enzymes, however, have shown to be useful analytical reagents.[13,14] One class in particular is the oxidoreductases, which often produce reactive oxygen species (ROS), such as peroxidase and hydrogen peroxide, respectively, being first reported in 1846.[15] In the case of sarcosine, sarcosine oxidase serves as a catalyst in the oxidative demethylation of sarcosine, an intermediate metabolite, to glycine, formaldehyde, and hydrogen peroxide. Direct determination of sarcosine can be difficult, but indirect quantification can be achieved by this reaction. Indeed, an established analytical assay for sarcosine includes the sarcosine oxidase-mediated demethylation of sarcosine to hydrogen peroxide, which reacts with 4-aminoantipyrine and phenol to yield quinoneimine dye (Scheme 1)[16]. The disadvantage is that such colorimetric analyses can be skewed in complex matrices like urine and blood.

Scheme 1. Colorimetric assay of sarcosine via production of quinoneimine dye.

$$Sarcosine + O_2 + H_2O + Sarcosine\ Oxidase \rightarrow Glycine + Formaldehyde + H_2O_2$$

$$2H_2O_2 + Phenol + 4\text{-}aminoantipyrine + Peroxidase \rightarrow 4H_2O + Quinoneimine\ Dye$$

A proposed solution to this skewing is the fluorimetric determination of sarcosine. One particular subset of fluorescein derivatives, including the commonly used 2',7'-dichlorofluorescein diacetate (DCFH-DA), is currently being recognized for its high specificity to reactive oxygen species. Many of these compounds have reported detection limits in the nanomolar concentration range, providing useful probes for oxidative stress in

cellular systems.[17,18,19,20] Their premise was based upon the theory that non-fluorescent fluorescein derivatives will fluoresce upon oxidation by hydrogen peroxide and other ROS, and that this fluorescence is directly proportional to the concentration of ROS present.[21]

Indeed, this subset of fluorescein derivatives is growing every day, and the specialized probes cited above formed the original basis of the proposed method for their relative structural and synthetic simplicity to newer compounds. Although this type of probe has the capability to indirectly quantify sarcosine by production of hydrogen peroxide via sarcosine oxidase, there exists several limitations: (1) as fluorescein derivatives, they are highly sensitive to pH changes and (2) hydrogen peroxide decomposition needs to follow a radical pathway. Under optimal conditions, the oxidation of sarcosine is significantly favorable to the further oxidation of formaldehyde to formic acid. The associated pH change from this was found to be more significant than that oxidation of fluorescein by radicals formed by hydrogen peroxide decomposition. In addition to this, the basic conditions under which sarcosine oxidation occurs seemed to have favored the decomposition of hydrogen peroxide into water and oxygen gas, not radicals. Furthermore, synthetic preparation of these specialized fluorescein derivatives is often complex, and the direct purchase is expensive, when compared with pure fluorescein. In this study, a novel and low cost enzymatic technique has been developed for determination of sarcosine in urine samples, not by measuring ROS formed from hydrogen peroxide decomposition, but the pH change associated with formaldehyde being oxidized to formic acid.

1.2. Reaction Mechanism

The developed method relies simply on the pH sensitivity of ordinary fluorescein. Under acidic conditions (pH<4), the non-fluorescent lactone form of fluorescein exists in high concentrations. Prepared in acetone, it is nearly the only form present and is furthermore readily soluble. Upon exposure to a basic reagent, lactoid fluorescein will readily change to the highly fluorescent monoanionic and dianionic forms. Thus, changes to the pH of a standard reagent can be quantitatively determined by the lactoid fluorescein, even with low concentrations and/or volumes. For the determination of sarcosine, this can

be achieved by an enzymatic oxidation of sarcosine to glycine, resulting in hydrogen peroxide and formaldehyde as byproducts, and subsequent oxidation of formaldehyde to formic acid and methanol (Scheme 2). In the presence of NaOH and heat, the Cannizzaro reaction proceeds converting the formaldehyde to formic acid and methanol while degrading the hydrogen peroxide without forming interfering radicals. The conditions of the Cannizzaro reaction catalyze the decomposition of hydrogen peroxide to water and oxygen gas with minimal ROS being formed. The resultant decrease in pH from the formic acid results in a quantifiable deviation from the standardized pH of the solution without interference from radicals from hydrogen peroxide. In this way, the coupling of an enzyme and ordinary fluorescein provides two advantages: (1) high specificity for sarcosine, and (2) a relatively cost-effective alternative to current methods such as DCFH-DA and LC-MS analysis.

Scheme 2. Proposed method for the determination of sarcosine.

$$Sarcosine + O_2 + H_2O + Sarcosine\ Oxidase \xrightarrow{37°C} Glycine + Formaldehyde + H_2O_2$$

$$Formaldehyde \xrightarrow{NaOH,\ 50°C} Formic\ acid + Methanol$$

$$H_2O_2 \xrightarrow{NaOH,\ 50°C} O_2 + H_2O$$

$$Lactoid\ Fluorescein\ in\ acetone\ (non\text{-}fluorescent) \xrightarrow{FormicAcid} Dianion\ Fluorescein\ (fluorescent)$$

The combination of the two steps, substrate-specific enzymatic production of formaldehyde followed by conversion to formic acid coupled with subsequent fluorimetric analysis by fluorescein, supplies an effective, indirect, analytical technique for metabolite-specific determinations without interference from ROS. This idea was demonstrated by the determination of sarcosine in urine and further comparison to the same samples being analyzed using LCMS/MS methods[9] where alanine is presumed to be an interference.

2. EXPERIMENTAL

2.1. Preparation of Sarcosine Oxidase

Sarcosine oxidase from *Corynebacterium* sp. (lyophilized powder, 5-10 units/mg) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). A 20 mmol·L^{-1} potassium phosphate solution was prepared from ultra-pure water and potassium phosphate monobasic, and the pH was adjusted to 8.3 with 1 mol·L^{-1} sodium hydroxide. To 1 mL of buffer was added 0.7 mg enzyme. The reagent was prepared fresh and chilled at 4 degrees Celsius.

2.2. Preparation of Sarcosine Stock Solution

The sarcosine and Tris-HCl buffer used in this study were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Ultra-pure water was generated using a Milli-Q Advantage A10 water purification system, 18MΩ (Millipore, Billerica, MA, USA). A 200 mmol·L^{-1} Tris-HCl buffer was prepared with ultra-pure water and Tris base, and the resulting pH was adjusted to 8.3 with NaOH. This represented the optimal pH of sarcosine oxidase and fluorescein. A stock 2.5 µmol·L^{-1} sarcosine solution was prepared in the buffer.

2.3. Analysis of Sarcosine Standard Solutions

Into a 250 µL Corning Costar 96 flat bottom plate were added insertions of the sarcosine stock solution. These insertions were based on the sarcosine concentration range (200-1400 nmol·L^{-1}) reported by LC-MS/MS analysis of urine samples diluted 750 times. This insertion was supplemented with enough Tris-HCl buffer to make 75 µL. The plate was then heated to 37 ^{o}C. To this was added 25 µL cold enzyme reagent, and the plate was then incubated at 37 ^{o}C for 30 minutes. Upon incubation, the plate was chilled to room temperature, 5 µL 500 mmol·L^{-1} NaOH inserted, and re-incubated at 50 ^{o}C for 10 minutes to ensure complete formation of formic acid. The plate was then cooled to room temperature again, and fluorescence readings were taken. Upon which 5 µL 15 nmol·L^{-1} fluorescein dissolved in acetone was added, resulting in a total volume of 110 µL per sample. The plate was then shaken in double orbital fashion for two minutes and analyzed two hours later.

2.4. Analysis of Urine Samples

Urine samples (*n*=10) were provided by the Central Missouri Urology Clinic. The set was comprised of five normal samples and five prostate cancer positive samples. Prior to subjection to the method outlined in this study, the samples were analyzed via liquid chromatography with tandem mass spectroscopy.[9] The supernatant of the samples were then properly diluted in buffer solution whereby 75 µL was inserted into the well plate. The procedure then followed that of the sarcosine standards.

2.5. Instrumentation

Fluorescence emission spectra were obtained with a BMG Labtech Fluorstar Omega (Ortenberg, Germany) plate reader equipped with a xenon lamp. Excitation and emission wavelengths were attuned to 485 nm and 520 nm, respectively. The lamp was allowed sufficient time to warm prior to measurement. Data was collected and analyzed via BMG Omega software.

3. RESULTS AND DISCUSSION

3.1. Spectral Properties

It is well observed that fluorescein can exist in several forms: the highly fluorescent dianion form, the moderately fluorescent monoanion form, and three neutral tautomers: the quinoid, lactone, and zwitterionic. Furthermore, fluorescein is highly sensitive to both pH and solution, and the proportion of the forms present is consequently dependent upon those as well. The colorless, nonfluorescent lactone form has the greatest proportion (>99%) in acidic conditions (pH<4.3), while the highly fluorescent dianion form is found best in pH conditions greater than 6.37.[22] Dissolution in acetone, however, achieves an equally high proportionality of the lactone form while providing a relatively acidic environment (pH=6.1) similar to that of urine. Therefore, addition of base raises the concentration of the fluorescent forms to the optimal conditions of fluorescein (pH=8.3). **Figure 1** illustrates the difference between fluorescein fluorescence in acetone and in buffered solution.

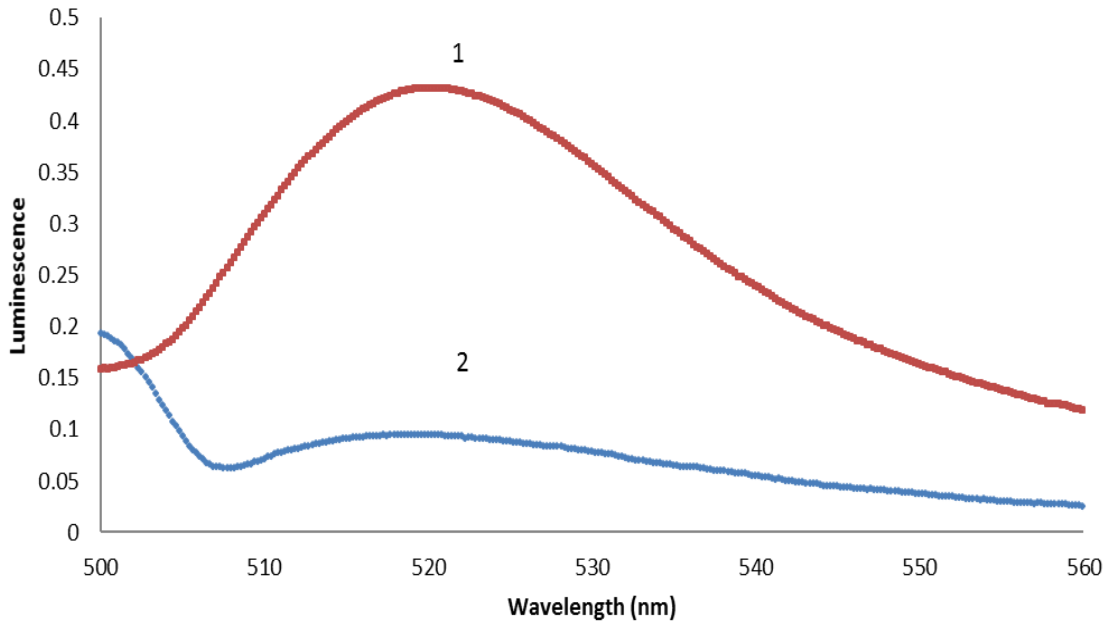

Figure 1. Emission spectra of 300 μmol·L^{-1} fluorescein in (1) acetone (pH= 6.1) and (2) in buffer solution (pH=8.3) at 25°C.

3.2. Linearity, detection limits, and reproducibility

Sarcosine standard solutions were incubated with sarcosine oxidase in basic conditions to generate formic acid in order to produce a measureable pH change. A stable calibration curve (**Figure 2**) was obtained from a set of sarcosine standards ranging from 23 nmol·L^{-1} to 1704 nmol·L^{-1}. A fairly linear relationship was observed over this range with a correlation coefficient of 0.9961. The limit of detection was determined to be 20 nmol·L^{-1} with a signal-to-noise (S/N) of 3. Reproducibility was measured (n=5), yielding a standard error of 4.2%. Based on this data, it was concluded that this technique is applicable over the given concentration range.

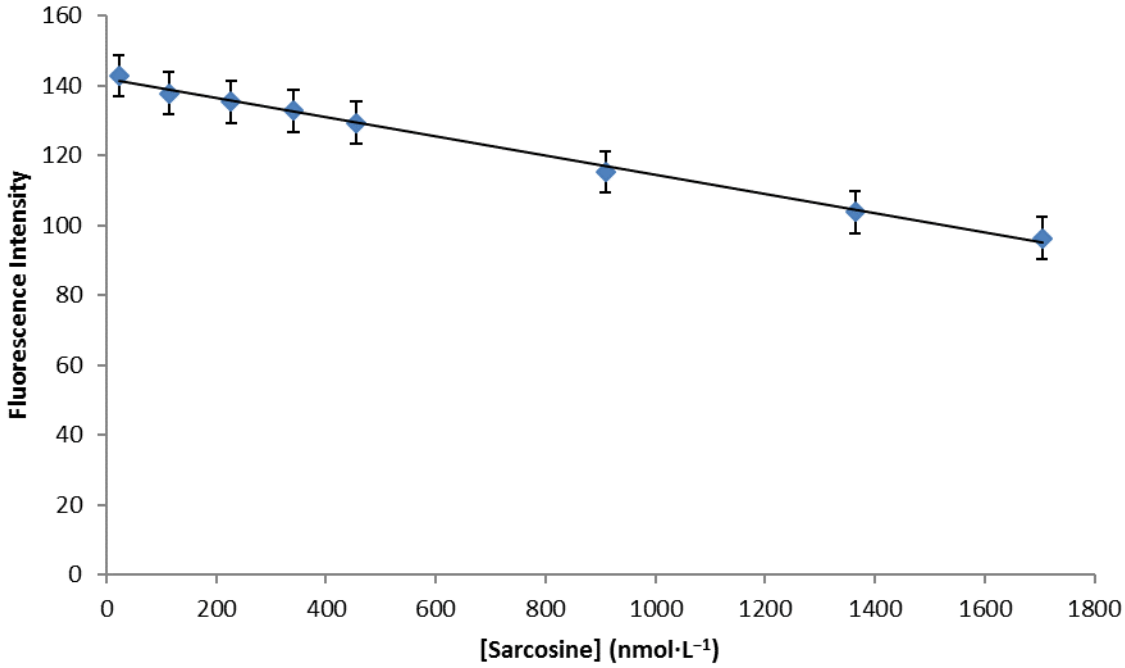

Figure 2. Calibration plot constructed from sarcosine standards ranging from 23 to 1704 nmol·L^{-1} (R^2 = 0.9961).

3.3. Determination of Sarcosine from Urine

Ten urine samples were analyzed via liquid chromatography with tandem mass spectroscopy (with known interferences from alanine) and via the proposed fluorimetric determination of sarcosine. Analyte concentrations were compared for the undiluted urine samples with the comparisons shown below (**Table 1**). Three preparations of each sample were prepared to provide validating statistical significance. Consequently an acceptable degree of reproducibility was established. All samples fell within the calibrated range except for one which initially yielded an undetectable amount of sarcosine. Similarly this sample produced a significantly low value by LC-MS/MS techniques as well. This zero value may be credited to the relatively high limit of detection (20 nmol·L^{-1}) of the fluorimetric method compared with that of LC-MS/MS (0.6 nmol·L^{-1}). In order to supply a non-zero value, the urine sample was diluted less until a detectable signal was achieved, and the base urine concentration was subsequently calculated. Nonetheless, the data

suggests the fluorimetric method generally produces lower sarcosine concentrations than the LC-MS/MS technique. This is expected because the LC-MS/MS technique utilized provides only a partial, if any, separation of alanine and sarcosine whereas the fluorimetric method, due to its enzymatic nature, displays a much higher specificity for sarcosine.

Table 1. Reported sarcosine concentrations in urine samples (n=10) by two methods: (1) LC-MS/MS and (2) fluorimetric method, and their corresponding percent differences.

LC-MS/MS (mmol·L^{-1})	Fluorimetric (mmol·L^{-1})	Percent Difference
342.53	320.18±10.13	-6.53
278.50	251.22±6.54	-9.80
695.90	545.84±22.43	-21.56
195.92	201.15±4.33	2.67
1061.19	572.39±32.11	-46.06
136.68	195.45±4.99	43.00
268.33	248.28±12.15	-7.47
185.71	136.37±7.36	-26.57
333.45	315.78±4.23	-5.30
149.89	45.82±4.97	-69.43

Although a general decrease in sarcosine concentrations has been observed, the decrease is not statistically significant (P-value = 0.23, paired t-test). This insignificance is actually expected; LC-MS/MS is a very precise analytical tool, much more so than fluorimetry. Despite its insignificance, the general decrease can reasonably be attributed to the utilization of the enzyme. The fluorimetric assay consisted of two general types of interferences: non-enzymatic oxidation of fluorescein, and inhibitors and substitutive substrates of sarcosine oxidase. The former, including urine acidity and oxidative components, was neglected due to the 750-fold dilution of the urine in 200 mmol·L^{-1} Tris-HCl buffer. These buffered urine solutions were observed to have an equivalent pH as the sarcosine standards. Furthermore, it was found that the average pH of the unbuffered urine samples varied only slightly to that of unbuffered sarcosine. Since the change in

fluorescence, not the final fluorescence, was used to determine sarcosine, the very minor deviations in buffered urine pH were negated. The employment of a strong buffer in very dilute samples also increases the application of the assay to other biological fluids, because the buffering capacities of said fluids can also be negated when compared with that of the strong buffer. Although inhibitors to sarcosine oxidase (including many inorganics) are likely present in urine to a small extent, sarcosine oxidase was provided in excess to the estimated sarcosine to counter this fact. One key substitutive substrate was present, however, d-Alanine. To assess the impact of its interference, d-alanine standard was compared with d-alanine treated with sarcosine oxidase under similar conditions using LC-MS/MS. An insignificant percentage (0.34%) of the d-alanine was found to be degraded by sarcosine oxidase. This determination corroborates previously reported numbers. Thus by eliminating possible interferences, it can reasonably concluded that the general decrease observed by the fluorimetric method can be attributed to the use of sarcosine oxidase.

A comparison plot of the two methods is shown below (**Figure 3**). It is evident that the rate of change reported by the fluorimetric assay is much more pronounced as sarcosine concentrations lower, compared with that of the LC-MS/MS method, implying increased relative sensitivity of the fluorimetric method to lower concentrations of sarcosine. This is reasonable: prior to fluorescein exposure, the pH of the sample was significantly above the pKa of the buffer (pKa=8.06). Thus, as the sample pH approached the pKa of the buffer by production of formic acid, the buffering capacity was increased, explaining why lower sarcosine concentrations experienced a greater respective change in fluorescence than higher concentrations. This certainly presents a limitation to the fluorimetric assay described herein, but also implies an enhanced sensitivity to sarcosine concentrations near the lower limit of quantitation.

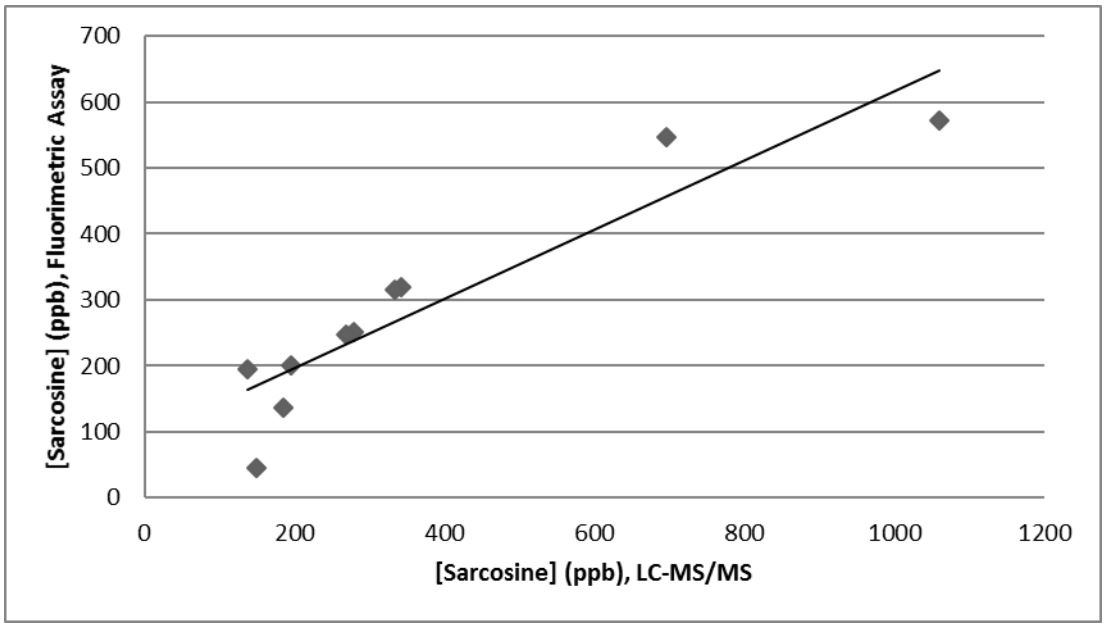

Figure 3. Correlation between the fluorimetric and LC-MS/MS techniques used to analyze sarcosine concentrations in 10 urine samples ($R^2 = 0.8466$).

Furthermore, sarcosine levels, as well as any other metabolite, can vary greatly among urine samples due to a large array of factors such as fluid intake, prevalent diseases, diet, and more. Thus for clinical purposes, sarcosine alone cannot be used as a marker. As such, a ratio of sarcosine levels to creatinine, analyzed with LC-MS/MS acting as a sort of internal standard, was constructed. Although creatinine was not determined using the fluorimetric method, there are a host of quantitative methods for creatinine, and furthermore, creatinine may also be determined by the fluorimetric assay by converting it to creatine with creatinine amidohydrolase, and subsequent conversion to sarcosine by creatine amidinohydrolase. This ratio can be used for cancer comparisons, and although diagnostic comparisons between cancer positive and cancer negative samples were outside the scope of this current study, this data was readily available and further illustrates that this simple fluorimetric method can be applied to diagnostic purposes (**Figure 4**).

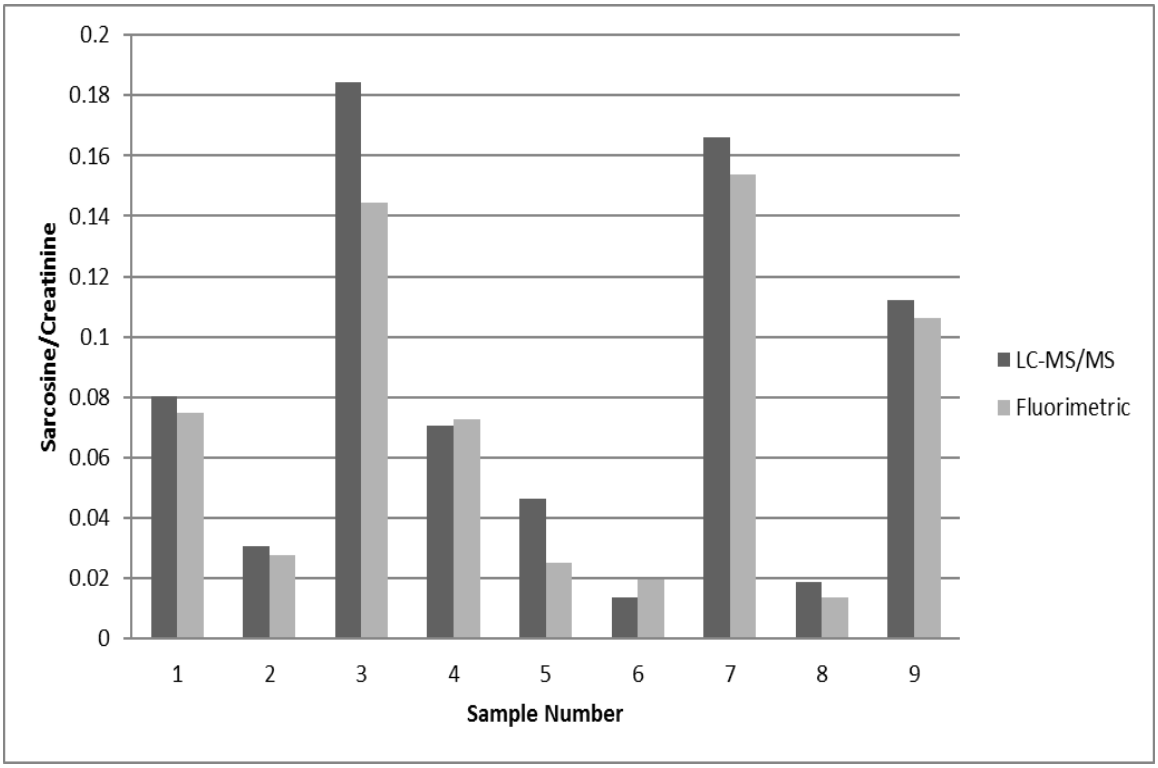

Figure 4. Comparison of sarcosine-to-creatinine ratio for LC-MS/MS and fluorimetric methods.

3.4. Recovery

Spiked recovery tests were conducted utilizing three different sarcosine concentrations. The sarcosine was added to the same urine sample prior to dilution in the following doses: 20 mmol·L^{-1}, 100 mmol·L^{-1}, and 500 mmol·L^{-1}. Analyte recovery in the low concentration was 86.45%, while recovery in the middle concentration was 106.32%, and recovery in the high concentration was 102.45%.

4. CONCLUSION

This study aimed to present a novel fluorimetric technique for determination of sarcosine in urine by enzymatic production of formic acid to produce a pH change subsequently measured by fluorescence change of fluorescein. The utilization of the highly specific sarcosine oxidase has eliminated interferences caused by the presence of alanine,

at least to a further extent than the LC-MS/MS technique used alongside the fluorimetric method. Although sarcosine has been successively separated in other LC-MS/MS techniques, the concept of a fluorimetric method provides a much more economical determination of sarcosine than these more advanced chromatographic techniques. The methodology presented here can further be applied to a large array of analytes which can act as substrates to enzymes, especially to oxidases. For example, d-amino acid oxidases (DAO) act on a similar mechanism as sarcosine oxidase, producing a keto acid, and ammonia and hydrogen peroxide as byproducts. In this case, the keto acid may be evaluated directly very similarly to the one described here. Even though the sensitivity of this technique is lower that the LC-MS/MS method, it provided a fast and low cost technique for sarcosine analysis. The real novelty of the study is the coupling of the highly-specific enzymatic approach with the economical fluorimetric analysis to provide an alternative technique for sarcosine analysis in complex matrices, such as urine; thus, the basis of a quick, accurate, and cheap clinical diagnostic tool has been established.

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II. PARTIAL ENZYMATIC ELIMINATION AND QUANTIFICATION OF SARCOSINE FROM ALANINE USING LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

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ABSTRACT

Since sarcosine and *D,L*-alanine co-elute on reversed-phase HPLC columns and the tandem mass spectrometer cannot differentiate them due to equivalent parent and fragment ions, derivatization is often required for analysis of sarcosine in LC/MS systems. This study offers an alternative to derivatization by employing partial elimination of sarcosine by enzymatic oxidation. The decrease in apparent concentration from the traditionally merged sarcosine-alanine peak associated with the enzymatic elimination has been shown to be proportional to the total sarcosine present (R^2=0.9999), allowing for determinations of urinary sarcosine. Sarcosine oxidase was shown to eliminate only sarcosine in the presence of *D,L*-alanine, and was consequently used as the selective enzyme. This newly developed technique has a method detection limit (MDL) of 1 µg/L (ppb) with a linear range of 3 ppb - 1 mg/L (ppm) in urine matrices. The method was further validated through spiked recoveries of real urine samples, as well as the analysis of 35 real urine samples. The averages recoveries for low, middle, and high sarcosine concentration spikes were 111.7%, 90.8%, and 90.1%, respectively. In conclusion, this simple enzymatic approach coupled with HPLC/MS/MS is capable of resolving sarcosine from *D,L*-alanine without the need for chemical derivatization or specialty chromatography columns.

KEY WORDS

Sarcosine, Sarcosine Oxidase, HPLC/MS/MS, Alanine, Enzymatic Elimination

1. INTRODUCTION

Metabolic diagnostics is a developing area in disease identification, deviating from the traditional antigenic-based diagnostics [1]. As the intermediates, final products, and byproducts of cellular processes, metabolites are highly representative of biochemical changes within the body. Likewise, it has been shown that metabolites are highly involved in carcinogenic processes through a number of modes, and consequently, they may be used as diagnostic analytes [2]. However, a number of analytical techniques lack the specificity required in the complex matrices, such as serum, blood, and urine, in which these metabolites are naturally found, presenting significant analytical difficulties to the elucidation of the diagnostic roles of the metabolites [3,4].

This was recently highlighted in 2009 by the metabolomic profiling of biopsy-positive prostate cancer patients and biopsy-negative prostate control individuals that suggested a correlation between urinary sarcosine levels and progression of the disease [5]. This claim was later refuted by two independent studies [6,7], which in the process placed further stress on the requirement of specificity for the analytical techniques employed. In particular, these sarcosine-based studies relied on a combination of liquid and gas chromatography with mass spectrometry. These methods, however, have inherent issues in the case of biological sarcosine. Gas chromatography is not well-suited for the separation of matrices involving non-volatiles, lending separation to liquid chromatography. In biological assays, *L*-alanine is co-present with sarcosine, and exists as an isomer of sarcosine. In untreated samples using reversed-phased columns, sarcosine and *D,L*-alanine co-elute, requiring further sample preparation for liquid chromatography such as chemical derivitazation [8]. Indeed, derivatization-based separation of sarcosine and *D,L*-alanine has been achieved by at least two independent studies [9,10], improving upon previous studies on sarcosine as a marker for prostate cancer [11]. The isomerism of the two compounds affects mass spectrometry as well, each possessing an identical precursor mass, 89.0932 Daltons. Furthermore, fragmentation in tandem mass spectrometry fails to resolve the two isomers due to equivalent fragmentation. Thus, without specialized, chemical preparation of the samples, sarcosine and *D,L*-alanine cannot be accurately determined by the methods outlined above [12].

One preparation technique, overlooked by these traditionally chemical-based studies, involves the use of biological catalysts as analytical reagents. Indeed, substrate-specific enzymes have been shown to be highly effective in analytical chemistry [13,14]. In the case of sarcosine, there already exists a fluorimetric assay for urinary sarcosine employing enzymatic oxidation of sarcosine to detectable byproducts [15]. Despite its success, this technique lacks the robustness of chromatographic - mass spectrometric techniques while maintaining a complex reaction scheme. It does, however, effectively demonstrate that enzymatic selection for sarcosine over *D,L*-alanine in urine samples can be used for sarcosine analysis.

Since alanine and sarcosine co-elute on reversed-phase columns in high performance liquid chromatography with tandem mass spectrometry (HPLC/MS/MS) techniques, one large, merged peak that represents the total sum of urinary alanine and sarcosine is observed. Hence, selective enzymatic degradation of one of the species, such as enzymatic oxidation of sarcosine by sarcosine oxidase, will result in a marked decrease from the original peak. If the percent sarcosine degraded is known, then it follows that the resulting decrease in apparent concentration is proportional to the original amount of sarcosine. In this way, analysis of sarcosine involves only partial enzymatic elimination of the analyte, not indirect determination from byproducts and subsequent reactions. This idea is examined herein by the determination of sarcosine in urine as well as further comparison with the fluorimetric method [15] (as shown in **Scheme 1**).

Scheme 1. Proposed reaction scheme for the determination of sarcosine.

$$Sarcosine + Alanine \xrightarrow{Sarcosine\ Oxidase,\ 37°C} Sarcosine\ byproducts + Alanine$$

Thus, an alternative, enzymatic approach combined with HPLC/MS/MS, has been proposed for urinary analysis of sarcosine without using either derivatization.

2. MATERIALS AND METHODS

2.1. Chemicals

Sarcosine oxidase from *Corynebacterium* sp. (lyophilized powder, 5-10 units/mg) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Glycylglycine used as an enzymatic buffer, sodium hydroxide as a pH modifier, as well as three standards, including sarcosine, *D,L*-alanine, and creatinine were also purchased from Sigma-Aldrich (Saint Louis, MO, USA). Additionally, acetonitrile (LC/MS grade) and formic acid (99.9%) used in the preparation of the mobile phase was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Ultra-pure water was generated using a Milli-Q Advantage® A10 water purification system (Millipore, Billerica, MA, USA). Synthetic urine was purchased from LoSo-UriSub™, CST TECHNOLOGIES, Inc., (Great Neck, NY, USA). *L*-glutamine isotope ($C_5H_{10}{}^{15}N_2O_3$) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA)

2.2. Preparation of Glycylglycine Buffer and Sarcosine Oxidase

A 0.2 M glycylglycine solution was prepared from ultra-pure water and glycylglycine, and the pH was adjusted to 7.4 with 1 $mol \cdot L^{-1}$ sodium hydroxide. A buffered sarcosine oxidase stock solution was prepared from these reagents from which standard reagents of 1.77×10^{-7} units/mL were prepared fresh and kept at 4 ^{o}C until use. This concentration of sarcosine oxidase was found to have the highest specific activity with its sarcosine substrate in the range of 1 ppb to 1 ppm (data not shown).

2.3. Preparation of Sarcosine Stock Solution

Stock solutions of sarcosine were prepared from ultra-pure water and sarcosine standard. The stock solutions were prepared at 10, 100, and 1000 ppb for no more than two weeks at 4 ^{o}C.

2.4. Analysis of Sarcosine Standards

Sarcosine and creatinine were added in equivalent concentrations ranging from 1 ppb to 1000 ppb as well as 50ppb *D,L*-alanine and 50ppb *L*-glutamine to a final volume to 500µL of ultra-pure water. Upon thorough mixing, the vial contents were subjected to

HPLC/MS/MS analysis to determine pre-enzymatic concentrations. After pre-analysis, the same sample was then heated to 37 ^{o}C, and 500 µL of cold enzyme reagent was added. The direct addition of the enzyme resulted in a 2-fold dilution. The vial was then incubated at 37 ^{o}C for 30 minutes. The sample was quickly cooled down to room temperature, and injected into HPLC/MS/MS for post-enzymatic sarcosine concentration. Samples were not run in parallel (pre- and post-enzymatic) because even minute differences in sample sarcosine concentrations significantly affected enzymatic activity toward the lower limit of the linear range.

2.5. Analysis of Urine Samples

Urine samples (*n*=35) were provided by the Central Missouri Urology Clinic. The samples were comprised of 15 normal samples and 20 prostate cancer positive samples. The urine samples were centrifuged to remove the particulates. The supernatant was diluted 250-fold with ultra-pure water whereupon 250 µL of these diluted urine samples were transferred into a sample vial with 250 µL ultra-pure water. This resulted in a final 500-fold dilution of the urine samples. Pre- and post-enzymatic analyses as well as enzymatic reaction followed similar protocol as that of the sarcosine standards.

2.6. HPLC/MS/MS Analysis

A Luna phenyl-hexyl column (3.0 µm, 3.0×150 mm, Phenomenex, USA) and a Shimadzu UFLC system (Columbia, MD) comprised of a degasser (DGU-30A3), two pumps (LC-20 AD XR), an autosampler (SIL-20AC XR) and a column oven (CTO-20A) were used for the separation of sarcosine. HPLC was performed at 25°C under a flow rate of 250 µL/min using a gradient system with the mobile phase consisting of A: 0.2% formic acid in water and B: 0.2% formic acid in acetonitrile (100%). The gradient program was: initial 98% A and 2% B; linear gradient to 60% A and 40% B in 5 min; return to initial conditions in 0.1 min at a flow rate of 250 µl/min, followed by equilibration for 10 min. Run-to-run time was 15 min. The sample injection volume was 10 µL. An API 4000Q trap MS/MS system (Applied Biosystems, Foster City, CA) was used for the quantification of sarcosine in urine samples**.** Sarcosine and alanine were detected in multiple-reaction monitoring (MRM) mode (m/z 89.9/44) with ESI-positive ionization along with *L*-

glutamine serving as an internal standard (m/z 148/130). Creatinine was also quantified in MRM positive mode (m/z 114/44.1) to account for the renal dilution [16]. Creatinine has been used in many clinical studies as an internal standard since its concentration strictly corresponds to urine dilution [8,16,17]. All species were determined simultaneously.

3. RESULTS AND DISCUSSION

3.1. Interference of Alanine

To assess the impact of its interference, an untreated *D,L*-alanine standard was compared with *D,L*-alanine treated with sarcosine oxidase under identical experimental conditions using the HPLC/MS/MS outlined above. An insignificant percentage (0.28% ± 0.12% for n=3 trials) of the *D,L*-alanine was found to be degraded by sarcosine oxidase. Such consistently low degradation suggests that sarcosine oxidase fails to act appreciably on *D,L*-alanine. Indeed, these results corroborate that reported in the fluorescence study [15]. It is therefore suggested that the sarcosine oxidase acts selectively toward sarcosine in the presence of *D,L*-alanine. Since *D,L*-alanine is being treated quantitatively as sarcosine, the proportionality of the molar signals was assessed through the ion affinities of the two species to identify possible correction factors. It was found that alanine possessed an equivalent ion affinity to sarcosine (n=5 for 5 ppb, 50 ppb, and 500 ppb) (**Figure 1**). This implies that no additional proportionality calculations are necessary since the mass spectrometer resolved both compounds identically.

3.2. Enzymatic Degradation of Sarcosine

Sarcosine standard solutions were incubated with sarcosine oxidase in order to partially eliminate it from the merged sarcosine-alanine peak. In order to accurately quantify sarcosine, the determination of the amount of sarcosine degraded is necessary. This was determined by treatment of standard sarcosine samples with sarcosine oxidase under similar conditions. The enzymatic degradation of the sarcosine standards demonstrated typical enzyme kinetics as shown by the sigmoidal curve in **Figure 2**.

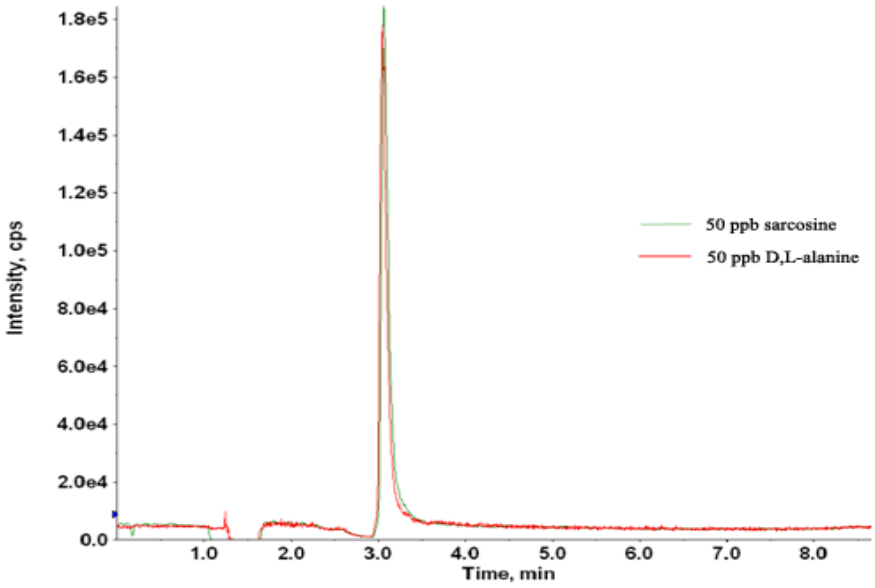

Figure 1. Extracted ion chromatogram (XIC) of the standard sarcosine and *D,L*-alanine under optimized conditions by HPLC/MS/MS.

Partial, constant degradation was observed between 50 ppb and 500 ppb Indeed, the curve flattens as the enzyme becomes saturated, implying degradation occurs as a function of available substrate. As an enzymatic assay, a time dependence exists, in addition to the concentration dependence; however, after the designated reaction time where the majority of the sarcosine has been degraded, slight variations in reaction times afford only negligible degradation differences. Indeed, this degradation curve, which showed decent reproducibility among repeated trials (N =3), was constructed with each data set to improve the analytical quality of the assay. Most importantly, these results reveal the relationship between the original amount of sarcosine and the absolute amount of sarcosine degraded shown as the following:

$$[Sarcosine\ Degraded] = [Original\ Sarcosine]x(Percent\ Degraded) \qquad (1)$$

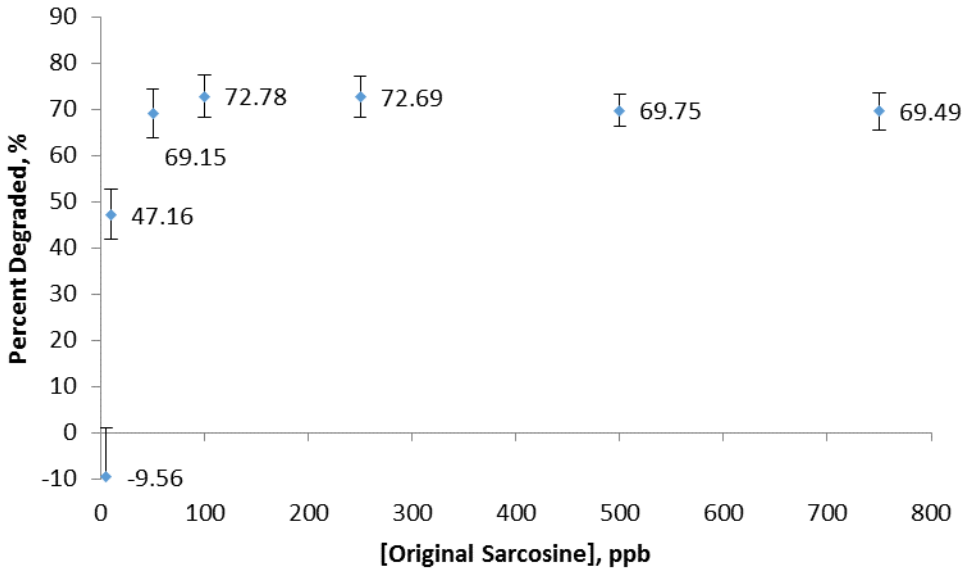

Figure 2. Percent degradation of sarcosine standard solutions from 5 ppb to 500 ppb with 8.85×10^{-8} units of sarcosine oxidase.

3.3. Linearity, Detection limits, and Reproducibility

Sarcosine was determined from known and unknown samples by first constructing an initial, pre-enzymatic calibration curve from sarcosine standards and the resultant peak areas. Since enzyme was added directly to the pre-enzymatic samples, all initial concentrations were consequently halved. Post-enzymatic peak areas were used in the pre-enzymatic calibration curve and subsequently doubled to yield dilution-corrected post-enzymatic concentrations. The difference between the pre-enzymatic and post-enzymatic concentrations, representing the absolute amount of sarcosine degraded, was then determined. The percent degradation corresponding to the absolute amount of sarcosine degraded was found from the enzymatic degradation data. Normalization of the observed decrease in sarcosine concentration by the percent degraded, as given by the equation above (**Equation 1**), then afforded the original sarcosine concentration. Thus, the analytical determination may be expressed by the following equation (**Equation 2**):

$$[Original\ Sarcosine] = \frac{[Sarcosine]_{Pre-enzymatic} - 2[Sarcosine]_{Post-enzymatic}}{\%Sarcosine\ Degraded} \quad (2)$$

A stable calibration curve was obtained from a series of sarcosine standards ranging from 1 ppb to 500 ppb spiked with 50ppb *D,L*-alanine. A highly linear relationship was observed over this range with a correlation coefficient of 0.9999. The method limit of detection was determined to be 1ppb at a signal-to-noise (S/N) ratio of 3. Reproducibility was measured (*n*=3), yielding a relative standard deviation (RSD) of 1.8% for these sarcosine standards. Based on this data, it is suggested that this technique is applicable over the given concentration range under the given chemical environment.

By considering the matrix effects of real samples, synthetic urine was used to replace the buffer solution in the preparation of the sarcosine standards. The pH was adjusted to physiological conditions (pH = 7.4) with 1.0 M NaOH. The synthetic urine was also diluted by a factor of 500 to mimic real urine sample analysis. This high dilution factor served to minimize matrix effects such as pH differences among real urine samples. ^{15}N isotope-labeled glutamine was added to vials at a concentration of 50 ppb as an internal standard. The calibration curve was reconstructed with samples ranging from 1 ppb to 1 ppm (R^2=0.9996). The limits of quantitation and detection were found to be 3 ppb and 1 ppb, respectively, in this new matrix. These limits correspond closely with those generated by the fluorimetric study (compare with limit of detection of 2 ppb). Despite its comparable limit of detection, the fluorimetric assay is limited by its sole analysis of sarcosine; the HPLC/MS/MS technique presented herein may be applied to the simultaneous analysis of numerous chemical species, such as creatinine and glutamine.

3.4. Recovery

Real urine matrices (*n*=3) were spiked with known concentrations of sarcosine standards, all of which fell within the linear range of the technique. The spiked concentrations were low (5 ppb), middle (50 ppb), and high (500 ppb). The averages of the recoveries for each level were determined to be 111.7% ± 3.7% (RSD 3.3%), 90.8% ± 1.1% (RSD 1.2%), and 90.1% ± 1.6% (RSD 1.8%), respectively, for the low, middle, and high injections. In the case of the low concentrations, a positive interference exists, which may be linked to marginal degradation of *D,L*-alanine by sarcosine oxidase and other chemical species or to positive modulation of sarcosine oxidase. Nevertheless, this level of

precision is sufficient for biological matrices like that of urine, and is primarily attributed to the high dilution (500 fold) of the samples. It is noted, however, that with the high dilution comes a loss in accuracy. Nevertheless, as shown by the recovery data, this uncertainty is reasonable, and the technique is considered analytically valid.

3.5. Real Urine Analysis

Real urine samples taken from healthy subjects (n=15) and prostate cancer-positive patients (n=20) were analyzed by the enzymatic technique. Samples were diluted 500 times with ultra-pure water in order to fall within the linear range as well as to minimize matrix effects including pH differences. These sample sets were run in triplicate to provide sufficient statistical validation. Standard solutions containing 50 ppb sarcosine, 50 ppb ^{15}N isotope-labeled glutamine, and 50 ppb *D,L*-alanine standard samples were evenly dispersed throughout the sample as a quality control measure. The calibrated sarcosine concentrations were corrected for dilution to provide the final urinary sarcosine concentration.

The experimental results suggest that the enzymatic approach (average urinary sarcosine concentration = 13.67 ppm) has a superior separation compared with the incomplete separation achieved by the optimized HPLC/MS/MS method alone (average urinary sarcosine concentration = 35.69 ppm). Indeed, the optimized HPLC/MS/MS method without enzymatic treatment failed to separate sarcosine and alanine due to their similar retention times in reversed-phase columns as expected. Assuming then, that the non-enzymatic method represented 100% of the sarcosine-alanine mixture, it can be deduced by the comparison of the two methods that average urinary sarcosine comprises approximately 40% of the sarcosine-alanine mixture. This concept may be extrapolated to concurrently estimate the amount of urinary *D,L*-alanine. This idea has not been extensively addressed in this study, however,

With sufficient separation, then, accurate comparisons between normal and prostate cancer-positive urine samples can be made using liquid chromatography with tandem mass spectrometry for sarcosine as a potential marker using this technique. Since analyte

concentrations can vary greatly among samples due to number of reasons, including dietary intake, genetic disposition, fluid intake and retention, among other factors, sarcosine concentrations were normalized to creatinine concentrations analyzed by HPLC/MS/MS [16, 17]. Indeed, creatinine is often used as a urinary normalization factor due to its high concentrations in urine samples. In this way, it is representative of the overall solute concentration of urine. Creatinine was analyzed simultaneously with sarcosine and its internal standard, ^{15}N isotope-labeled glutamine.

This study reports a negligible difference ($p = 0.115$, two-tailed t-test) of urinary sarcosine concentrations between normal and prostate cancer-positive urine samples (**Figure 3**). Despite this, the validation of sarcosine as a prostate cancer biomarker was not the intent of this study. Indeed, a more detailed clinical study with more control over the cancer patients and healthy subjects would be required to afford more conclusive findings. Nevertheless, this method provides an accurate analytical platform for the analysis of sarcosine in urine and other biological samples as an alternative to HPLC/MS/MS analysis utilizing derivatization. Moreover, the sarcosine-alanine isomers are but one of many metabolic isomers that have presented difficulties to HPLC/MS/MS techniques. Others include amino acid isomers, such as leucine and isoleucine, as well as isomeric fatty acids and oligopeptides, all of which possess selective enzymes. With non-invasive diagnostic techniques increasingly being developed, demand for high-throughput analyses that can discern among interferences is critical. In this way, the study more broadly demonstrates the novel application of selective enzymes to isomeric interferences using high-throughput HPLC/MS/MS techniques for quantitative bioanalysis.

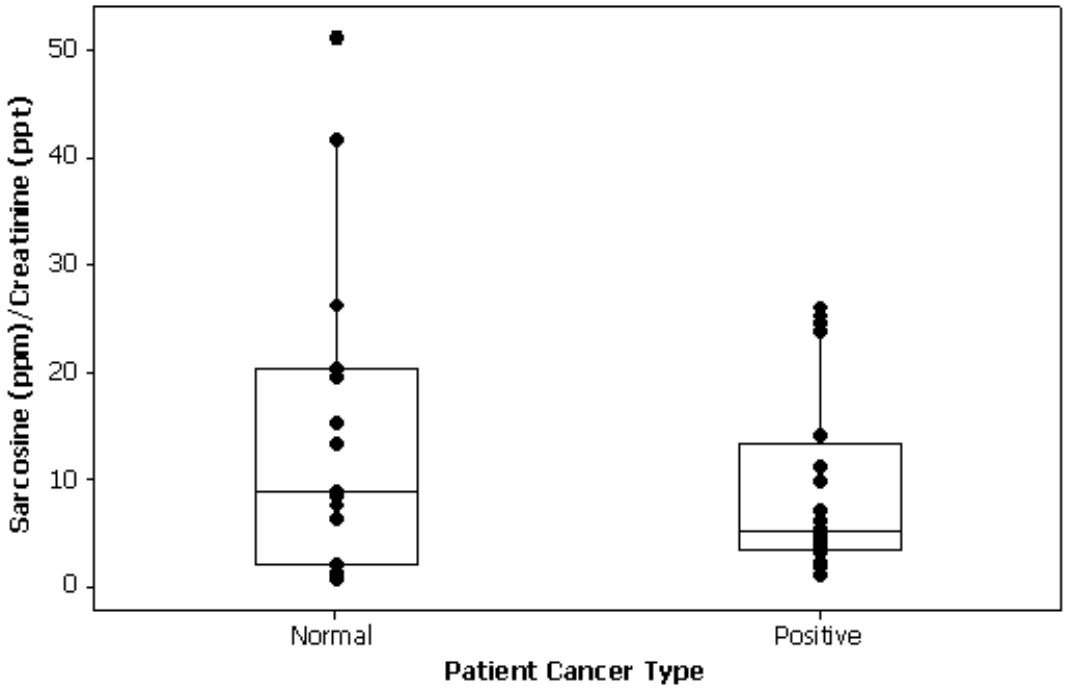

Figure 3. Comparison of urinary sarcosine concentrations adjusted to urinary creatinine concentrations in healthy individuals (*n*=15) and prostate cancer patients (*n*=20).

4. CONCLUSIONS

As an alternative solution to derivatization for the separation of sarcosine and *D,L*-alanine under HPLC/MS/MS analysis, a simple, enzymatic method has been developed and validated in this study. The premise of the technique rests on the fact that selective enzymatic elimination from an isomeric pair that traditionally generates a merged peak on reversed-phase HPLC/MS/MS will result in a marked decrease in apparent concentration proportional to the original amount of the eliminated species. Sarcosine oxidase was used as the selective enzyme in this study, and was shown to be selective for sarcosine in the presence of *D,L*-alanine. The difference in calibrated sarcosine concentrations, before and after enzymatic treatment, when normalized to the percent degraded, yielded the original concentration of sarcosine (R^2=0.9999) within the linear range of 1 ppb – 1ppm. The method is simple, sensitive (MDL is 1 ppb), and shows a good reproducibility (RSD = 1.8% for sarcosine standards in synthetic urine). It is comparable with current fluorimetric studies, but offers a more robust solution as a potential screening technique through the

simultaneous analysis of other analytes. In short, this study presents the coupling of high-throughput HPLC/MS/MS with the selectivity of enzymes to form an analytical platform for the broad analysis of biological samples including metabolic isomers.

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III. SIMULTANEOUS DETECTION OF SIX URINARY PTERIDINES AND CREATININE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY FOR CLINICAL BREAST CANCER DETECTION

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ABSTRACT

Recent preliminary studies have implicated urinary pteridines as candidate biomarkers in a growing number of malignancies including breast cancer. While the development of capillary electrophoresis – laser induced fluorescence (CE-LIF), high performance liquid chromatography (HPLC), and liquid chromatography – mass spectroscopy (LC-MS) pteridine urinalyses among others have helped to enable these findings, limitations including poor pteridine specificity, asynchronous or non-existent renal dilution normalization, and a lack of information regarding adduct formation in mass spectrometry techniques utilizing electrospray ionization (ESI) have prevented application of these techniques to a larger clinical setting. In this study, a simple, rapid, specific, and sensitive high-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) method has been developed and optimized for simultaneous detection of six pteridines previously implicated in breast cancer and creatinine as a renal dilution factor in urine. In addition, this study reports cationic adduct formation of urinary pteridines under ESI-positive ionization for the first time. This newly developed technique separates and detects the following six urinary pteridines: 6-biopterin, 6-hydroxymethylpterin, *d*-neopterin, pterin, isoxanthopterin, and xanthopterin, and as well as creatinine. The method detection limits for the pteridines are between 0.025 and 0.5 µg/L and 0.15 µg/L for creatinine. The method was also validated by spiked recoveries (81-105%), reproducibility (RSD: 1-6%), and application to 25 real urine samples from benign and malignant breast cancer samples through a double-blind study. The proposed technique was finally compared directly with a previously reported CE-LIF technique, concluding that additional or alternative renal dilution factors are needed for proper investigation of urinary pteridines as breast cancer biomarkers.

KEY WORDS

HPLC-MS/MS, urinalysis, pteridines, breast cancer biomarkers

1. INTRODUCTION

Improvements in early cancer detection techniques for breast cancer, including clinical breast examination (CBE), mammography, ultrasonography (US), and magnetic resonance imaging (MRI), have contributed to an approximately 25% increase in incidence detection rates in the United States since the 1970s.[1] Despite the increased awareness, confident diagnosis is non-ideal, instead relying on a combination of the above techniques. For example, routine CBEs, which are considerably more cost-effective than mammography as preliminary assessments of malignancy, are characteristically poor indicators of malignancy, with positive predictive values and diagnostic sensitivities reported at 1-10% and 25-60%, respectively.[2-6] Hence, the practical value of CBEs is often questioned since CBEs fail to improve mortality rates as many cases go undetected until later stages and high false positive rates lead to additional anxiety, cost, and the possible need for invasive techniques.

Mammography as a preliminary assessment for malignancy offers significantly improved diagnostic sensitivity over CBE with reported sensitivities ranging from 63% to 98%.[7-9] Such values can be deceptive, however, since mammographic sensitivity decreases to 30-48% in cases of dense and heterogeneously dense breasts, which have been shown to have up to a fivefold increased risk of cancer compared with fatty breasts.[10-12] Generally large interradiologist variability further obscures accurate cancer detection rates in imaging techniques like mammography.[13] While US and MRI have been found to be more sensitive than mammography alone in cases of invasive cancer, only the combination of CBE, mammography, and confirmational MRI has been reported to have nearly 100% diagnostic sensitivity.[12] Thus, while confident breast cancer detection can be achieved with available techniques, the development of a simple and rapid biomarker-based analytical technique with comparable diagnostic sensitivity would be vastly superior to the relatively complex and uneconomical combination of analyses currently used. One possible biomarker-based solution to this involves urinary pteridines.

Urinary pteridines are an emerging class of metabolites that may be monitored non-invasively and are increasingly being preliminarily implicated in a number of malignancies, including breast cancer.[14] Pteridines as biomarkers are not without challenges, however. Despite the diversity of the pteridine family, with such wide implications, the possibility of urinary pteridines serving as ambiguous indicators of malignancy must be considered. Thus, adequately large panels need to be examined with special attention placed on ratios of the individual pteridines toward specific malignancies. Pteridine urinalysis in particular has overcome significant analytical challenges over the past decade enabling the recent flurry of studies investigating pteridines as potential and novel biomarkers.[15-21] Examples of typical pteridine urinalysis difficulties include: thermally and photocatalytically labile compounds, poor solubilities in common solvents, the presence of three oxidation species, and relatively low urinary clearance. Nevertheless, capillary electrophoresis – laser induced fluorescence (CE-LIF) has emerged as a capable analytical platform to quantify urinary pteridines.[15, 17, 19] Indeed, the minimal sample preparation required by CE-LIF urinalysis and its high cost-effectiveness compared with more advanced chromatographic techniques are certainly appealing advantages. However, current CE-LIF techniques are limited by two fundamental problems: (1) the inability to simultaneously quantify non-fluorescent creatinine as a renal dilution factor, and (2) the platform is inherently non-specific and consequently subject to relatively high interference levels from the complicated matrices in real urine samples.

High-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) presents an alternative to CE-LIF that can sufficiently address these two problems. Indeed, the high-throughput nature of HPLC-MS/MS is better suited for the identification and validation of urinary pteridines as indicators of malignancy while CE-LIF may represent the most sensible and economic choice only *after* such potential confirmations. These considerations are not novel, either, as a couple of HPLC and LC-MS/MS techniques for selected pteridines have recently been developed.[18, 22] Nevertheless, these studies have thus far been limited in scope by either considering a narrow panel of urinary pteridines, failing to examine possible adduct formation in ESI-MS/MS techniques, or incorporating either impractical asynchronous or no corrections for renal dilution. By

considering these limitations, in this study, an optimized HPLC-MS/MS has been developed to separate and quantify six pteridines and creatinine (**Figure 1**) simultaneously in urine samples as an alternative technique to a CE-LIF technique previously used to implicate urinary pteridines in breast cancer.[15] Since this previous CE-LIF technique reported positive correlations between urinary pteridines in positive and negative breast cancer samples, rather than corroborate these findings, this study further examined the ability of the urinary pteridines to discern benign from malignant breast cancer urine samples.

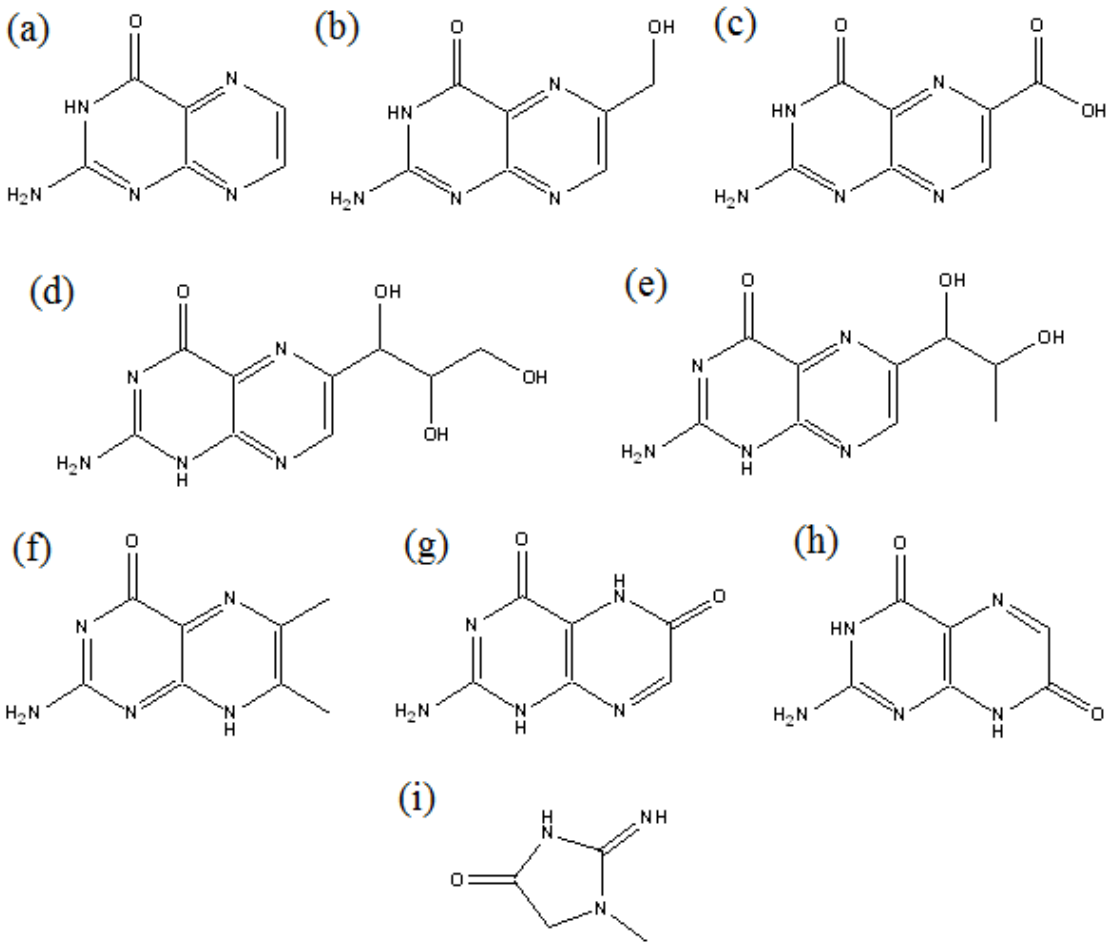

Figure 1. Chemical structures of eight urinary pteridines (fully oxidized) and creatinine: (a) pterin, (b) 6-hydroxymethylpterin, (c) pterin-6-carboxylic acid, (d) *d*-neopterin, (e) 6-biopterin, (f) 6,7-dimethylpterin, (g) xanthopterin, (h) isoxanthopterin, (i) creatinine.

2. EXPERIMENTAL

2.1. Chemicals and Materials

Isoxanthopterin, pterin, *D*-neopterin, xanthopterin, 6-biopterin, pterin-6-carboxylic acid, ammonium oxalate, ammonium hydroxide, and sodium hydroxide were purchased from Sigma-Aldrich (St.Louis, USA). 6,7-Dimethylpterin and 6-hydroxymethylpterin were obtained from Schircks Laboratory (Jona, Switzerland). Potassium iodide and iodine were purchased from Fisher Scientific (New Jersey, USA). Synthetic urine was obtained from CST Technologies Inc. (New York, USA). Ultrapure water was generated by a Milli-Q Advantage® A10 and Millipore Elix® water purification system.

2.2. Instrumentation

A Luna phenyl-hexyl column (3.0 μm, 3.0×150 mm, Phenomenex, USA) and a Shimadzu UFLC system (Columbia, MD) comprised of a degasser (DGU-30A3), two pumps (LC-20 AD XR), an autosampler (SIL-20AC XR) and a column oven (CTO-20A) were used for the separation of the urinary pteridines. HPLC was performed at 25°C under a flow rate of 0.3 mL/min using a gradient system with the mobile phase consisting of A: 0.1% formic acid in water and B: 0.2% formic acid in acetonitrile. The gradient program was characterized by an initial 2 minute pre-equilibrium of 20% A followed by a linear increase to 37% A in 5.5 minutes. Run-to-run time was 7.5 minutes with a sample injection volume of 10 μL.

An API 4000Q trap MS/MS system (Applied Biosystems, Foster City, CA) was used for the quantification of urinary pteridines**.** All ions were monitored in scheduled multiple-reaction monitoring (MRM) mode with ESI-positive ionization. Optimized flow injection parameters include: ion spray voltage: 5500V; curtain gas: 14 psi; collision gas: 6 psi; GS1: 35 psi; GS2: 35 psi; and source temperature: 425°C.

2.3. Standard Preparation

Approximately 2 mg of each pteridine and creatinine was accurately weighed and dissolved in 15 mL of ultrapure water and 500 μL of 1 M ammonium hydroxide. Upon complete dissolution by sonication and vortexing, all components were added together with

enough ultrapure water and 500 µL of oxidizing solution (4:2% w/v potassium iodide to iodine in ultra-pure water) to generate a stock solution of 10 mg/L of each component. Calibration standards were prepared from the stock solution by serial dilution in ultra-pure water and enough synthetic urine to generate a 0.5% solution of synthetic urine to mimic a 200-fold dilution in real sample analysis.

2.4. Urine Samples and Preparation

Real urine samples (*N*=25) were provided by Mercy Hospital (Springfield, MO) by consenting women (age range: 25-80) referred to the Mercy Hospital Breast Cancer Treatment Center as a double-blind study. Pathologies (benign or malignant) were independently determined by Mercy Hospital through a combination of CBE, mammography, US, MRI, and biopsy methods.

Real urine samples were stored in a freezer at a temperature of -80°C. Upon fully thawing the samples to room temperature, a 100 µl aliquot of urine sample was placed in a yellow centrifuge tube containing 850 µL ultrapure water, 40 µl of oxidizing solution, and 10 µl of 2.0 M ammonium hydroxide. The sample was then thoroughly mixed and was incubated for 30 minutes at 4°C followed by centrifugation at 5000 rpm (2571 g) for 20 min at 4°C. Upon centrifugation and filtration with a 0.22 µm membrane, a 10-fold dilution was made by adding 100 µL of the filtrate to 870 µL of ultra-pure water and 30 µL of 440 mg/L ammonium oxalate, giving a final dilution factor of 100.

3. RESULTS AND DISCUSSION

3.1. Oxidation Pre-treatment

Most pteridine urinalyses reported thus far have incorporated an oxidation pre-treatment step with the rationale being that the fully oxidized species are substantially more stable than the reduced dihydro- and tetrahydro-pterin species and that the fully oxidized species also affords the strongest fluorescence response in CE-LIF techniques.[17, 23] While a variety of oxidizing agents have been investigated, including tri-iodide ions via potassium iodide-iodine solutions, permanganate, and manganese oxide, potassium iodide-iodine was chosen in order to represent an easier adaptation of the model CE-LIF technique. Since

previous studies had concluded that a 1:2 proportion of 4%:2% (w/v) potassium iodide-iodine solution to urine was sufficient to fully oxidize all pteridines, this study maintained these oxidation conditions. To further consider adverse reactions with the tri-iodide ions, untreated and treated pteridine standards (which were shown to be 95-99% oxidized as labeled) were examined via direct infusion. The presence of tri-iodide ions did not significantly alter pteridine response under these oxidation conditions.

3.2. MS/MS Optimization

ESI-positive ionization with 0.1% formic acid yielded the highest ionization efficiencies for all oxidized pteridines and creatinine. Formic acid content above 0.2% suppressed pteridine ionization for xanthopterin, isoxanthopterin, 6-biopterin, and pterin-6-carboxlyic acid. The adduct formation potentials of the oxidized pteridines and creatinine were then examined under various conditions via direct infusion into the MS/MS unit at a concentration of 100 µg/L prepared in 0.1% formic acid in ultra-pure water. Q1 ions were monitored under Multiple Channel Analyzer (MCA) mode with a flow rate of 0.2 mL/hour. The scan range was set from 5 m/z below the expected molecular ion to 200 m/z above to monitor singly charged ions as no doubly charged ions were preliminarily observed. Dissolution with sodium hydroxide afforded significant sodium, $[M+Na]^+$, and in some cases disodium, $[M-H+2Na]^+$, and tri-sodium, $[M-2H+3Na]^+$, adducts in 6,7-dimethylpterin, *d*-neopterin, 6-hydroxymethylpterin, pterin, and pterin-6-carboxylic acid. Sodium adducts in these standards comprised over 60% of the total signal, and in the case of fully unsubstituted pterin, 83% of the total signal, thereby affecting pteridine detection sensitivities by splitting the signal among various adducts. In contrast, dissolution with ammonium hydroxide favored the protonated adduct, $[M-H]^+$, in all pteridines but xanthopterin and pterin-6-carboxylic acid with resulting ammonium adducts, $[M+NH_3]^+$ and $[M-H+2NH_4]^+$, representing only 32% of total xanthopterin signal and 31% of the total pterin-6-carboxylic acid signal, respectively. The Q1 scans for pterin dissolved in sodium hydroxide and ammonium hydroxide were shown in **Figure 2** to demonstrate the formation of sodium adduct ions of pteridines. In addition, a significant creatinine dimer (48% of total signal) was found at 227 m/z. Because real urine has significant sodium and ammonium cation populations, all adducts with relative abundances of over 30% for each

respective compound were considered as quantitative precursor ions. MS/MS ions pairs were then systematically optimized with direct infusion and flow injection analysis for all quantification and confirmation pairs including all major adducts (sodium and ammonia) for the eight fully oxidized pteridines and creatinine. The optimized MS/MS parameters were summarized in **Table 1**.

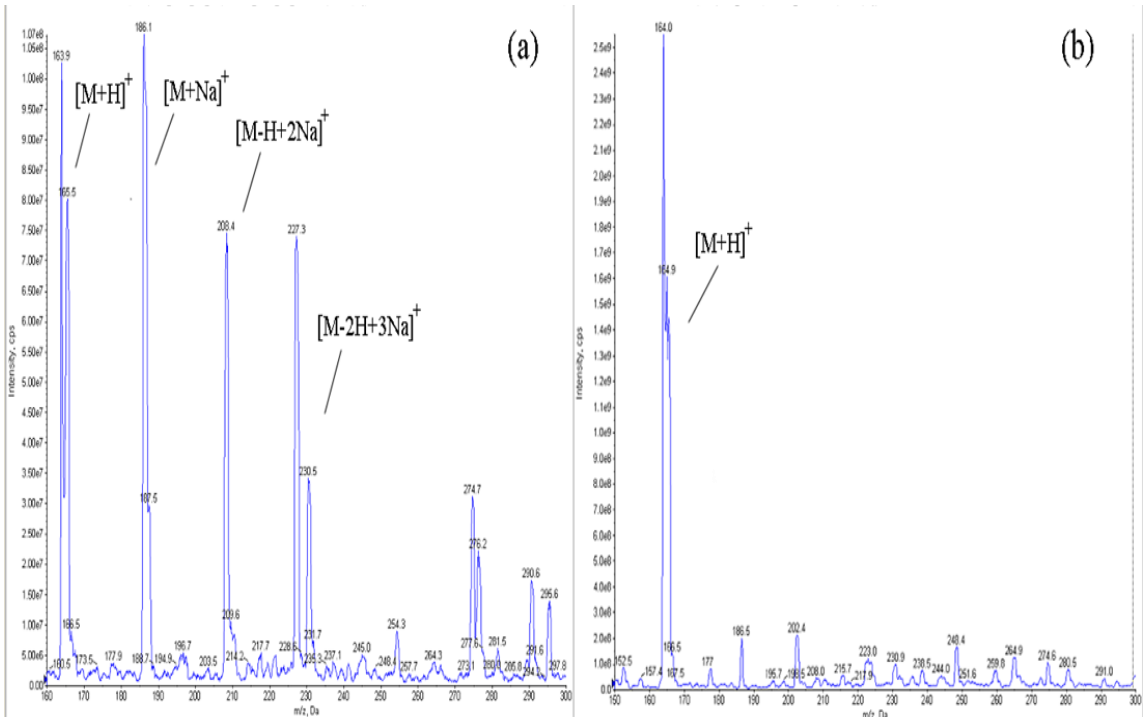

Figure 2. Q1 scans of 100 µg/L pterin in 0.1% formic acid in ultra-pure water dissolved with (a) sodium hydroxide and (b) ammonium hydroxide.

Table 1. Optimized MS/MS conditions of 8 urinary pteridines, creatinine, and their common adducts.

Compounds	Ion Pairs (m/z)	Declustering Potential (DP, V)	Collision Energy (CE, V)	Collision Cell Potential (CXP, V)
Creatinine	114.034/44.000	56	33	6
	114.034/86.000	56	19	14
Creatinine Dimer	227.503/113.900	1	21	4
6,7-Dimethylpterin	192.534/165.300	96	31	10
	192.534/147.000	96	31	12
6,7-Dimethylpterin+Na	213.999/196.200	101	19	12
	213.999/86.300	101	27	14
6-Biopterin	238.423/220.300	31	21	14
	238.423/178.200	31	29	10
D-Neopterin	254.156/206.200	51	27	12
	254.156/190.200	51	31	12
D-Neopterin-Na	276.088/216.200	81	27	14
	276.088/234.100	81	27	24
6-Hydroxymethylpterin	194.293/176.300	31	23	14
	194.293/106.100	31	39	10
6-Hydroxymethylpterin+Na	216.210/204.000	86	25	12
	216.210/199.100	86	15	26
Pterin	164.431/119.000	76	31	8
	164.431/91.900	76	41	4
Pterin-Na	185.891/114.100	71	35	8
	185.891/86.200	71	45	16
Isoxanthopterin	180.113/135.400	76	33	10
	180.113/163.100	76	27	10
Xanthopterin	180.113/135.400	76	33	10
	180.113/163.100	76	27	10
Xanthopterin+NH_3	196.115/168.500	91	23	10
	196.115/112.200	91	35	6
Xanthopterin—H+2NH_4	213.754/141.200	66	21	8
	213.754/141.200	66	31	6
Pterin-6-carboxylic acid	208.110/190.100	66	19	12
	208.110/180.300	66	23	10
Pterin-6-carboxylic acid+Na	229.787/186.300	61	25	18
	229.787/212.200	61	17	14
Pterin-6-carboxylic acid+NH_3	225.084/208.100	36	17	14
	225.084/190.100	36	23	12

3.3. Adduct Reduction

Although adduct speciation was analytically considered by ultimately summing the calibrated concentrations, sodium scavenging additives were considered as an additional safe-measure in real urine samples where high sodium and relatively low pteridine levels may obfuscate pteridine detection through adduct formation. Indeed, this concern was substantiated by monitoring adduct formation in 100 µg/L fully oxidized pteridine standards dissolved with ammonium hydroxide and prepared in 0.5% synthetic urine (representing a 200-fold dilution of real urine), which afforded sodium adduct formation comparable to pteridine dissolution with sodium hydroxide. Thus, in real urine samples, active measures to minimize sodium adduct formation must be taken for ideal pteridine detection.

While desalting and trap columns may be used to remove free sodium cations, these techniques are indiscriminate and alter the oxidative environment by also removing the tri-iodide ions, thereby possibly and more detrimentally affecting pteridine detection. Therefore, in this study, *in situ* sodium scavenging additives including ascorbic acid, oxalic acid, and ammonium oxalate were examined based on their previously reported cationic adduct reduction in MALDI techniques.[24] The sodium adduct reduction capabilities of the three compounds were evaluated by direct infusion of 100 µg/L fully oxidized pteridine standards prepared in 0.5% synthetic urine and spiked with 10 mg/L additive under MCA scan mode and a 200 m/z scanning window. In comparison to the control that contained no additives, combined sodium and ammonia adducts for all eight pteridines were decreased 53% by ammonium oxalate, 37% by ascorbic acid, and oxalic acid instead promoted ammonia adduct formation without significantly affecting sodium adduct formation to yield an overall 10% increase in total adduct formation. In addition to cationic adduct reduction, the mild reducing agents, ascorbic acid and oxalic acid, also afforded minor reduction (3-12%) of the quantitation ions for xanthopterin, isoxanthopterin, and 6-biopterin. More importantly, the sodium scavenging capabilities of ammonium oxalate seem to be accentuated in real urine samples, up to 99.4% in some samples. Indeed, the MRM transitions for sodium and ammonia adducts in some real samples were entirely undetected. While this phenomenon was not thoroughly investigated, the authors presume

this is caused by the association of free sodium and ammonia with excess organic matter and the highly charged, filtered and precipitated proteins. Excess ammonium oxalate was therefore added during the final dilution of real urine samples to counteract adverse cationic adduct formation. The final concentration of ammonium oxalate used (13 mg/L ≈ 0.1 mM) was found to afford the maximum adduct reduction with minimal pteridine ion suppression in 0.5% synthetic urine.

3.4. UFLC Optimization

The structural isomers, xanthopterin and isoxanthopterin, must be chromatographically resolved for quantitation with MS/MS. A Phenomenex Luna phenyl-hexyl column was found to sufficiently separate the two isomers under a wide range of liquid chromatographic conditions including flow rate (0.15-0.40 mL/min) and mobile phase constitution. (0.0-0.2% formic acid in water, acetonitrile, and methanol). In comparison, the previously utilized LUNA amine column provided inferior resolution under the conditions described above.[22] Upon column selection, chromatographic conditions were systematically optimized for all eight pteridines and creatinine (see Experimental for optimized conditions). The resulting chromatogram, including separation of xanthopterin and isoxanthopterin, was shown in **Figure 3**.

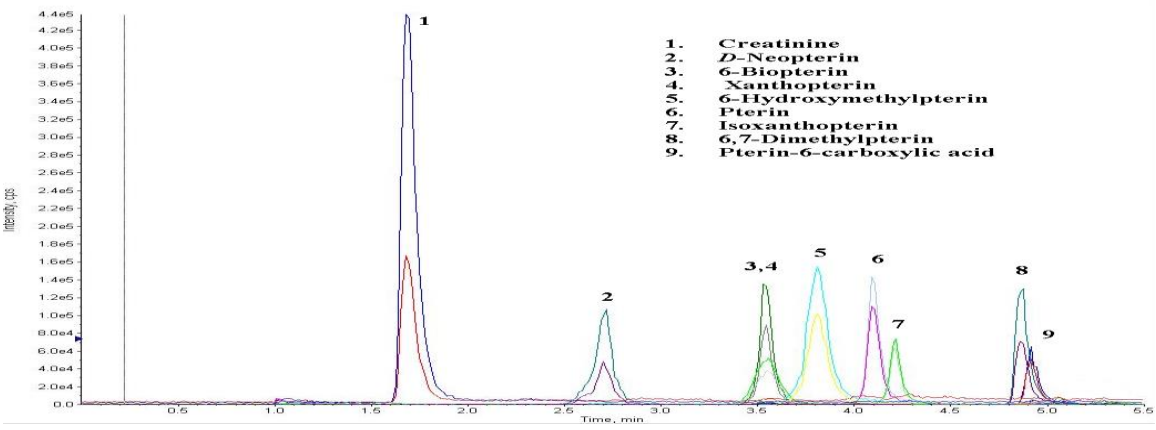

Figure 3. Overlaid extracted ion chromatograms (XIC) of eight urinary pteridines, creatinine, primary adducts, and confirmation pairs with nominal concentrations of 100 µg/L under the optimized UFLC-MS/MS conditions.

3.5. Method Performance

Under the optimized HPLC-MS/MS conditions, calibration curves for all pteridines and creatinine were constructed to demonstrate linearity under an appropriate analytical range. Because some pteridines exhibit significant adduct formation in 0.5% synthetic urine, even after treatment with ammonium oxalate, and fragmentation efficiencies were furthermore widely distributed among the various adducts, calibration curves were produced from standards by monitoring MRM transitions alongside an accompanying Q1 scan. The Q1 scan allowed for absolute concentration determination by considering the signal proportionality among the various adducts for each respective pteridine. For example, a nominal 100 µg/L pteridine standard with a 20% monosodium adduct formation potential represented 80 µg/L $[M+H]^+$ and 20 µg/L $[M+Na]^+$, from which appropriate calibration curves may be produced. The desired calibration range was set to 1-1000 µg/L based on previous studies indicating that the monitored pteridines should fall within this range after a 200-fold dilution.[15, 17] As shown in **Table 2**, all compounds were highly linear with linear regression coefficients above 0.9998 under the selected analytical range. Moreover, the method was found to be highly sensitive with detection limits ranging from 0.025 to 0.5 µg/L. These sensitivities are comparable to the model CE-LIF technique and marginally better than previously reported HPLC and LC-MS/MS techniques. Because urinary creatinine concentrations are on average 1000-fold higher than that of pteridines, creatinine analysis varied from that of pteridines. Creatinine levels in 200-fold diluted urine range from 500 to 5000 µg/L. At these levels, creatinine ionization saturation produced a sigmoid calibration curve, requiring the construction of a non-traditional calibration curve in order to simultaneously quantify it with pteridines. Linearization was achieved by doubly inverting creatinine concentrations and detector response (cps), providing a new linear regression coefficient of 0.9972 for this elevated range. This approach, in addition to pteridine analysis, was validated through spiked recoveries in real urine samples (**Table 3**). The data in **Tables 2 and 3** clearly demonstrate that the method is highly sensitive and reproducible for all eight pteridines and creatinine in real urine samples. Finally, this method compares well with other pteridines studies, as shown in **Table 4**.

Table 2. Linear Range, IDLs, MDLs, retention Times, and encountered ranges in urine of the HPLC-MS/MS method.

Compounds	Linear Range (µg/L)	R^2	IDLs in DI water (µg/L)	MDLs in Synthetic Urine (µg/L)	Retention Time (min)	Encounter Range (µg/L)
Creatinine	0.15-500 500-5000	0.9998 0.9972	0.1	0.15	1.74	20-174*
***D*-Neopterin**	0.025-1000	0.9999	0.025	0.025	2.74	115-3816
6-Biopterin	0.2-1000	0.9999	0.1	0.2	3.43	158-2933
Xanthopterin	0.5-1000	0.9999	0.1	0.15	3.57	35-2796
6-Hydroxymethylpterin	0.25-1000	0.9999	0.1	0.1	3.76	50-413
Pterin	0.3-1000	0.9999	0.25	0.3	4.08	25-854
Isoxanthopterin	0.4-1000	0.9999	0.4	0.4	4.20	22-2714
6,7-Dimethylpterin	0.1-1000	0.9998	0.05	0.1	4.82	0-20**
Pterin-6-carboxylic acid	0.5-1000	0.9999	0.5	0.5	4.88	0-4695**

*Creatinine has been displayed in terms of mg/dL.

**6,7-Dimethylpterin and Pterin-6-carboxylic acid were undetected in most real samples at 200-fold dilutions. Dilutions of 100-fold were used to quantify the two pteridines in other samples.

Table 3. Summarized results of triplicate recoveries (%) of three varying concentrations of eight urinary pteridines and creatinine in a spiked real urine sample.

	Low (1 µg/L)		Medium (10 µg/L)		High (100 µg/L)	
Compound	**Recovery**	**RSD**	**Recovery**	**RSD**	**Recovery**	**RSD**
†Creatinine	95.4	2.4	97.1	3.0	101.1	1.8
D-Neopterin	102.4	4.2	97.4	2.3	96.4	2.1
6-Biopterin	97.6	3.0	104.7	3.2	102.3	2.7
Xanthopterin	81.3	4.2	91.4	2.7	93.4	2.5
6-Hydroxymethylpterin	103.0	2.1	99.2	2.3	98.6	1.6
Pterin	97.8	2.7	96.5	2.4	93.1	2.2
Isoxanthopterin	104.5	4.5	100.3	3.8	102.8	4.0
6,7-Dimethylpterin	89.4	3.6	89.7	2.4	93.7	1.8
Pterin-6-carboxylic acid	84.1	5.7	91.3	5.5	93.4	5.3

†Creatinine spiked recoveries were made at 500, 1000, and 3000 µg/L in to better represent creatinine concentrations in real urine samples.

Table 4. Comparison of the proposed HPLC-MS/MS technique with the model CE-LIF technique and two previously reported LC and LC-MS/MS techniques.

Technique	**Proposed HPLC-MS/MS**	**CE-LIF**[15]	**LC-MS/MS**[22]	**LC**[18]
Pteridines Investigated	Pterin, Xanthopterin, Isoxanthopterin, Neopterin, 6-Biopterin, Pterin-6-Carboxlyic acid, 6,7-Dimethylpterin, 6-Hydroxymethylpterin	Pterin, Xanthopterin, Isoxanthopterin, Neopterin, 6-Biopterin, Pterin-6-Carboxlyic acid, 6,7-Dimethylpterin, 6-Hydroxymethylpterin	Pterin, Isoxanthopterin, 6-Biopterin, 7-Biopterin, 6-Neopterin, 7-Neopterin	Pterin, Xanthopterin, Isoxanthopterin, Neopterin, Biopterin, 7-Biopterin, L-Monapterin, Pterin-6-Carboxylic acid, 6-Hydroxymethylpterin, Lumazine, 6-Hydroxylumazine, 7-Hydroxylumazine, Biolumazine
Simultaneous Creatinine Quantification	Yes	No	No	Yes
Oxidation Method	KI-I_2	KI-I_2	MnO_2	KI-I_2 and $KMnO_4$
Run-to-Run Time (minutes)	7.5	20	19	39
Limits of Detection (µg/L)	0.025-0.5	0.035-0.100	0.007-0.360	0.2-6.1

3.6. Real Urine Analysis

The newly developed HPLC-MS/MS method was ultimately validated in 25 real urine samples provided by Mercy Hospital (Springfield, MO) through a double-blind study. The sample set consisted of 13 benign breast cancer samples and 12 malignant breast cancer samples, with pathologies determined independently by Mercy Hospital. At 200-fold dilution, six of the eight pteridines were detected with 6,7-dimethylpterin and pterin-6-carboxylic acid remaining undetected except for in concentrated specimens (N=6). At 100-fold dilution, the compounds were detected in 16 of the samples. Lower dilution folds resulted in significant ion suppression caused from ion saturation. This phenomenon is especially evident for creatinine which experienced complete saturation near 10 µg/L. Therefore, 100-fold dilutions were considered as the absolute lowest dilution factor in order to simultaneously quantify the pteridines and creatinine. Moreover, without further sample preparation or the addition of a desalting or trap column, lower dilution factors risked the longevity of the column and the mass spectrometer. Other studies have also reported low urinary concentrations for these two pteridines, although the authors expected to detect these two compounds at a 200-fold dilution based on previously reported ranges and the supporting spiked recovery data.[15] The apparent inability to monitor these two pteridines in urine is currently pending further investigation. Hence, the proposed technique may not be valid for 6,7-dimethylpterin and pterin-6-carboxylic under the conditions described herein. Nevertheless, the remaining six pteridine and creatinine concentrations were found in similar ranges to those previously reported. These encountered pteridine ranges have been summarized in **Table 3**.

Despite being a method development study, we further examined the potential ability of urinary pteridines to discern benign from malignant breast cancer samples. This limited real sample study, designed solely to validate the presented method, should not be considered conclusive; rather, more detailed clinical trials are needed to confirm urinary pteridines as useful breast cancer biomarkers. Pathological correlations were calculated with Minitab Statistical Software by rejecting or accepting the null hypothesis that mean pteridine levels in malignant breast cancer samples are lower than or equal to those from benign specimens. Interestingly, the HPLC-MS/MS technique failed to show any

pathological correlations with any of the creatinine-normalized pteridines. These findings were validated by analyzing the same 25 samples with the model CE-LIF technique. As shown in **Table 5**, neither technique afforded positive pathological correlations with any of the eight pteridines. Thus, urinary pteridines may not be able to differentiate benign and malignant breast cancers. Since only an age range for the samples analyzed herein were provided through the double blind study, it possible that the control (benign) population differed significantly from the positive (malignant) population. While baseline urinary pteridine levels are currently unknown in large populations, urinary creatinine levels are known to be age- and gender-dependent where levels are substantially elevated in younger individuals.[25, 26] Moreover, urinary creatinine by itself may not be an appropriate normalization factor for breast cancer since breast cancer has been reported to adversely affect both creatinine production and clearance.[27, 28] To at least preliminarily address this issue, pathological correlations for unnormalized urinary pteridine levels were determined (**Table 5**). Although neglecting creatinine afforded no statistically significant correlations, nearly all correlations improved with the HPLC-MS/MS technique, while sparse improvements were seen with the CE-LIF technique, supporting this conjecture to some extent.

Table 5. Pathological correlations (*P*-values) of eight creatinine-normalized and unnormalized urinary pteridines determined by UFLC-MS/MS and CE-LIF techniques.

	UFLC-MS/MS		**CE-LIF**	
Pteridine	**Normalized**	**Unnormalized**	**Normalized**	**Unnormalized**
6-Biopterin	0.604	0.608	0.732	0.728
Pterin	0.638	0.275	0.546	0.549
Neopterin	0.529	0.413	0.47	0.845
Xanthopterin	0.274	0.149	0.759	0.931
Pterin-6-carboxylic Acid	N/A	N/A	0.995	0.73
6,7-Dimethylpterin	N/A	N/A	0.725	0.474
6-Hydroxymethylpterin	0.837	0.11	0.636	0.755
Isoxanthopterin	0.234	0.22	0.704	0.471

Moreover, direct comparison of the two techniques revealed that xanthopterin and isoxanthopterin concentrations determined by the CE-LIF technique were on average 5- and 30-fold higher, respectively, than those reported by the proposed HPLC-MS/MS technique. Based on the high quality method validation data for the proposed HPLC-MS/MS technique and the inherently non-specific nature of CE-LIF, it is possible that these concentration discrepancies arise from co-eluting interferences or different oxidation states of pteridines in the CE-LIF approach. This phenomenon is under further investigation. Nevertheless, the new method may be used to determine urinary pteridines and creatinine simultaneously for biomedical studies, in which the pteridines need to be quantified.

4. CONCLUSION

A simple, rapid, specific, and sensitive HPLC-MS/MS method was developed for simultaneous analysis of six urinary pteridines and creatinine, which has not been previously reported to the best of our knowledge, especially toward application of breast cancer detection. Additionally, cationic adduct formation for urinary pteridines analyzed with ESI-positive ionization have been reported for the first time. The HPLC-MS/MS platform allows for greatly enhanced run times and increased specificity over previously reported CE-LIF approaches. The method was validated through evaluation of spiked recoveries, reproducibility (represented by RSD), MDLs, and application in 25 real urine samples from breast cancer positive and negative samples. Results from the real samples have finally led to the conclusion that improved validation studies with sufficient statistical power and the utilization of an alternative renal dilution factor to creatinine are needed for clinical confirmation of urinary pteridines as breast cancer biomarkers. Finally, the disclosed technique may be applied to other diagnostic studies in addition to breast cancer.

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IV. NORMALIZATION OF URINARY PTERIDINES BY URINE SPECIFIC GRAVITY FOR EARLY CANCER DETECTION

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ABSTRACT

Background: Urinary biomarkers, such as pteridines, require normalization with respect to an individual's hydration status and time since last urination. Conventional creatinine-based corrections are affected by a multitude of patient factors whereas urine specific gravity (USG) is a bulk specimen property that better resists those same factors. In this study, we examined the performance of traditional creatinine adjustments relative to USG to six urinary pteridines in aggressive and benign breast cancers in addition to high grade bladder cancers.

Methods: 6-Biopterin, neopterin, pterin, 6-hydroxymethylpterin, isoxanthopterin, xanthopterin, and creatinine were analyzed in 66 urine specimens with a previously developed liquid chromatography – tandem mass spectrometry technique. Creatinine and USG performance was evaluated with non-parametric Mann-Whitney hypothesis testing.

Results: USG and creatinine were moderately correlated ($r = 0.857$) with deviations occurring in dilute and concentrated specimens. In 48 aggressive and benign breast cancers and 16 bladder cancer and healthy specimens, normalization by USG significantly outperformed creatinine adjustments which marginally outperformed uncorrected pteridines in predicting pathological status. In addition, isoxanthopterin and xanthopterin were significantly higher in pathological specimens when normalized by USG.

Conclusion: USG, as a bulk property, can provide better performance over creatinine-based normalizations for urinary pteridines in cancer detection applications.

KEY WORDS

Pteridine, Urine Specific Gravity, Creatinine, Breast Cancer, Renal Dilution, Urine Normalization

1. INTRODUCTION

The past five years have witnessed a renewed interest in urinary pteridines as noninvasive, metabolic biomarkers for early cancer detection [1]. Recent advances aimed at improving pteridine urinalysis have overcome many of the early challenges facing pteridine research including molecular speciation and physiologically low urinary concentrations [2-6]. Equipped with powerful, new analytical techniques, exploratory studies continue to associate elevated pteridine levels with cancer, although a biological premise for these associations remains unclear [7-9]. Despite these encouraging preliminary findings, pteridine urinalyses have not yet matured to a level acceptable for comprehensive clinical assessment. Particularly, urinary biomarkers like pteridines must be adjusted to reflect an individual's hydration level and time since last urination [10]. While many pteridine studies employ creatinine-based adjustments, one study recently questioned the validity of normalizing urinary pteridines to creatinine after reporting that pteridines could not distinguish benign and aggressive breast cancers [2].

Creatinine is an endogenous byproduct of muscle activity produced with supposedly little day-to-day variation in a given individual [11]. In addition, serum creatinine is constantly cleared from the bloodstream by the kidneys, leading many to consider creatinine as an acceptable measure of renal dilution [12]. This has resulted in the common practice of reporting urinary analytes as a ratio of the analyte to creatinine (mol analyte / mol creatinine). There is growing evidence, however, that creatinine excretion is not consistent, following repeated challenges to creatinine's usefulness as a normalization factor [13-15]. In particular, creatinine has been linked to: age, race, sex, and gender [16-18]; physical activity and muscle mass [19]; diet [20-22]; normal physiological functions including menstrual cycles [23]; and an increasing number of pathological conditions including diabetes and breast cancer [24, 25]. Hence, creatinine may not be an appropriate renal dilution factor for certain sample populations.

Urine specific gravity (USG) is the dimensionless ratio between the density of a urine specimen and that of pure water under given conditions. As a bulk property that is representative of the entire urine specimen, USG is presumably less affected by the same

individual factors that influence creatinine [26]. Importantly, the urine density which provides a useful measure of total urine concentration (g/L), is based not only on the number of solute particles, but also on their molecular mass. Thus, abnormal urinary constituents such as protein and glucose, in addition to physiological albumin, sulfates, phosphates, and other heavy species can erroneously inflate USG [27, 28]. Conversely, low density ketones, which are excreted under states of dehydration, starvation, or high fat metabolism common to diabetic patients suffering from ketoacidosis, can deflate USG values [29, 30]. Unlike creatinine, however, USG may be easily corrected with routine clinical urinalyses for many of these factors [31].Moreover, USG can withstand multiple freeze/thaw cycles and long-term storage at -20°C or colder [32, 33]. Consequently, USG has been extensively used as an alternative renal dilution factor to creatinine in primarily toxicological applications [34-36]. In contrast USG corrections have currently found limited utility in clinical settings, presumably from a lack of relevant performance evaluations [10, 37].

Methods employing USG normalizations have generally adopted the correction protocol proposed by Levine and Fahy [38]:

$$C_{corrected} = C_{raw} \times \frac{USG_{reference} - 1}{USG_{experimental} - 1}$$

where $C_{corrected}$ is the adjusted analyte concentration, C_{raw} is the uncorrected analyte concentration, $USG_{reference}$ is a reference USG for a given population, and $USG_{experimental}$ is the experimentally determined USG. A $USG_{reference}$ value of 1.020 is typically applied to U.S. populations, although minor variations can be made to account for differing salts and ingested fluids [35, 38].

To the best of our knowledge, the performance of USG corrections relative to creatinine has not been thoroughly studied in candidate biomarkers for cancer, and specifically, urinary pteridines. In this study, breast cancer was identified as a useful paradigm for performance evaluations based on the recent conflicting reports concerning the ability of urinary pteridines to distinguish benign and aggressive breast cancers in sample populations possibly inappropriate for creatinine normalization [2, 7]. Creatinine

and USG adjustments of urinary pteridines in benign and aggressive breast cancers were therefore compared to evaluate their performance in this double-blind study. We further applied the study to a small scale specificity study involving bladder cancers to demonstrate the utility of USG normalized pteridines beyond breast cancer.

2. MATERIALS AND METHODS

2.1. Samples and specimens

Fifty women 35-85 years of age (mean age: 61 years; standard deviation: 13 years) were recruited by the Mercy Hospital Breast Cancer Treatment Center in Springfield, Missouri [2]. All participants provided written consent, and the collection was approved by the Institutional Review Board at Mercy Hospital in Springfield, Missouri. No monetary compensation was provided. All participants had been recently referred to the Breast Cancer Treatment Center following positive indications by routine clinical breast examinations. Urine specimens were collected early, before 10:00 a.m., but did not necessarily constitute first morning voids. New specimens were stored up to two weeks in a freezer at -20°C at Mercy Hospital. Urine specimens were shipped weekly by frozen ground freight whereby they were transferred to a -80°C freezer until thawing, aliquoting, centrifugation, and assaying one to three months later. Between sample collection and final pteridine assays, samples underwent 2-3 freeze-thaw cycles. No dietary restrictions were placed on the participants prior to sample collection. Pathologies were independently determined by Mercy Hospital pathologists using a combination of clinical breast examinations, mammography, ultrasound and MRI imaging, and biopsy methods [2]. Anonymous pathological reports including diagnoses were disclosed in a double-blind manner at the end of the study.

Sixteen additional specimens from men and women 26-70 years of age (mean age: 52 years; standard deviation: 14 years) were collected in spring 2010 by the Rolla Urology Clinic for the bladder cancer component of the study [7]. All participants provided written consent, and the collection was approved by the Institutional Review Board at Missouri University of Science and Technology. No monetary compensation was provided. Four men and women had untreated, high grade bladder cancers that were diagnosed within the

past five years. Twelve more specimens were collected from healthy, age-matched men and women. Urine specimens were thawed immediately, aliquoted, and centrifuged at 5000 rpm (2571 g) at 4°C for 20 minutes. Centrifuged aliquots were immediately stored at -80°C for three years. Between sample collection and final pteridine assays, samples underwent 2-3 freeze-thaw cycles.

2.2. Chemicals and materials

Isoxanthopterin, pterin, *D*-neopterin, xanthopterin, 6-biopterin, ammonium oxalate, and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). 6-Hydroxymethylpterin was obtained from Schircks Laboratories (Jona, Switzerland). Potassium iodide and iodine were purchased from Fisher Scientific (New Jersey, USA). Synthetic urine was obtained from CST Technologies Incorporated (New York, USA). Ultrapure water was generated by a Milli-Q Advantage® A10 and Millipore Elix® water purification system.

2.3. Assays

The pteridine assay that was used has been described elsewhere [2]. Briefly, a liquid chromatography – tandem mass spectrometry (LC-MS/MS) technique was used to separate and quantify urinary pteridines. Separation was achieved with a Phenomenex Luna phenyl-hexyl column (3.0µm, 3.0 x 150 mm) and a Shimadzu UFLC system (Columbia, MD, USA) comprised of a degasser (DGU-30A3), two pumps (LC-20 AD XR), an autosampler (SIL-20AC XR), and a column oven (CTO-20A) operating under HPLC conditions. An API 4000Q trap MS/MS system from Applied Biosystems (Foster City, CA, USA) quantified urinary pteridines under scheduled multiple-reaction monitoring (MRM) mode with ESI-positive ionization. Pteridines were quantified over a linear range of approximately 0.25 µg/L to 1000 µg/L. Spiked recoveries in real urine samples ranged from 81% to 105% with relative standard deviations (RSDs) between 1% and 6% [2]. Urine specimens were centrifuged at 5000 rpm (2571 g) at 4°C for 20 minutes and pre-treated with 2M ammonium hydroxide and a 4:2% w/v potassium iodide to iodine solution in order to promote fully oxidized pteridines. Ammonium oxalate was used as a sodium adduct

suppressor at a final concentration of 10 µg/L. Urine specimens were diluted a total of 200-fold prior to analysis. All samples were run as true triplicates.

Creatinine was quantified simultaneously with pteridines by the same LC-MS/MS technique using a non-linear calibration curve [2]. The non-linear calibration curve produced spiked recoveries ranging from 95.4% to 101.1% and RSDs between 1.8% and 3.0% for creatinine concentrations between 500 µg/L and 5,000 µg/L. Overly concentrated specimens in which creatinine was unquantifiable by the non-linear calibration curve were diluted 2000-fold and reanalyzed.

Because USG is most accurately determined refractometrically [31], a Carl Zeiss Abbe refractometer. (No. 51865) was used for USG quantification. Prior to analysis, thawed, centrifuged urine specimens were equilibrated at 25°C for 20 minutes. Refractive indices were converted to USG values via a Reichert total solids refractometer conversion table. Ultra-pure water and synthetic urine of known specific gravity were additionally used as quality control standards. The USG assay had inter- and intra-assay CVs of 0.18% and 0.07%, respectively. USG normalizations followed Levine-Fahy protocol using a reference value of 1.020.

2.4. Statistical analysis

Final pteridine concentrations were reported in molar units. Creatinine-corrected pteridines were expressed as mol pteridine/mol creatinine while USG-corrected pteridines remained in molar units. Minitab 16 Statistical Software was used to perform all statistical analyses. Pearson correlations were used to describe the relationship between USG and creatinine. Regular outliers found in aggressive cancers in this study (normality *P*-value = 0.001) and previous findings suggested a Student's t-Test, which assumes a normal distribution, would erroneously inflate statistical significance [2, 7]. Therefore, non-parametric, one-tailed Mann-Whitney hypothesis testing that offers improved efficiency for non-normal distributions was used to evaluate mean differences among normalized pteridines and pathologies at a 5% significance level (*P*-value < 0.05). Finally, two overly

dilute specimens in which pteridines were unquantifiable were excluded from statistical analysis.

3. RESULTS AND DISCUSSION

Spot urine samples, like those taken during clinical tests, come from a patient of unknown hydration status and void time. Analytes of interest must therefore be adjusted to consider the dilution-concentration of the urine specimen. In this study, we examined the performance of traditional creatinine adjustments relative to USG. A direct comparison between urinary creatinine and USG from 48 urine specimens in the breast cancer study was made in Figure 1. The Pearson correlation (r = 0.857, *P*-value = 0.0000, SE = 0.0058 USG) indicated a moderately strong relationship between urinary creatinine and USG, in agreement with previous findings [34]. Moreover, as a primary component of urine that is presumably constantly excreted, creatinine was expected to model USG in a linear fashion. Significant deviations were nevertheless observed in overly concentrated (creatinine > 250 mg/dL) and overly dilute (creatinine < 50 mg/dL) specimens. Similarly, it has become common practice, particularly in toxicological applications, to reject overly dilute (creatinine < 50 mg/dL, USG < 1.010) and overly concentrated (creatinine > 300 mg/dL, USG > 1.030) urine specimens [10, 13, 35]. However, these conventional creatinine and USG requirements may be too restrictive for clinical practice; as shown in Figure 1, 26% of our specimens had unacceptable creatinine levels and 42% failed to meet these USG requirements. Such a considerable number of low urinary creatinine and USG specimens in this study may be explained by a recent finding that women 50 years or older have unusually low urinary creatinine and USG [35]. In addition, such sample discarding is impractical in a clinical setting and creates additional economic and emotional burden on the patient by requiring another specimen provided at a later date. For these reasons, some studies have proposed relaxed concentration-dilution requirements down to 10 mg/dL and 1.002 for the minimum acceptable creatinine and USG levels, respectively [34]. In contrast this study excluded samples with unquantifiable pteridines rather than based on creatinine and USG values resulting in the exclusion of only two urine specimens. Finally, it is important to note that we did not correct USG to conditions including glucosuria, proteinuria, and ketonuria. In addition, study participants were not screened for diabetes.

Thus, these USG-based results may be further optimized in future studies by considering these pathological conditions.

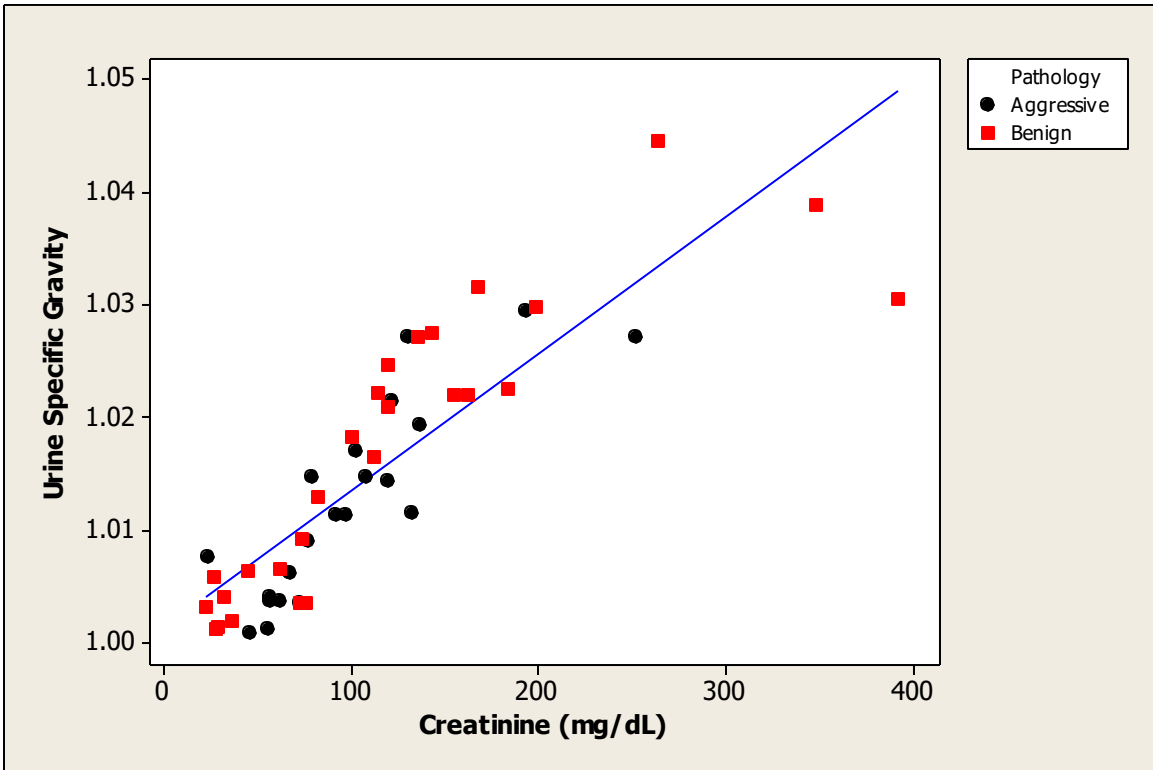

Figure 1. Relationship between USG and creatinine in benign (n = 27) and aggressive breast cancers (n = 21).

USG and creatinine normalization performance were evaluated by statistical hypothesis testing that assumed mean pteridine levels were higher in aggressive cancers based on earlier clinical studies [7]. *P*-values from non-parametric Mann-Whitney mean difference tests for each pteridine in aggressive and benign breast cancers were summarized in Table 1. Summed *P*-values for all six pteridines were provided for simple comparisons among normalization methods, although each pteridine must be considered individually for clinical assessment.

Table 1. *P*-values from non-parametric Mann-Whitney one-tailed mean difference tests between aggressive breast cancer (n = 21) and benign breast cancer (n = 27) urine specimens.

Normalization Method	Uncorrected	Creatinine	USG
6-Biopterin	0.4787	0.4111	0.3776
Pterin	0.2094	0.1277	0.1968
Neopterin	0.5000†	0.5000†	0.3935
Xanthopterin	0.4736	0.3956	0.0090
6-Hydroxymethylpterin	0.1799	0.1586	0.0767
Isoxanthopterin	0.1651	0.1417	0.0011
Summed *P*-values	**2.0067**	**1.7347**	**1.0547**

† Uncorrected and creatinine adjusted mean neopterin values in aggressive cancers were less than those in benign samples.

Interestingly, creatinine normalization (summed *P*-value = 1.7347) provided only a marginal 13.6% improvement over uncorrected pteridines (summed *P*-value = 2.0067). In addition, no pteridines adjusted by creatinine were found to have significantly different (*P*-value < 0.05) mean levels between benign and aggressive breast cancers, rendering any clinical utility useless. These results corroborated similar findings published in a recent study evaluating pteridines adjusted to creatinine in benign and aggressive breast cancers [2]. In comparison USG normalization (summed *P*-value = 1.0547) yielded an overall 47.4% improvement in pathologically correlating pteridines over uncorrected pteridines and outperformed creatinine by a factor of 3.5. Statistical mean differences for all six pteridines except pterin increased with USG normalization, which is reflected by its significantly lowered summed *P*-value. Isoxanthopterin (*P*-value = 0.0011) and xanthopterin (*P*-value = 0.0090) had significantly elevated levels in aggressive breast

cancers compared with benign breast cancers. In addition, 6-hydroxymethylpterin, which was unquantifiable in 22% of samples, also demonstrated some ability to differentiate benign and aggressive breast cancers, although the difference in 6-hydroxymethylpterin values was not statistically significant (*P*-value = 0.0767). Neopterin, which had lower uncorrected mean values in aggressive breast cancers than in benign, highlights the need for urine concentration-dilution normalization. Mean neopterin levels became marginally biased toward aggressive cancers when normalized with USG. Basic statistical information for USG normalized pteridines from the breast cancer study was summarized in Table 2.

Table 2. Basic statistical summaries for six urinary pteridines normalized to USG as well as raw creatinine and USG values in aggressive and benign breast cancer urine samples.

	Mean Aggressive (n = 21)	**Mean Benign (n = 27)**	**Variance Aggressive**	**Variance Benign**
6-Biopterin (M)	1.55E-05	1.00E-05	5.62E-10	1.17E-10
Pterin (M)	2.89E-06	1.99E-06	1.64E-11	6.86E-12
Neopterin (M)	1.42E-05	9.65E-06	4.28E-10	8.70E-11
Xanthopterin (M)	9.05E-06	5.62E-06	7.40E-11	1.31E-11
6-Hydroxymethylpterin (M)	4.73E-06	3.37E-06	6.16E-11	4.07E-11
Isoxanthopterin (M)	3.55E-06	1.03E-06	3.25E-11	8.43E-13
Creatinine (mg/dL)	99.3	122.2	52.4	94.0
USG	1.012	1.017	0.009	0.013

The implications of xanthopterin and isoxanthopterin in aggressive breast cancers in this study prompted further investigation of their potential clinical utility. Boxplots detailing the pathological differences observed in this study for xanthopterin and isoxanthopterin were shown in Figures 2 and 3, respectively. As seen in Figure 2, xanthopterin values associated with aggressive breast cancers were defined as several significantly elevated outliers with a majority of values similar to benign breast cancers. Hence, xanthopterin may not serve as a particularly reliable standalone biomarker for discerning aggressive and benign breast cancers. In contrast isoxanthopterin values from aggressive breast cancers were visibly elevated indicating isoxanthopterin may have superior predictive power which is reflected by its lower *P*-value. Where previous pteridine

studies have focused on individual pteridine diagnostic utilities, we additionally coupled xanthopterin and isoxanthopterin to produce a bivariate indicator of aggressive breast cancers with enhanced diagnostic utility. A corresponding scatterplot of this approach has been provided in Figure 4. A remarkably clear distinction between benign and aggressive breast cancers was observed with the bivariate approach in comparison with those presented in the individual boxplots shown in Figures 2 and 3.

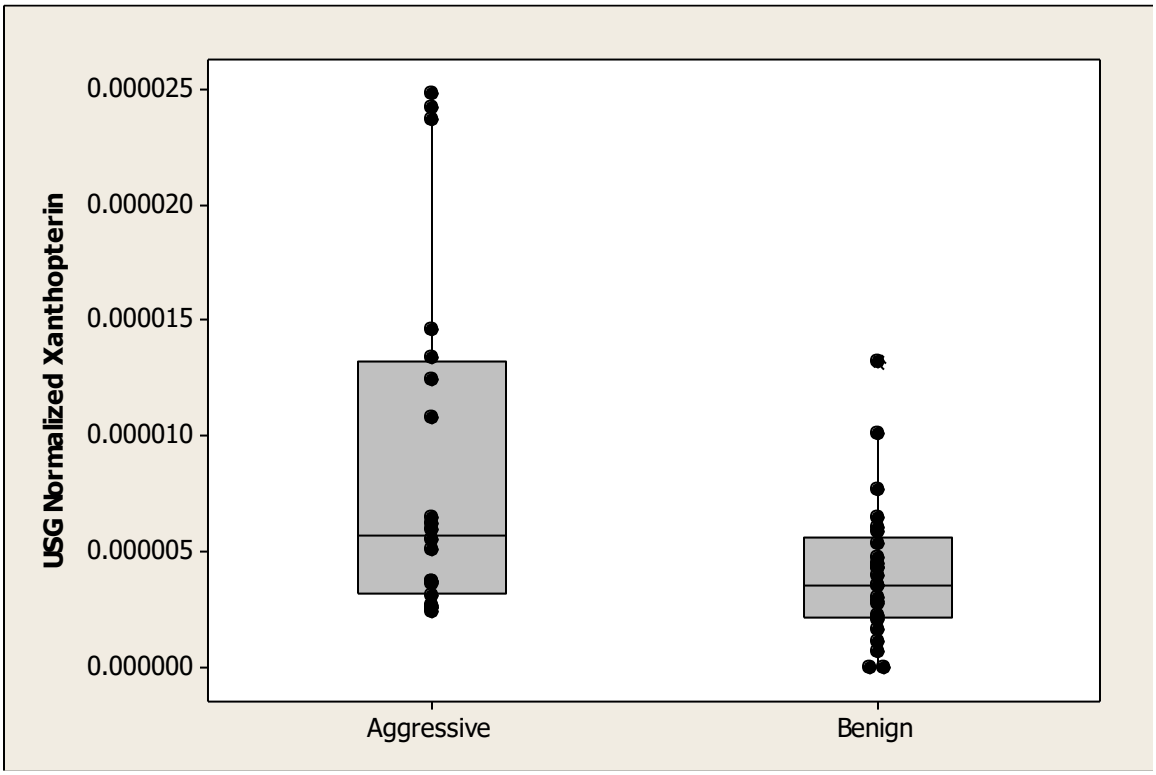

Figure 2. Boxplot of USG normalized xanthopterin levels in aggressive (n = 21) and benign (n = 27) breast cancer urine samples.

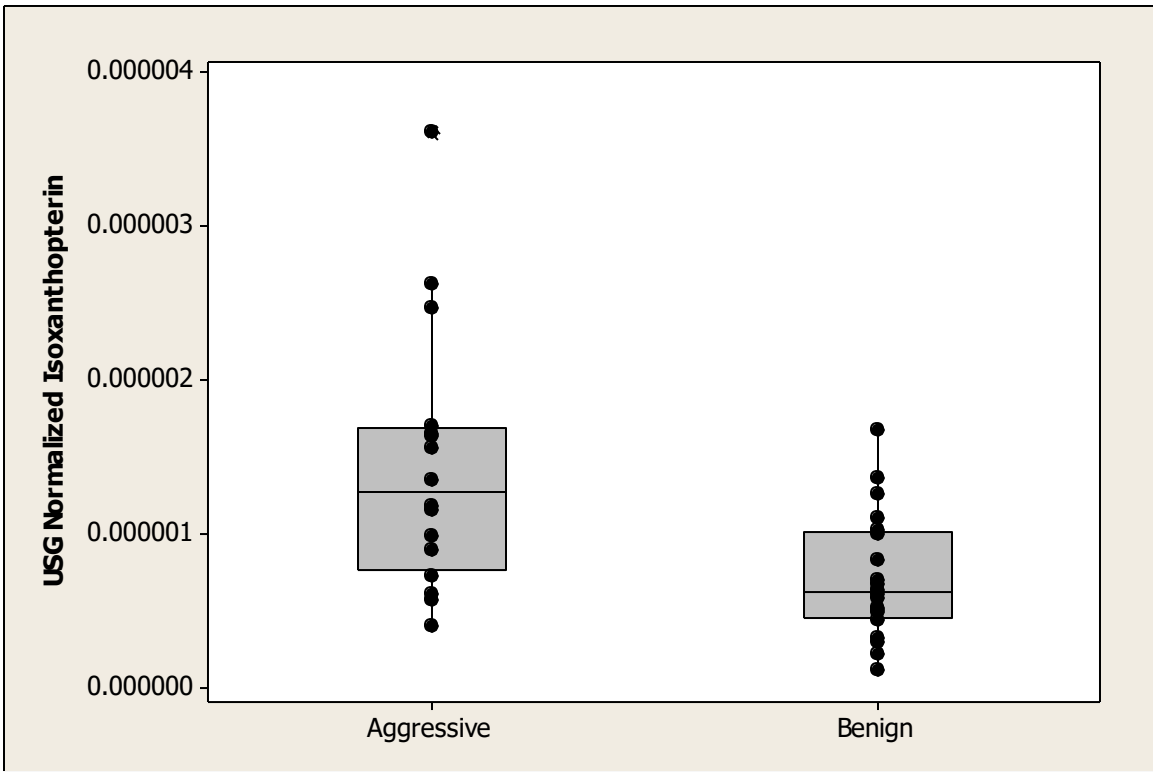

Figure 3. Boxplot of USG normalized isoxanthopterin levels in aggressive (n = 21) and benign (n = 27) breast cancer urine samples.

Applying arbitrary thresholds at 5 µM xanthopterin and 1.5 µM isoxanthopterin, this bivariate approach afforded 87.5% diagnostic sensitivity, 70% specificity, 70% positive predictive value, and 87.5% negative predictive value in discerning aggressive from benign breast cancers. While the individual implications of xanthopterin and isoxanthopterin were supported by numerous other implications of xanthopterin and isoxanthopterin in a variety of pathological conditions, to the best of our knowledge, similar bivariate approaches have not been considered before for pteridines [4-7]. In addition, the isoxanthopterin and xanthopterin results from this study differed significantly from an earlier study using capillary electrophoresis – laser-induced fluorescence (CE-LIF) that also implicated urinary isoxanthopterin and xanthopterin in breast cancers [7]. While it been suggested that the CE-LIF technique may have co-eluting interferences for xanthopterin and isoxanthopterin, an observation supported by unusually high urinary

concentrations compared with LC-MS/MS methodologies, it is also important to note the differences in normalization methods and sample exclusion protocol between this study and that study [2]. Importantly, the CE-LIF study utilized healthy controls that were not gender- and age-matched. As Carrieri and co-workers have demonstrated, creatinine varies considerably more with age and gender than does USG [35]. These considerations alongside new reports indicating that creatinine is affected by menstrual cycles and breast cancer suggest that creatinine may not be an appropriate normalization factor for urinary breast cancer biomarkers [10, 23-25]. While benign and aggressive breast cancers in this study were gender- and age-matched, a clinically wide range of ages were encountered which merited additional correlation studies. Pearson correlations were made for USG, creatinine, xanthopterin, and isoxanthopterin with respect to patient age. Urinary xanthopterin ($r = 0.048$, P-value = 0.754) and isoxanthopterin ($r = -0.113$, P-value = 0.500) clearance were largely age-independent. In contrast, creatinine ($r = -0.321$, P-value = 0.026) was moderately correlated with age with a relationship similar to that reported by Rowe and colleagues [39]. Interestingly, USG ($r = -0.185$, P-value = 0.224) was also weakly correlated with age but did not conflict with larger USG studies. In addition, this study had a significant bias toward women over 50 years of age (mean age = 61 years), which represents a subpopulation in which USG has not been sufficiently studied. Nevertheless, the relatively age-independent nature of xanthopterin, isoxanthopterin, and USG in comparison with creatinine provided a useful interpretation of the observed normalization performance parameters. Because xanthopterin and isoxanthopterin has now been shown to not vary with age within these demographics, their normalization by age-dependent creatinine was reasonably inappropriate. Although these findings were highly encouraging, it should be stressed that a biological premise for pteridines like xanthopterin and isoxanthopterin in cancer has not been firmly established. More importantly, the study results indicated that USG normalization may provide superior performance to creatinine-based adjustments, particularly to age-independent biomarkers like pteridines.

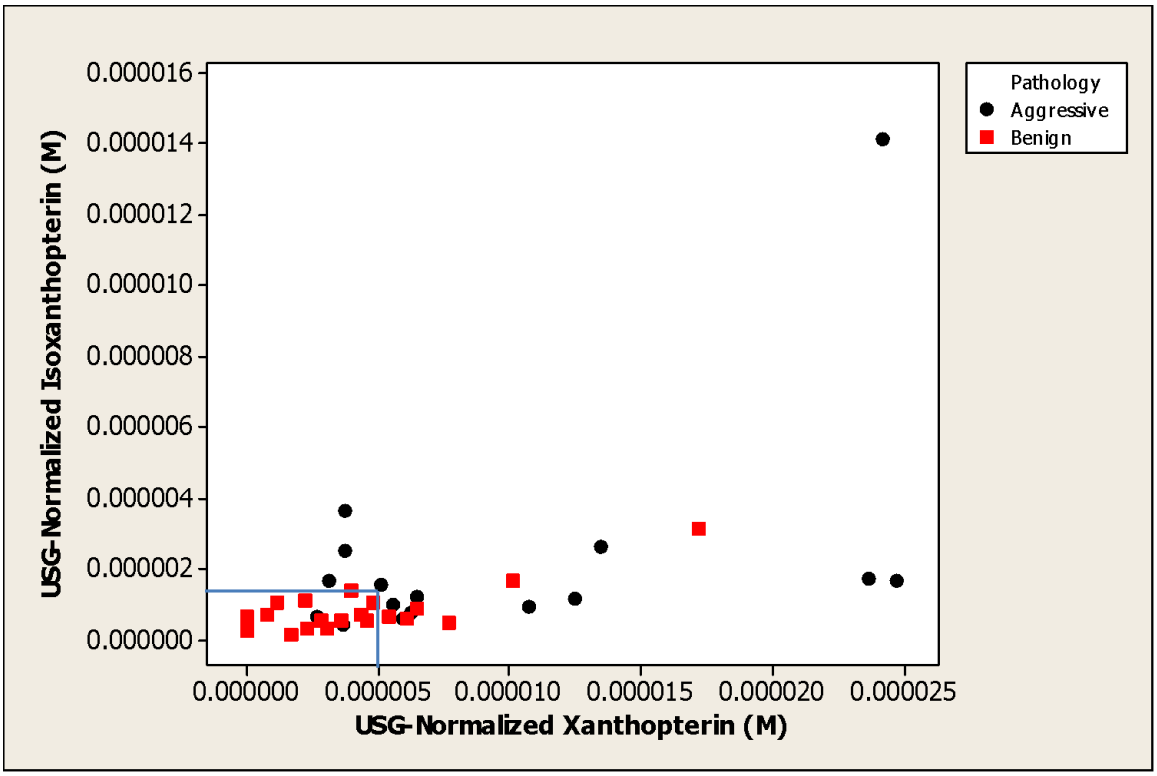

Figure 4. Scatterplot of urinary xanthopterin and isoxanthopterin in 48 benign and aggressive breast cancers.

Finally, the applicability of USG normalization of urinary pteridines was considered through a small-scale bladder cancer study. The results of that study were summarized in Table 3 in the form of *P*-values. Similar to breast cancers, USG normalization afforded optimal *P*-values (summed *P*-value = 0.9109) compared with creatinine normalization (summed *P*-value = 1.8224) which again provided only a marginal improvement over uncorrected pteridines (summed *P*-value = 1.9714). Isoxanthopterin (*P*-value = 0.0105) and xanthopterin (*P*-value = 0.0220) were again implicated as potentially useful biomarkers. It should be noted that 6-hydroxymethyl pterin was strongly uncorrelated with pathology whereas pterin showed some ability, although not statistically significant (*P*-value = 0.0736). Hence, 6-hydroxymethylpterin and pterin may be useful in discerning breast and bladder cancers, although further study is required. As a final note, the bladder cancer study, which was comprised of four untreated, high grade bladder cancer

specimens and 12 normal, healthy specimens, lacked sufficient statistical power to form any conclusions. For this reason, basic statistical information from this study has not been provided and the study is purely demonstrative. A more robust sample set was not pursued based on a lack of appropriate urine specimens. Nevertheless, the preliminary results from this comparison study again suggest that USG normalization of urinary pteridines may be more efficient than creatinine adjustments.

Table 3. *P*-values from non-parametric Mann-Whitney one-tailed mean difference tests between pathological bladder cancer (n = 4) and normal (n = 12) urine specimens.

Normalization Method	Uncorrected	Creatinine	USG
6-Biopterin	0.4274	0.3784	0.2463
Pterin	0.2144	0.4485	0.0736
Neopterin	0.3797	0.2683	0.1285
Xanthopterin	0.2011	0.1400	0.0220
6-Hydroxymethylpterin	0.4844	0.3422	0.4300
Isoxanthopterin	0.2644	0.2450	0.0105
Summed *P*-values	**1.9714**	**1.8224**	**0.9109**

4. CONCLUSION

In this study, we compared the performance of USG and creatinine normalizations of six urinary pteridines in discerning aggressive from benign breast cancers in 50 gender- and age-matched urine specimens. The basis of the study was premised on reports that commonly used creatinine normalizations may be inappropriate for clinical biomarkers due to its influence by a variety of patient factors whereas USG may be a more tolerant urine concentration-dilution indicator. Urinary creatinine was found to be well correlated with USG with deviations occurring in overly dilute and overly concentrated specimens. In addition, creatinine was found to be moderately correlated with patient age while USG, xanthopterin, and isoxanthopterin were reported to be relatively age-independent. This age

dependence was observed in a clinical performance comparison where USG-adjusted pteridines significantly outperformed creatinine-adjusted pteridines which only marginally outperformed uncorrected pteridines in discerning aggressive from benign breast cancers. In addition, isoxanthopterin and xanthopterin were reported to be significantly elevated in aggressive cancers when normalized by USG in comparison to creatinine. Moreover, these pathological correlations were enhanced by coupling xanthopterin and isoxanthopterin to form a bivariate indicator of cancer aggression with remarkable diagnostic sensitivity and specificity. These trends were also replicated in a similar small scale study involving bladder cancers. Hence, these findings indicated that USG normalization offers improved performance for clinical biomarkers and particularly urinary pteridines. In addition, these results strongly recommend that biomarker studies consider sample population demographics in order to choose the most appropriate normalization factor. Finally, larger studies are required to confirm the conclusions made by this study.

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V. HIGH-THROUGHPUT INTRACELLULAR PTERIDINIC PROFILING BY LIQUID CHROMATOGRAPHY – QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY

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ABSTRACT

Pteridines are a diverse family of endogenous metabolites that may serve as useful diagnostic biomarkers for disease. While many preparative and analytical techniques have been described for analysis of selected pteridines in biological fluids, broad intracellular pteridine detection remains a significant analytical challenge. In this study, a novel, specific and sensitive extraction and high performance liquid chromatography – quadrupole time-of-flight – mass spectrometry (HPLC-QTOF MS) method was developed to simultaneously quantify seven intracellular pteridines and monitor 18 additional, naturally-occurring intracellular pteridines. The newly developed method was validated through evaluation of spiked recoveries (84.5% - 109.4%), reproducibility (2.1% – 5.4% RSD), method detection limits (0.1 – 3.0 µg/L) and limits of quantitation (0.1 – 1 µg/L), and finally application to non-small cell lung cancer A549 cells. Twenty-three pteridine derivatives were successfully detected fromcell lysates with an average RSD of 12% among culture replicates. Quantified intracellular pteridine levels ranged from 1 to 1000 nM in good agreement with previous studies. Finally, this technique may be applied to cellular studies to generate new biological hypotheses concerning pteridine physiological and pathological functions as well as to discovery new pteridine-based biomarkers.

KEY WORDS

HPLC-QTOF MS, A549, pteridinic profiling, pteridines, pteridine extraction

1. INTRODUCTION

Pteridines are endogenous metabolites that function broadly in the human body as cofactors and intermediates for an array of metabolic pathways. Reported associations between pteridine levels and actively proliferating cells have prompted efforts to evaluate pteridine derivatives as novel indicators of disease and especially cancer [1]. Consequently the clinical significance of a handful of pteridine derivatives has been established over the past several decades [1-3]. Neopterin in particular has been comprehensively studied leading to an improved understanding of its pathomechanistic production and related diagnostic utility. Briefly, neopterin production has been linked to the stimulation of human monocytes by T-lymphocytes- and natural killer cell-derived cytokines and is related to a variety of pathological conditions such as infection, cancer, autoimmune diseases and rejected organ transplants [4-7]. Similarly, the cytokine-induced synthesis of tetrahydrobiopterin (BH4), a cofactor for nitric oxide synthase that is further related to vascular dysfunctions, has also been widely reported [8-10]. Hence, some pteridine derivatives like neopterin and BH4 appear to reflect activated immune systems in response to disease and associated inflammation. While such pteridine biomarkers fail to constitute classical tumor markers, other pteridine derivatives like xanthopterin, isoxanthopterin, oncopterin, pterin-6-carboxylic acid, 6-biopterin, among others have been widely speculated as candidate cancer biomarkers with pathomechanistic production thought to be related to perturbed cancer cell metabolisms. However, the lack of rigorous clinical validation and a poor understanding of how these pteridine derivatives specifically relate to cancer suggest further study is urgently required [1, 3, 11]. Moreover, with a large number of pteridine derivatives relatively unstudied and multiple, previously studied pteridine derivatives already having reported diagnostic value, the inclusion of broader pteridine panels in new analytical methods is urgently needed. These serious issues facing general pteridine research and efforts to establish pteridine derivatives as useful biomarkers for disease have arisen in part from complex bioanalytical and clinical challenges related to the accurate and specific detection of pteridine derivatives under native speciation [11, 12].

Pteridine analysis in biological fluids has progressed significantly in the past several years. These method development studies have met varied challenges ranging from physiochemical properties and sample preparation issues to clinical considerations and limited pteridine profiling. Physiochemical complications include trace concentrations in biological fluids, pteridine derivatives with multiple oxidation states, and species labile to heat, light, and auto-oxidation [11, 12]. For these reasons, various oxidative pre-treatments have often been utilized by a wide range of techniques and sample matrices to oxidize any residual reduced pteridine derivatives to facilitate the analysis of more stable and more fluorescent, oxidized forms [13-19]. The resulting destruction of biologically useful information related to the reduced pteridine derivatives by these oxidative pre-treatments has prompted recent efforts to develop new techniques capable of simultaneously determining pteridines in their native oxidation states[20-22]. This trend toward total, native pteridine detection has become increasingly important as efforts to clinically validate pteridines continue to expand. In addition, the role of creatinine as an appropriate normalization factor in noninvasive urine-based strategies has been recently questioned for applications involving certain populations [14]. In response, urine specific gravity has been proposed as a more tolerant indicator of urine concentration-dilution for pteridine-based discrimination of benign and aggressive breast cancers [23]. Finally, new methods continue to improve detection limits and overcome these and other bioanalytical challenges following the rapid development of multiple, sensitive and specific pteridine detection techniques utilizing a variety of platforms including capillary electrophoresis – laser-induced fluorescence (CE-LIF) [19], high-performance liquid chromatography – fluorescence detection (HPLC-FD) [13, 15, 22, 24], high-performance liquid chromatography – mass spectrometry (HPLC-MS) [25], high-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) [14], and synchronous fluorescence spectroscopy [26]. These techniques have expanded beyond urinary pteridine detection [13, 14, 19, 24-26] to include serum [15] and cerebrospinal fluid [22] analyses as well, although these additional matrices remain relatively underdeveloped.

In comparison, intracellular pteridinic profiling, central to both elucidating pathomechanistic production of pteridine derivatives and discovering new, clinically useful

pteridine biomarkers, remains remarkably underdeveloped. Current techniques have been generally limited to analysis of biopterin and neopterin derivatives using a combination of HPLC-ED, HPLC-FD and ELISA methods with the exception of a recent HPLC-MS/MS technique for biopterin derivatives [20, 27-29]. Specific analytical difficulties such as polar metabolite extraction, interference minimization, and instrument compatibility especially with mass spectrometry, have complicated efforts to further intracellular pteridinic profiling. For example, while fluorescence-based techniques, such as capillary electrophoresis – laser-induced fluorescence (CE-LIF) and liquid chromatography – fluorescence detection (LC-FD), have remained popular for pteridine detection due to their high sensitivities and effective separations, such techniques require chemical standards for compound confirmation and are particularly sensitive to fluorescent interferences and UV-absorbing material. Similarly, conventional quadrupole tandem mass spectrometry techniques (e.g. LC-MS/MS) offer competitive sensitivities without the need for complete separation, but lack the high mass resolution currently to reliably semi-quantitatively monitor additional pteridine derivatives without the use of often expensive and otherwise difficult-to-obtain chemical standards. In comparison, high resolution, high-performance liquid chromatography – quadrupole time-of-flight mass spectrometry (HPLC-QTOF MS) provides adequate sensitivity, greatly improved selectivity, and the ability to perform MS/MS experiments in combination with comprehensive database integration to provide a platform suitable for both quantitative and semi-quantitative profiling of a robust pteridine panel.

In this study, a combination pteridine extraction and high resolution HPLC-QTOF MS detection method was developed for the quantitative determination of seven selected pteridines (**Figure 1**) and the semi-quantitative analysis of 18 additional pteridines to profile a complete panel of 25 pteridines in cell lysate matrices (**Figure 2**). Basic information for the 25 selected pteridine derivatives have been provided in **Table 1**. The validated method was finally applied to non-small cell lung cancer A549 cells to demonstrate the robustness of the newly developed method.

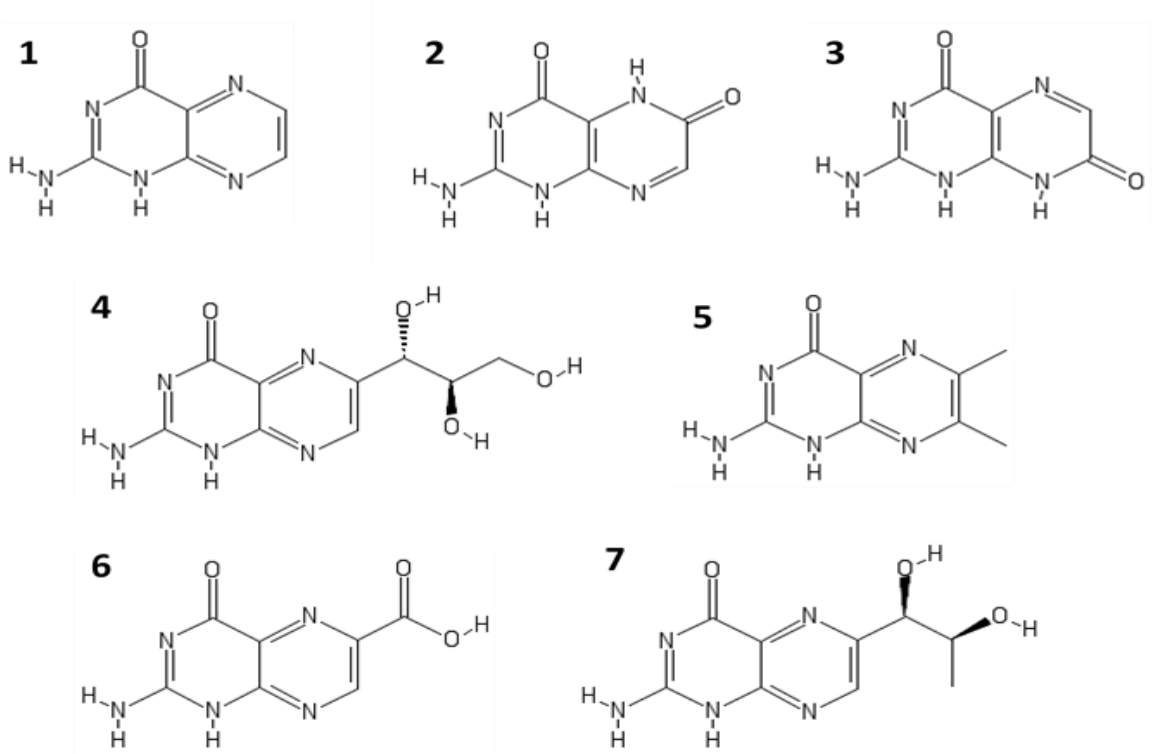

Figure 1. Chemical structures of seven quantitatively measured cellular pteridine derivatives: (1) pterin, (2) xanthopterin, (3) isoxanthopterin, (4) *d*-neopterin, (5) 6,7-dimethylpterin, (6) pterin-6-carboxylic acid, and (7) 6-biopterin.

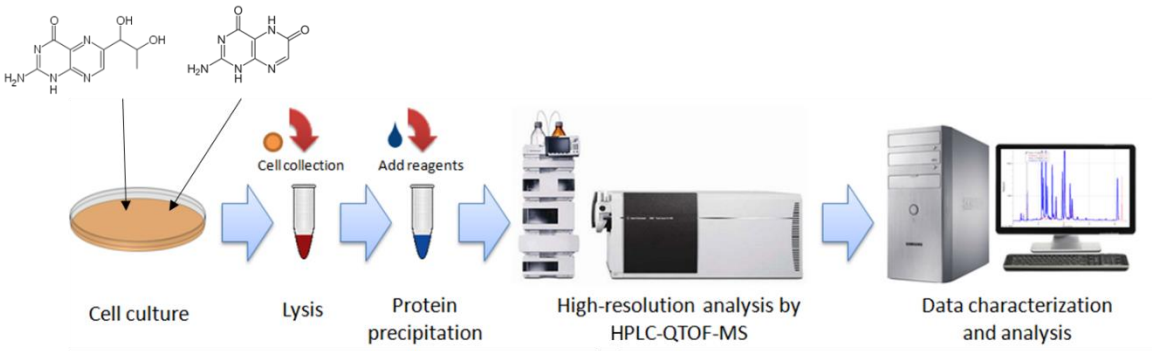

Figure 2. General schematic of the newly developed extraction and quantitative techniques for broad intracellular pteridinic profiling.

Table 1. Basic information for 25 monitored intracellular pteridine derivatives

Compound	CAS Number	Formula	$[M+H]^+$ Ion (m/z)	Analysis Type
Pterin	218-799-1	$C_6H_5N_5O$	164.0567	Quantitative
Lumazine	487-21-8	$C_6H_4N_4O_2$	165.0407	Semi-quantitative
7,8-Dihydropterin	17838-80-1	$C_6H_7N_5O$	166.0723	Semi-quantitative
5,6,7,8-Tetrahydropterin	1008-35-1	$C_6H_9N_5O$	168.0880	Semi-quantitative
6-Methylpterin	708-75-8	$C_7H_7N_5O$	178.0723	Semi-quantitative
Xanthopterin†	119-44-8	$C_6H_5N_5O_2$	180.0516	Quantitative
Isoxanthopterin†	529-69-1	$C_6H_5N_5O_2$	180.0516	Quantitative
6-Hydroxylumazine†	2577-35-7	$C_6H_4N_4O_3$	181.0356	Semi-quantitative
7-Hydroxylumazine†	2577-38-0	$C_6H_4N_4O_3$	181.0356	Semi-quantitative
7,8-Dihydroxanthopterin	1131-35-7	$C_6H_7N_5O_2$	182.0672	Semi-quantitative
6-Formylpterin	23663-21-0	$C_7H_5N_5O_2$	192.0516	Semi-quantitative
6,7-Dimethylpterin	611-55-2	$C_8H_9N_5O$	192.0880	Quantitative
6-Hydroxymethylpterin	712-29-8	$C_7H_7N_5O_2$	194.0672	Semi-quantitative
6,7-Dimethyltetrahydropterin	167423-51-0	$C_8H_{13}N_5O$	196.1193	Semi-quantitative
Pterin-6-carboxylic Acid	948-60-7	$C_7H_5N_5O_3$	208.0462	Quantitative
Sepiapterin†	17094-01-8	$C_9H_{11}N_5O_3$	238.0935	Semi-quantitative
6-Biopterin†	22150-76-1	$C_9H_{11}N_5O_3$	238.0935	Quantitative
Biolumazine	94591-24-9	$C_9H_{10}N_4O_4$	239.0775	Semi-quantitative
7,8-Dihydrobiopterin	6779-87-9	$C_9H_{13}N_5O_3$	240.1091	Semi-quantitative
5,6,7,8-Tetrahydrobiopterin	17528-72-2	$C_9H_{15}N_5O_3$	242.1248	Semi-quantitative
Monapterin†	2009-64-5	$C_9H_{11}N_5O_4$	254.0884	Semi-quantitative
Neopterin†	2009-64-5	$C_9H_{11}N_5O_4$	254.0884	Quantitative
7,8-Dihydroneopterin	1218-98-0	$C_9H_{13}N_5O_4$	256.1040	Semi-quantitative
5,6,7,8-Tetrahydroneopterin	25976-00-5	$C_9H_{15}N_5O_4$	258.1197	Semi-quantitative
Oncopterin	143460-23-5	$C_{12}H_{18}N_6O_3$	295.1513	Semi-quantitative

† These pteridines are structural isomers with equivalent precursor ion exact masses.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials

Isoxanthopterin, pterin, *D*-neopterin, xanthopterin, 6,7-dimethylpterin, and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 6-Biopterin, pterin-6-carboxylic acid, ammonium hydroxide, formic acid, and ascorbic acid were purchased from J&K Scientific, LTD (Beijing, China). Trypsin and Dulbecco's Modified Eagel's Medium (DMEM) used in the cell culture preparation were also purchased from Sigma-Aldrich.

2.2. Instrumentation

A Venusil XBP phenyl-hexyl column (5.0 μm, 4.6×150 mm, Bonna-Agela Technologies Inc., USA) and an Agilent LC 1200 HPLC system (Santa Clara, California, USA) comprised of a degasser (G1322A), a binary pump (G1312B), an autosampler (G1367D), an autosampler thermostat controller (G1330B), and a column oven (G1316B) were used for the separation of the selected cellular pteridines. Separation was carried out at 25 °C with a flow rate of 0.25 mL/min using mobile phase that had the following composition, A: 0.1% (v/v) formic acid in water and B: 0.1% (v/v) formic acid in acetonitrile. A gradient program was used to enhance separation that initialized at 50% B followed by a linear decrease to 20% B at 3 minutes followed by a linear increase to 80% B at 8 minutes. This composition was held for the remainder of the run. Run-to-run time was 25 minutes with a sample injection volume of 5 μL.

An Agilent 6530 high resolution QTOF mass spectrometer with positive-ion ESI was used to detect and quantify the selected pteridines. The QTOF mass spectrometer was operated under untargeted MS/MS mode for characterization and semi-quantitative analysis of selected pteridines lacking standard reagents. Optimized operational parameters included: gas temperature: 325 °C; drying gas: 6 L N_2/min; nebulizer pressure: 30 psig; sheath gas temperature: 350 °C; sheath gas flow: 7.5 L N_2/min; ESI voltage: +3500 V; TOF fragmenter voltage: 130 V; skimmer voltage: 65 V; quadrupole RF Vpp voltage: 750 V.

2.3. Standard Preparation

Approximately 4 mg of each pteridine standard was accurately weighed to 0.01 mg precision and dissolved in a 0.1% (w/v) dithiothreitol (DTT) solution comprised of 9.8 mL ultrapure water and 200 µL 2 M ammonium hydroxide. Ultrasonication and vortexing were used together to facilitate the dissolution process. A stock mixture containing 10 mg/L of each pteridine standard was prepared daily and maintained at 4 °C under dark conditions. Calibration standards were prepared from the stock mixture by serial dilution in a 0.1% (w/v) DTT solution prepared from ultrapure water.

2.4. Cell Cultures and Preparation

A549 adenocarcinoma human alveolar basal epithelial cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cells were suspended in DMEM with 10% fetal bovine serum (FBS). Cells were cultivated in 10 cm Petri dishes in a controlled incubator maintained at 37 °C and 5% carbon dioxide under dark conditions. Cells were harvested by incubating at 37 °C and 5% carbon dioxide with 1 mL trypsin solution and immediately counted with a hemocytometer. After centrifuging the trypsinized cells at 200 g for 5 minutes at 4 °C, the trypsin supernatant was carefully aspirated and the cell pellets were washed twice with ultrapure water. The cells were then resuspended in 500 µL chilled ultrapure water containing 0.1% DTT followed by manual homogenization to lyse cellular membranes. The homogenized cells were then further snap-frozen with liquid nitrogen for 1 minute and allowed to gradually thaw to 4 °C. A 500 µL addition of cold (-20 °C) methanol containing 0.1% DTT and 10 mM ammonium hydroxide was made to the thawed lysate and the extract was allowed to incubate for 10 minutes on ice. Insoluble portions were subsequently removed by centrifugation at 20,000 g for 20 minutes at 4 °C. A 500 µL aliquot of the undiluted supernatant was transferred to a 1.5 mL vial and placed in a dark, thermostated (4 °C) autosampler. A 5 µL aliquot was injected for pteridinic profiling of the cell lysate.

3. RESULTS AND DISCUSSION

3.1. Pteridine Extraction

Similar to pteridine analysis, intracellular pteridine extraction is complicated by the unique physiochemical properties of pteridines, specifically poor solubility in many aqueous and organic solvents, trace biological concentrations, and high polarity. While pteridine extraction from insect tissue and animal irises using basic buffers followed by fluorimetric detection has been widely reported, pteridine extraction protocol for either mammalian cells or MS-compatible detection has remained limited [30-32]. Fismen et al reported biopterin derivative extraction from cell lysates using a combination of trichloroacetic acid, acetic acid, and EDTA, but did not consider other extraction solvents or other pteridine derivatives [20]. Therefore, previously reported comparisons made among common metabolite extraction techniques for similarly polar nucleotide-derived compounds and amino acids were used to develop candidate pteridine extraction techniques [33]. The selected extraction solvents included 100% cold (-20 °C) methanol, 50% cold (-20 °C) aqueous methanol, and hot (60 °C) water, all of which contained 0.1% DTT to prevent auto-oxidation of reduced pteridine species [13] and 5 mM ammonium hydroxide [14] to improve pteridine solubility. Systematic comparison of DTT concentrations on a variety of reduced pteridines has previously demonstrated 0.1% DTT effectively maintains reduced pteridine stability in complex biological matrices [13]. It should be noted that 50% aqueous acetonitrile was not examined due to pteridine solubility concerns. A standard extraction volume of 1000 µL was used, although this parameter remains to be optimized for given cell counts. It was noted that extraction volumes larger than 5000 µL for approximately $1 \bullet 10^7$ A549 cells resulted in non-detection of some pteridine derivatives. In addition, cryogenic cell lysis through liquid nitrogen exposure significantly improved pteridine extraction over manual homogenization alone which was indicative of more complete lysis. Normalized extraction efficiencies were determined by normalizing the extracted concentrations of the seven, quantitatively monitored pteridines to the highest performing extraction solvent.

Pure organic solvent (100% cold methanol) failed to appreciably extract pteridines with an average extraction efficiency of 41%. The resulting poor extraction efficiency may

be attributed to the limited solubility of polar pteridines in cold, pure organic solvent. Pteridine extraction by hot water provided 122% extraction efficiency, but was characterized by high inter-experimental variability and markedly decreased reduced pteridine derivatives. These data indicate hot water extractions may promote the non-specific oxidation of reduced pteridine derivatives to more oxidized forms. Meanwhile, 50% cold methanol afforded optimal extraction efficiencies without significantly affecting reduced pteridine species, although it remains unclear to what extent these oxidation processes may have occurred. These extraction comparisons supported similar findings for the extraction of polar nucleotide-derived compounds and amino acids [33]. In addition, 5 mM ammonium hydroxide significantly improved pteridine extraction for all extraction solvents studied. Oxidative influence on reduced pteridine derivatives by ammonium hydroxide was not appreciably observed in the presence of 0.1% DTT. Higher ammonium hydroxide concentrations posed MS compatibility concerns and often resulted in the formation of a viscous precipitate after centrifugation that rendered removal of the supernatant difficult and resulted in decreased pteridine levels. This phenomenon has been reported with similar potassium hydroxide-assisted extractions [33]. Finally, a wider range of inorganic solvents may further improve extraction efficiencies, but are not compatible with HPLC-QTOF MS analysis.

3.2. MS/MS Optimization

Seven, quantitatively monitored pteridines were mass optimized with respect to their precursor and two most abundant product ions. Quantitative analysis of these seven pteridines implemented a 20 ppm symmetric expansion about these optimal masses to provide high-resolution extracted ion chromatograms for each pteridine derivative. Previous work has reported optimal positive-ion ESI using 0.1% (v/v) formic acid in aqueous and organic mobile phases [14, 25]. Therefore, 1000 µg/L pteridine standards were prepared in 0.1% (v/v) aqueous formic acid and injected into the high resolution QTOF mass spectrometer via direct infusion at a rate of 0.3 mL/hour. Ions were monitored under full scan mode between 150 m/z and 300 m/z for one minute to determine the crude average ion mass. The pteridine standard was reexamined under Selected Ion Monitoring (SIM) scan mode for one minute centered on the crude average ion mass with a 200 ppm

symmetric expansion. The new centroid mass from the SIM scan was used as the experimentally optimized precursor ion mass. To optimize product ion masses, targeted MS/MS acquisition was used on the infused standard centered on the optimized precursor ion mass with a 20 ppm symmetric expansion and a collision energy of 20 eV which was preliminarily found to afford the optimal product ion intensities. MS/MS spectra were acquired for one minute, and the average masses for the two most abundant product ions were used as the optimized product ion masses for confirmation purposes. The precursor and production ion masses determined were similar to those reported by studies using lower mass resolution instruments [14]. Notably, primarily $[M+H]^+$ ions were observed with negligible formation of ammonium adducts, $[M+NH_3]^+$ and $[M-H+2NH_4]^+$, in xanthopterin and pterin-6-carboxlyic acid. These differences may be attributed to different positive-ion ESI conditions, such as a lower capillary voltage (3500 V vs 4500 V) and significantly higher gas flow rates. Hence, under the given MS/MS conditions, the assumption that similar pteridine derivatives will form $[M+H]^+$ appeared reasonable. The optimized MS/MS parameters were summarized in **Table 2**.

3.3. HPLC Optimization

The separation of the seven, quantitatively monitored pteridines was based on chromatographic conditions described in our previous work with some notable modifications [14]. In this study, a larger Venusil XBP phenyl-hexyl (5.0 μm, 4.6×150 mm) column, in comparison with a Phenomenex Luna phenyl-hexyl (3.0 μm, 3.0×150 mm) column, was selected to separate the seven pteridines based on column availability. Resulting differences in retention time, retention order, and peak shape were noted in comparison with the previously described method. Therefore, the separation was evaluated and optimized under a range of flow rates from 0.10 mL/min to 0.40 mL/min with a variety of mobile phase compositions (0.0-0.2% formic acid in water, methanol, and acetonitrile). Briefly, it was observed that initially high aqueous mobile phase (>50%) compositions resulted in loss of retention and the occurrence of significant peak tailing while very high organic compositions (>80%) resulted in complete retention. The resulting optimized conditions may be found in Section 2.

Table 2. Monitored masses, linear range, linearity, method detection limits, and retention times of seven selected pteridines for quantitative analysis

Compounds	Precursor Ion (m/z)	Confirmation Ion #1 (m/z)	Confirmation Ion #2 (m/z)	Linear Range (µg/L)	R^2	Method Detection Limit (µg/L)	Retention Time (min)
Pterin-6-carboxylic acid	208.0462	190.0334	162.0398	0.5-5,000	0.9994	0.5	8.61
Isoxanthopterin	180.0516	163.0256	135.0263	3-10,000	0.9994	3	8.87
D-Neopterin	254.0884	206.0815	190.0747	0.1-10,000	0.9997	0.1	9.42
Xanthopterin	180.0516	163.0256	135.0263	0.8-10,000	0.9999	0.8	9.50
6-Biopterin	238.0935	220.0789	178.0788	0.5-10,000	0.9994	0.5	9.55
Pterin	164.0567	119.0674	91.0745	0.5-10,000	0.9996	0.5	9.97
6,7-Dimethylpterin	192.0880	147.0672	175.0576	0.5-5,000	0.9985	0.5	10.96

A representative chromatogram, including separation of the structural isomers, xanthopterin and isoxanthopterin, was shown in **Figure 3**. In addition, significant equilibriation volumes were required for reproducible separations involving real cell lysates, necessitating longer 25 minute run-to-run times. Peak resolution also decreased as a result of the larger column, indicating that smaller HPLC and UPLC columns with more efficient packing may further improve method separation. Nevertheless, it is clear from **Figure 3** that the quantitatively measured, structural isomers xanthopterin and isoxanthopterin remained completely resolved. Moreover, matrix interference concerns were further minimized through high resolution QTOF MS and MS/MS monitoring with 20 ppm symmetric expansions to facilitate compound identification and confirmation.

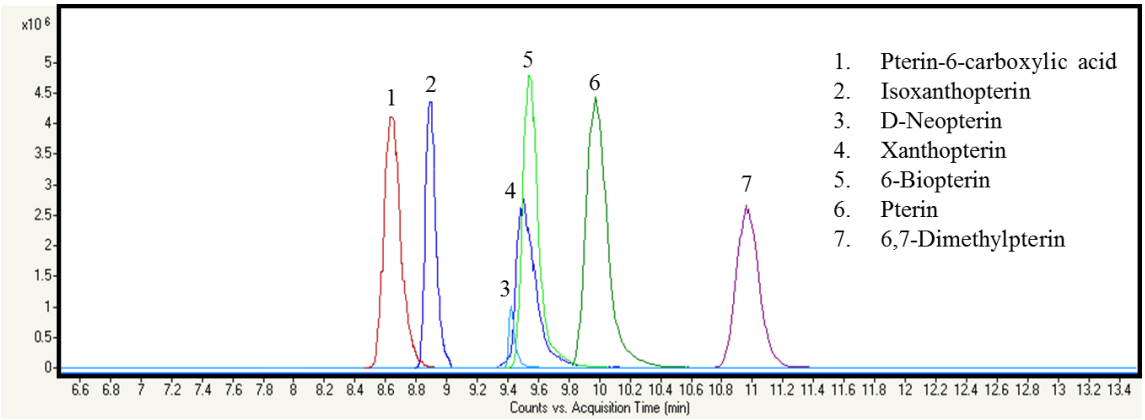

Figure 3. Overlaid extracted ion chromatograms (EIC) of seven quantitatively monitored pteridine derivative standards at nominal concentrations of 100 µg/L.

3.4. Method Performance

Method performance parameters for the seven quantitatively monitored pteridine derivatives were determined in similar extraction solvent matrices without the presence of cell lysate material which presented matrix reproducibility challenges. Hence, possible matrix effects were not present for this component of the study, but were quantitatively

evaluated later in the real cell study. Under the optimized HPLC-QTOF MS conditions, calibration curves for all seven quantitatively monitored pteridines were constructed to demonstrate linearity under a broad analytical range. As shown in **Table 2**, all seven pteridines performed linearly over multiple orders of magnitude with linear regression coefficients (R^2) above 0.9985. Moreover, the method was found to be highly sensitive with method detection limits ranging from 0.1 to 3 µg/L and limits of quantitation ranging from 0.1 to 10 µg/L. These detection limits were comparable to previously reported pteridine detection limits with fluorescence detectors and mass spectrometry techniques. In addition, spiked recoveries were performed at low (10 µg/L), medium (100 µg/L) and high (1,000 µg/L) concentrations to determine technique precision and reproducibility. The data in **Table 3** demonstrated high recovery accuracies ranging from 94.5% to 109.4% with deviations ranging from 1.2 to 5.4% RSD. These figures of merit indicated the quantitative HPLC-QTOF MS technique is both accurate and precise as well as comparable to other pteridine analyses developed for biological fluid analysis.

Table 3. Triplicate spiked recovery results of seven selected pteridines at three varying concentrations.

	Low (10 µg/L)		**Medium (100 µg/L)**		**High (1000 µg/L)**	
Compound	**Recovery**	**RSD**	**Recovery**	**RSD**	**Recovery**	**RSD**
D-Neopterin	109.4	4.0	105.4	3.5	102.9	2.8
6-Biopterin	104.5	3.8	105.1	3.2	101.7	2.4
Xanthopterin	94.7	3.4	102.4	4.4	97.8	2.7
Pterin	98.7	2.1	95.1	3.0	95.6	3.4
Isoxanthopterin	100.2	3.7	102.1	4.3	103	3.9
6,7-Dimethylpterin	105.7	5.4	103.2	5.2	97.8	4.2
Pterin-6-carboxylic acid	94.5	4.2	95.7	3.4	95.9	4.7

†All values are presented as a percentage (%).

3.5. Application to Real Cell Cultures

The newly developed extraction and HPLC-QTOF MS methods were applied to real cell cultures to demonstrate method validation and feasibility. A549 adenocarcinomic human alveolar basal epithelial cells were selected as a useful model due to their widespread occurrence in biomedical research, consistent cell growth and size, and relative ease of culturing. Biological and analytical triplicate cell cultures were examined to provide quantitative figures of merit for the combined extraction and quantitative techniques. Summarized results have been shown in **Table 4**. Under the optimized conditions, all 25 selected pteridine derivatives except oncopterin (expected 295.15131 m/z) were detected. The presence of an unidentified system peak centered at 294.13091 m/z with significant [A+1] and [A+2] isotopic ions obfuscated possible oncopterin signals. Moreover, all seven quantitatively monitored pteridines were quantifiable from the cell lysate except neopterin and isoxanthopterin which was generally detected but unquantifiable with an average signal-to-noise ratio (SNR) of 2.1. Among the semi-quantitatively monitored pteridine derivatives, 12 were identified by the elution of a single peak at the expected precursor ion using a 20 ppm symmetric expansion as well as confirmation by MS/MS fragmentation and comparison with MS/MS spectra provided in the Human Metabolome Database. Multiple elution peaks and lacking relevant MS/MS fragmentation spectra complicated the confirmation of six additional pteridine derivatives. For these compounds, all elution peaks for a respective precursor ion were monitored for the purpose of this study. The inability to confidently confirm these particular pteridine derivatives with MS/MS spectra reflects the often poorly studied nature of many of these compounds within broader mass spectrometry applications. Further study of these compounds is therefore urgently required. Combined technique reproducibility among the pteridine derivatives was nevertheless generally good with an average RSD of 12%. Accuracy limitations related to the cell hemocytometer and incomplete trypsin solution removal during the extraction process may further improve technique reproducibility and accuracy. Nevertheless, these data indicated the newly developed technique was suitable and useful for broad intracellular pteridine profiling from cell lysates.

Table 4. Summarized results of 25 intracellular pteridine derivatives in A549 cells

Compound	Retention Time (min)	Average Peak Area	RSD[2]	Intracellular Conc. (nM)
Oncopterin	N/A	N/A	N/A	-
Pterin-6-carboxylic Acid	8.61	6280	22.32	111
6-Hydroxymethylpterin	8.64	66838	19.76	-
6-Hydroxylumazine[b]	8.66	67828	1.98	-
6-Formylpterin[1]	8.75	21702	5.06	-
Monapterin[d]	8.87	14224	4.92	-
7,8-Dihydroneopterin[1]	8.92	39599	13.31	-
7-Hydroxylumazine[b]	8.95	52077	19.81	-
Lumazine[1]	9.00	21733	1.03	-
Sepiapterin[c]	9.05	23446	30.68	-
6-Formylpterin-2[1]	9.41	5410	68.29	-
Neopterin[d]	9.42	4199	6.92	<MDL
7,8-Dihydroneopterin-2[1]	9.62	361	17.61	-
Isoxanthopterin[a]	9.76	4426	24.27	<MDL
5,6,7,8-Tetrahydroneopterin	9.78	2050489	5.27	-
6-Biopterin[c]	9.87	20780	4.69	379
Biolumazine	9.98	2928486	9.56	-
5,6,7,8-Tetrahydrobiopterin	9.99	195769	10.82	-
7,8-Dihydrobiopterin	10.00	7386079	8.92	-
7,8-Dihydropterin	10.10	20807	8.45	-
6-Methylpterin[1]	10.22	2599	79.85	-
Xanthopterin[a]	10.22	8371	3.25	475
7,8-Dihydroxanthopterin[1]	10.25	84546	14.99	-
Pterin	10.27	13786	13.85	280
6-Formylpterin-3[1]	10.27	82467	8.81	-
Lumazine-2[1]	10.28	7098	27.79	-
6,7-Dimethylpterin	10.96	4107	15.36	106
5,6,7,8-Tetrahydropterin[1]	11.39	8844	8.15	-
6-Methylpterin-2[1]	11.54	19914	4.67	-
6,7-Dimethyltetrahydropterin[1]	13.32	44649	17.46	-
6-Methylpterin-3[1]	13.42	12646	14.59	-
5,6,7,8-Tetrahydropterin-2[1]	13.49	55897	22.62	-
7,8-Dihydroneopterin-3[1]	13.49	16328	9.61	-
6,7-Dimethyltetrahydropterin-2[1]	14.92	211973	7.67	-
7,8-Dihydroxanthpterin-2[1]	19.43	1607940	27.08	-

[a-d]These pteridines are structural isomers with equivalent precursor ion exact masses.

[1]The identities of these compounds were not confirmed after database lookup.

[2]All values are presented as a percentage determined from analytical triplicates.

In addition, the intracellular concentrations of the seven quantitatively monitored pteridine derivatives were determined. Molar concentrations within the 1 mL extract were converted to moles pteridine derivative and normalized by the estimated number of cells determined by the manual hemocytometer to yield average moles pteridine per cell. An average A549 cytosolic volume of 1204 μm^3 was finally used to determine average molar pteridine concentrations per cell.[34] Selected intracellular pteridine concentrations ranged from 1 nM to 1000 nM. Limited data concerning absolute intracellular pteridine concentrations rendered literature comparisons difficult. Total biopterin has been reported in hepatocytes using HPLC-FD near 9.0 µM with BH4 comprising nearly 8.6 µM and dihydrobiopterin (BH2) comprising the remaining 0.4 µM [35]. Fismen et al reported similar BH4 values in HUVEC cells with an HPLC-MS/MS technique while also reporting negligible BH2 and biopterin content [20]. The BH4, BH2, and biopterin levels reported herein appeared to be in agreement with these previous studies. For example, while this study successfully detected biopterin from the cell lysates, the reported levels were beneath the detection limits reported by these previous studies. Similarly, BH2 and BH4 were also reported herein with vastly higher peak areas than biopterin, although the semi-quantitative nature of their analysis and their co-elution under the given conditions limited further comparisons. While co-elution and differences in ionization efficiencies may limit the extent of this observation, there is some evidence of non-specific oxidation of BH4 to BH2 based on reportedly higher BH2 peak areas in this study. EDTA may therefore serve as a useful addition to the extraction solvent to chelate free metal ions to improve reduced pteridine derivative quantitative analyses [20, 36]. In addition to biopterin, reported intracellular xanthopterin levels compared well with previously reported xanthopterin levels in red blood cells [37]. With respect to neopterin, Werner and colleagues also reported undetectable neopterin levels in A549 cells using HPLC-FD and oxidative treatment [29]. Curiously, fully reduced 5,6,7,8-tetrahydroneopterin generated a significant peak, although its occurrence merits further study. The abundant prevalence of biolumazine was also unexpected and is most likely a result of its co-elution with BH2 and BH4. The general agreement between the intracellular pteridine levels reported in this study and others supported the accuracy and precision of the newly developed extraction and quantitation techniques. Moreover, the intracellular pteridine detection method described

herein provides several distinct advantages over previous techniques. First, these data indicate significantly broadened application potential by enabling quantitative analysis of reduced and oxidized pteridine derivatives. This marks a substantial improvement over previous intracellular pteridine techniques that typically examine no more than three pteridine derivatives. This technique may therefore be applied to multiple cell lines to generate novel pteridine profiles to generate new biological hypotheses and to discover new pteridine-based biomarkers for pathological conditions. In addition to quantitatively monitoring selected pteridine derivatives in perturbed metabolic pathways, additional pteridine derivatives may now be semi-quantitatively monitored without the need for expensive and difficult-to-obtain chemical standards. Second, the utilization of a chemical stabilizer through DTT absolves the need for oxidative pre-treatments that may destroy useful reduced pteridine derivative information as well as minimizes the introduction of procedural artifacts that may lead to inaccurate measurements. Finally, the authors note that this component of the study did not aim to generate any biological hypotheses concerning pteridine derivatives in pathological conditions.

4. CONCLUSIONS

A novel, specific, and sensitive extraction and HPLC-QTOF MS method was developed for simultaneous analysis of seven intracellular pteridines with the ability to semi-quantitatively monitor 18 additional, naturally-occurring pteridines which has not been previously reported to the best of our knowledge. Additionally, multiple extraction solvents and conditions were evaluated to provide an optimized pteridine extraction protocol from cell lysates. Method validation through evaluation of spiked recoveries, reproducibility (represented by RSD), MDLs, and application in A549 cell cultures indicated the newly developed method was suitable for the detection of various pteridine derivatives in endothelial cells with high precision and accuracy. This technique has significantly advanced intracellular pteridine detection by substantially expanding the number of pteridine derivatives that may be simultaneously detected, either quantitatively or semi-quantitatively. Finally, this technique may be applied to cellular studies to generate new biological hypotheses concerning pteridine physiological and pathological functions as well as to discovery new pteridine-based biomarkers.

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VI. URINARY METALLOMICS AS A NOVEL BIOMARKER DISCOVERY PLATFORM: BREAST CANCER AS A CASE STUDY

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ABSTRACT

Background: Urinary metallomics is presented here as a new "omics" approach that aims to facilitate personalized cancer screening and prevention by improving our understanding of urinary metals in disease.

Methods: Twenty-two urinary metals were examined with inductively-coupled plasma – mass spectrometry in 138 women newly diagnosed with breast cancer and benign conditions. Urinary metals from spot urine samples were adjusted to renal dilution using urine specific gravity.

Results: Two urinary metals, copper (*P*-value = 0.036) and lead (*P*-value = 0.003), were significantly elevated in the urine of breast cancer patients. A multivariate model that comprised copper, lead, and patient age afforded encouraging discriminatory power (AUC: 0.728, *P*-value < 0.0005), while univariate models of copper (61.7% sensitivity, 50.0% specificity) and lead (76.6% sensitivity, 51.2% specificity) at optimized cutoff thresholds compared favorably with other breast cancer diagnostic modalities such as mammography. Correlations found among various metals suggested potential geographic and dietary influences on the urine metallome that warrant further investigation.

Conclusions: In summary, this proof-of-concept work introduces urinary metallomics as a noninvasive, potentially transformative "omics" approach to early cancer detection. Urinary copper and lead have also been preliminarily identified as potential breast cancer biomarkers.

KEY WORDS

Breast cancer metallomics, urinary metallomics, ICP-MS, urinary lead, urinary copper, noninvasive breast cancer screening

1. INTRODUCTION

Metallomics is an emerging field concerned with the comprehensive analysis of metal and metalloid species within a biological system [1, 2]. The extensive and essential roles of metal and metalloids in pathophysiology lend suitability to metallomics as a novel approach to disease detection and monitoring. For example, metalloproteins represent approximately one third of the known proteome and have wide-ranging roles in biologically important processes such as oxygen and electron transport, biosynthesis and biodegradation, and hydrolysis of amides and esters, and others [3]. Metalloprotein dysregulation is frequently associated with pathological status, such as metallothionein overexpression in certain invasive ductal breast cancers [4-6]. Moreover, metal ions are presumed to be highly regulated [7, 8] owing to their critical roles in maintaining cellular redox status and regulating protein expression [9-11]. A comprehensive understanding of metal content, speciation, localization and function under various pathophysiological conditions is therefore becoming increasingly important in understanding disease mechanisms and discovering novel diagnostic, prognostic, and therapeutic targets.

The role of metallomics in understanding breast cancer has led to novel insights into metal functionality in breast cancer carcinogenesis and metastasis. For example, copper hyperaccumulation in cancer cells is required for angiogenesis and tumor growth [12]. Similarly, dysregulated copper transport leads to oxidative stress and perturbed cell signaling pathways via the copper transport protein CTR1 while copper transport protein CTR2 expression has been linked to breast cancer prognosis and cisplatin drug resistance [13]. Intracellular copper additionally stimulates breast cancer metastasis via redox regulation such as inducing reactive oxygen species generation in cellular structures associated with cell motility [14]. Consequently, novel chemotherapy sensitizers that function as redox modulators via copper(II) chelation have emerged [15]. Similarly, intracellular zinc is physiologically highly regulated in order to maintain cellular redox status [16], but becomes deregulated in breast tumors. Metallothioneins, antioxidant proteins with high binding affinities for essential metals like copper and zinc, are also poorly regulated in invasive ductal carcinomas [17], resulting in increased free cytosolic zinc and copper ions [18]. Zinc importer protein overexpression and zinc

hyperaccumulation have similarly been well documented in heterogeneous breast cancers [18-22]. For these reasons, researchers have explored the utility of copper and zinc as diagnostic biomarkers for breast cancer, such as low serum zinc [23] and high serum Zn/Cu serum ratios [24]. Furthermore, divalent transition metals including copper, cobalt, lead, mercury, tin, and chromium have been shown to activate estrogen receptor-α and consequently cell proliferation [25]. Other metals, like lead, have been associated with selenium antagonism, which minimizes the anti-carcinogenic effect of selenium, leading to higher risk for developing breast cancer [26]. Together, these pathophysiological metallomic changes provide a new molecular modality for earlier detection of aggressive breast cancer.

Comprehensive metallomic screening techniques essential to discovering new clinically useful metallomic biomarkers have become popular following advances in advanced analytical techniques that have enabled researchers to study otherwise trace metals in biologically relevant matrices. Inductively-coupled plasma – mass spectrometry (ICP-MS) in particular has emerged as a powerful platform based on its unparalleled sensitivity and throughput. Recent efforts have already used this approach to implicate high lead, uranium [27] in addition to antimony and cadmium [28] in hair samples of breast cancer patients. Urinary cadmium, another selenium antagonist, has recently been proposed as a noninvasive indicator of breast cancer through multiplicative interactions with selenium [29]. However, application of metallomics to urinalysis remains underdeveloped, despite its distinct advantages that include reduced sample preparation and noninvasive detection modality. While concentration ranges of many urinary metals in healthy populations have been widely reported, other trace elements and concentration ranges in clinically relevant populations, such as women with newly diagnosed breast cancer or with suspected breast cancer, have yet to be described or critically reviewed. This study therefore examined 22 urinary metals using ICP-MS in a proof-of-concept study involving 138 women with newly diagnosed breast cancer and benign conditions in order to quantify the clinical applicability of urinary metals in breast cancer detection and prognosis.

2. MATERIALS AND METHODS

2.1. Patients and specimens

A total of 138 women 33-84 years of age were recruited to provide urine specimens for this proof-of-concept study and other studies at the Mercy Breast Center - Springfield (Springfield, Missouri) between July 2013 and December 2014. Study protocol was approved by the Mercy Medical Research Institute Institutional Review Board (IRB) and participants were required to provide informed consent. Inclusion criteria for participants included new referrals to the Mercy Breast Center - Springfield following possible indication for breast cancer that required further biopsy and immunohistochemical characterization. Exclusion criteria included individuals with a previous history of cancer and/or any known comorbidities. All women resided in the Southwest Missouri region which is a predominate lead and zinc mining area. Additional demographic, metal exposure, and patient health information were not collected owing tothe retrospective nature of this study. Spot urine specimens comprising first morning and second morning voids were collected for the study and immediately stored at -20°C for 1-6 days at Mercy Breast Center – Springfield followed by shipment to Missouri University of Science and Technology via next-day frozen ground freight for analysis. Urine aliquots were stored at -80°C and underwent 2-4 freeze/thaw cycles prior to analysis which occurred 1-6 months after specimen collection. Urinary metals were shown to be freeze/thaw resistant with minimal metal adsorption on container walls after five freeze/thaw cycles across six months storage at -80°C. Patient diagnoses were independently determined by qualified Mercy Breast Center – Springfield staff using a combination of ultrasound-guided core biopsies and pathological stains. The study was conducted in a double-blind manner. Data and resources including patient urine specimens, pathological reports, and metallomic results were anonymized with numerical identifiers.

2.2. ICP-MS Urinary Metal Assay

Twenty-two urinary metals including vanadium (V), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), gallium (Ga), arsenic (As), selenium (Se), rubidium (Rb), strontium (Sr), molybdenum (Mo), silver (Ag), cadmium (Cd), tin (Sn), antimony (Sb), cesium (Cs), barium (Ba), tellurium (Tl), lead (Pb), and uranium (U)

were quantified using a previously described ICP-MS technique with several significant modifications [30]. Briefly, 2 mL urine specimen aliquots were equilibrated to room temperature and diluted fivefold with 1% Optima grade nitric acid (Fisher Scientific Inc, #A467-1) and internal metal standards ^{45}Sc, ^{89}Y, and ^{159}Tb (PerkinElmer Inc, N9303834) in nitric acid pretreated sample tubes. Two calibration standard mixtures (PerkinElmer Inc, N9300233 and N9301721) comprising the 22 metals were used for instrument calibration. Samples were injected into a NexION 350D ICP-MS (PerkinElmer Inc) using an autosampler and peristaltic pumps. The ICP-MS was operated in kinetic energy discrimination (KED) mode using ultra-high purity helium with a flow rate of 3.9 mL/min for As and Se, and 4.7 mL/min for other metals to minimize polyatomic interferences that may arise in complex urine matrices. Quantitation isotopes included ^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{69}Ga, ^{75}As, ^{77}Se, ^{85}Rb, ^{88}Sr, ^{97}Mo, ^{107}Ag, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{133}Cs, ^{137}Ba, ^{205}Tl, ^{208}Pb, and ^{238}U, while confirmation isotopes included ^{53}Cr, ^{62}Ni, ^{65}Cu, ^{68}Zn, ^{82}Se, ^{86}Sr, ^{95}Mo, ^{110}Cd, ^{117}Sn, ^{123}Sb, ^{135}Ba, ^{203}Tl, ^{206}Pb, and ^{235}U. ICP-MS operational parameters included: RF power, 1600 W; plasma gas flow, 18 L/min; auxiliary gas flow, 1.20 L/min; and nebulizer gas flow: 1.06-1.08 L/min. Urinary metal concentrations were adjusted by internal standard responses and dilution.

Urinary metals were quantified over an element dependent linear range from 0.01 µg/L to 100 µg/L using matrix-matched calibration standards. Matrix-matched calibration standards were prepared using fivefold synthetic urine (CST Technologies Inc, UriSub®) to mimic the high salt content and formation of polyatomic interferences in real urine specimens. A freeze-dried reference urine standard (UTAK Laboratories Inc, Product #12110) with certified metal concentrations was also used as an indicator of trueness. Duplicated samples and spiked recoveries of urine specimens were additionally used to measure reproducibility and accuracy of the technique. Method accuracy ranged from 87% to 127% at biologically relevant concentrations (**Table 1**) while intra- and inter-run reproducibility were calculated as 1-7% and 2-12% relative standard deviation, respectively. Metal concentrations measured below the limit of quantitation (LOQ) were taken as one half the quantitation limit (LOQ/2) for statistical analysis.

Table 1. Method performance parameters for the detection of 22 urinary metals using ICP-MS operating in KED mode.

Analyte	Internal Standard	Quantification Limit[a] (µg/L)	UTAK Certified Value (µg/L)	UTAK Measured Value[b] (µg/L)	Accuracy (%)	Spiked Recovery (%)
^{51}V	^{45}Sc	0.09	0.08	<LOQ	<LOQ	98
^{52}Cr		0.07	1.18	1.33 (0.11)	113	96
^{55}Mn		0.32	0.84	1.07 (0.09)	127	98
^{59}Co		0.01	0.68	0.69 (0.04)	101	97
^{60}Ni		0.09	2.29	2.40 (0.13)	105	98
^{63}Cu		0.14	25.2	25.78 (1.20)	102	96
^{66}Zn		0.29	517	498.60 (17.58)	96	107
^{69}Ga		0.19	N/A	N/A	N/A	105
^{75}As		0.11	3.96	5.64 (0.21)	142	153
^{77}Se		0.30	55.3	48.01 (4.92)	87	80
^{85}Rb	^{89}Y	0.04	N/A	N/A	N/A	100
^{88}Sr		0.26	N/A	N/A	N/A	95
^{97}Mo		0.35	71.9	67.62 (1.69)	94	99
^{107}Ag		0.02	N/A	N/A	N/A	88
^{111}Cd		0.03	0.04	<LOQ	<LOQ	102
^{118}Sn		0.02	N/A	N/A	N/A	103
^{121}Sb		0.03	N/A	N/A	N/A	103
^{133}Cs		0.01	N/A	N/A	N/A	100
^{137}Ba		0.34	N/A	N/A	N/A	102
^{205}Tl	^{159}Tb	0.002	N/A	N/A	N/A	98
^{208}Pb		0.01	0.29	0.30 (0.04)	103	100
^{238}U		0.002	N/A	N/A	N/A	98

[a] Quantification limits were calculated as 10×STD of seven replicates and not adjusted for dilution.

[b]Measured values are expressed as mean (SD) for triplicate samples.

2.3. Urine specific gravity assay

Urinary metals were adjusted for patient hydration-dilution status using urine specific gravity (USG). Conventional creatinine normalizations were excluded since urinary creatinine varies with age [31], diet [32], physical activity [33], and presence of breast cancer [34]. USG was measured using a temperature-corrected Reichert TS 400 clinical refractometer. Bulk urine specimens were allowed to equilibrate to room temperature followed by analysis of a 200 µL aliquot. Ultra-pure water (USG = 1.000) and synthetic urine (USG = 1.022) of known specific gravity were used as reference standards. USG was measured with inter- and intra-assay RSDs of 0.12% and 0.04%, respectively. Biomarker concentrations were adjusted to USG using the Levine-Fahy equation and a reference USG of 1.020:

$$C_{corrected} = C_{raw} \times \frac{USG_{reference} - 1}{USG_{experimental} - 1}$$

where $C_{corrected}$ is the adjusted analyte concentration, C_{raw} is the uncorrected analyte concentration, $USG_{reference}$ is a reference USG for a given population, and $USG_{experimental}$ is the measured USG.

2.4. Statistical analyses

Anderson-Darling normality tests indicated non-normal distributions for all 22 urinary metals (Anderson-Darling > 3.5, $P < 0.005$), while $\log_{10}$ transformation failed to approximate the normal curve (Anderson-Darling > 3.0, $P < 0.005$). For this reason, nonparametric analyses were performed on untransformed USG-adjusted metal concentrations and the covariates USG and patient age. Mann-Whitney *U* analyses were used to compare women newly diagnosed with breast cancer and benign conditions. Kruskal-Wallis and Dunn's multiple comparisons tests were used to compare urinary metal concentrations across individual carcinoma types and grades. Correlations among different urinary metals and clinicopathological factors were measured with Pearson correlations. Logistic regression analyses were used to generate classification models, compute odds-ratios, and construct receiver-operating characteristic (ROC) curves for metals that were found to be associated with breast cancer. Odds-ratios were computed using interquartile range (IQR) increments for each covariate. The ROC curves evaluated the potential of each

classification model to distinguish invasive breast cancer across all thresholds and were constructed by plotting sensitivity vs. 1-specificity. Statistical uncertainty was quantified with 95% confidence intervals where appropriate. A *P*-value below 0.05 was considered statistically significant.

3. RESULTS

3.1. Patient Population

One hundred thirty-eight urine specimens were collected among which seven were excluded for being overly dilute or concentrated, defined as having a USG value below 1.003 or above 1.030, respectively. These cutoff thresholds were selected based on the diminished ability of USG to accurately model patient hydration-dilution status beyond these points [35]. The remaining 131 eligible patients were classified as 79 (60%) women diagnosed with benign fibroadenomas, fibrocystic changes, benign papillomas, and stromal fibrosis, and 52 (40%) women with newly diagnosed breast carcinomas. Carcinomas were further characterized as comprising 12 ductal carcinomas in situ (DCIS), 38 invasive ductal carcinomas (IDC), and 2 invasive lobular carcinomas (ILC) (**Fig. 1**).

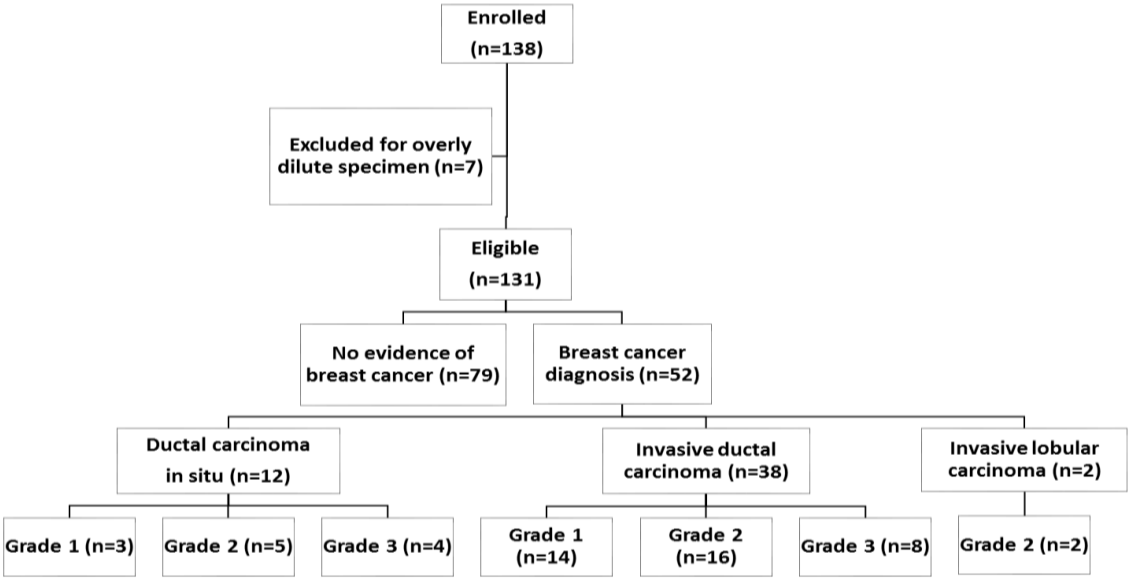

Figure 1. Patient enrollment flowchart including excluded specimens.

Low-grade DCIS cases (n = 5) were considered pre-invasive, indolent disease [36], which resulted in their classification as a benign condition. The retrospective nature of this study, whereby samples were originally collected for a different purpose, precluded the inclusion of healthy, age-matched controls. As a proof-of-concept study, the benign cases, which lacked known comorbidities, previous history of cancer, and had pathologically confirmed absence of breast cancer were used as approximate controls. However, future studies should include healthy control populations since urinary metals may associate with benign conditions of the breast.

3.2. Association of Urinary Metals with Breast Cancer

Urinary metal concentrations encountered in the two patient groups were summarized in **Table 2**. All 22 urinary metals were reliably detected by the ICP-MS analysis with the exceptions of V, Mn, Ag, and U. Trace levels of Cr and Ga were also unquantifiable (signal-to-noise < 10) in a majority of samples. Notably, the heavy metals Cu (*P*-value = 0.036) and Pb (*P*-value = 0.003) were significantly elevated in the women with newly diagnosed breast cancer. Weak associations from Cd (*P*-value = 0.163) in addition to non-significant increases in Zn, Ba, and Rb were also observed. Patient age was additionally considered a disease correlate ($P < 0.0005$) with means (SD) of 64.7 (10.9) years in the breast cancer group and 56.7 (11.8) years in the benign group. Patient age was not found to correlate with any of the urinary metals, which indicated that patient age was not a confounding factor for the observed relationships between metals and breast cancer. USG was not associated with presence of breast cancer and had a pooled mean (SD) of 1.013 (0.001).

Logistic regression models were used to evaluate the clinical performance of various classification models in distinguishing invasive breast cancer. Univariate models were independently developed for Cu (odds-ratio increment = 4.48 µg/L) and Pb (odds-ratio increment = 0.368 µg/L) owing to their significant elevation in breast cancer patients. Both Cu (odds-ratio: 1.77, 95% CI: 1.15-2.72, *P*-value = 0.008) and Pb (odds-ratio: 1.65, 95% CI: 1.14-2.40, *P*-value = 0.005) were found to be significant factors for having breast cancer. A multivariate model that comprised Cu, Pb, and patient age (odds-ratio increment

= 19 years) was additionally constructed where Cu (odds-ratio: 1.52, 95% CI: 0.95-2.43, *P*-value = 0.079) and Pb (odds-ratio: 1.46, 95% CI: 0.97 – 2.18, *P*-value = 0.064) did not significantly contribute to the regression line which was dominated by patient age (odds-ratio: 2.77, 95% CI: 1.44-5.32, *P*-value < 0.0005).

Table 2. Comparison of USG-adjusted urinary metal levels in women with benign conditions (n=84) and breast cancer (n=47). All data are expressed as median (IQR).

Metal	**LOQ[a] (µg/L)**	**Cancer Patients (µg/L)**	**Benign Patients (µg/L)**	***P*-value[b]**
^{51}V	0.45	<LOQ	<LOQ	N/A
^{52}Cr	0.35	0.175 (0.175-0.580)[c]	0.175 (0.175-0.424)[c]	0.541
^{55}Mn	1.60	<LOQ	<LOQ	N/A
^{59}Co	0.05	0.316 (0.235-0.439)	0.254 (0.191-0.431)	0.405
^{60}Ni	0.45	1.28 (0.99-2.75)	1.54 (0.99-2.39)	0.730
^{63}Cu	0.70	9.35 (7.43-13.61)	8.47 (6.92-10.46)	0.036
^{66}Zn	1.45	447 (286-757)	386 (263-664)	0.272
^{69}Ga	0.95	0.475 (0.475-1.152)[c]	0.475 (0.475-1.047)[c]	0.310
^{75}As	0.55	4.15 (2.94-6.34)	4.28 (2.74-6.44)	0.981
^{77}Se	1.50	36.66 (29.07-53.26)	38.92 (29.64-49.10)	0.726
^{85}Rb	0.20	1318 (1104-1747)	1239 (958-1819)	0.330
^{88}Sr	1.30	123.43 (84.27-162.41)	123.00 (80.45-167.46)	0.814
^{97}Mo	1.75	38.43 (26.43-56.69)	42.33 (29.85-70.92)	0.420
^{107}Ag	0.10	<LOQ	<LOQ	N/A
^{111}Cd	0.15	0.669 (0.440-1.262)	0.611 (0.472 – 0.885)	0.163
^{118}Sn	0.10	0.482 (0.191-1.475)	0.469 (0.137-1.005)	0.467
^{121}Sb	0.15	0.163 (0.075-0.234)	0.075 (0.075-0.210)	0.348
^{133}Cs	0.05	6.01 (5.25-8.57)	6.58 (4.56-9.00)	0.950
^{137}Ba	1.70	2.82 (0.85-4.71)	2.19 (0.85-3.38)	0.205
^{205}Tl	0.01	0.165 (0.131-0.223)	0.175 (0.129-0.256)	0.781
^{208}Pb	0.05	0.578 (0.395-0.876)	0.388 (0.260-0.597)	0.003
^{238}U	0.01	<LOQ	<LOQ	N/A

[a] LOQs and urinary concentrations were adjusted by a 5-fold dilution factor.

[b] *P*-values represent group comparisons between women newly diagnosed with breast cancer and benign conditions using nonparametric Mann-Whitney *U* analyses.

[c] Unquantifiable levels of Cr, Ga, Sb, and Ba in some samples were taken as LOQ/2..

ROC analysis suggested clinical performance of individual models was ordered as follows: Cu < Pb < Patient Age < Multivariate Model (**Fig. 2**). Specifically, Cu poorly distinguished invasive breast carcinomas across all thresholds (AUC: 0.611, 95% CI: 0.510-0.712, *P*-value = 0.035) while Pb demonstrated slightly improved discriminatory power (AUC: 0.659, 95% CI: 0.562-0.756, *P*-value = 0.003). Although patient age possessed marginally better discriminatory power (AUC: 0.685, 95% CI: 0.593 – 0.778, *P*-value < 0.0005), the combined multivariate model provided optimal results (AUC: 0.728, 95% CI: 0.641-0.816, *P*-value < 0.0005). The univariate Cu and Pb models both had 19.2% sensitivity (95% CI: 9.2%-33.3%) and 91.2% specificity (95% CI: 83.6%-96.6%) while the multivariate model afforded 36.2% sensitivity (95% CI: 22.7%-51.5%) and 88.1% specificity (95% CI: 79.2%-94.1%). While characteristic performance permits objective classification model ranking, clinical applicability is better assessed at optimal cutoff thresholds. To this end, Cu had 61.7% sensitivity (95% CI: 46.4%-75.5%) and 50.0% specificity (95% CI: 38.9%-61.1%) using a cutoff of 8.50 µg/L, while Pb had 76.6% sensitivity (95% CI: 62.0%-87.7%) and 51.2% specificity (95% CI: 40.0%-62.3%) using an optimal threshold of 0.400 µg/L.

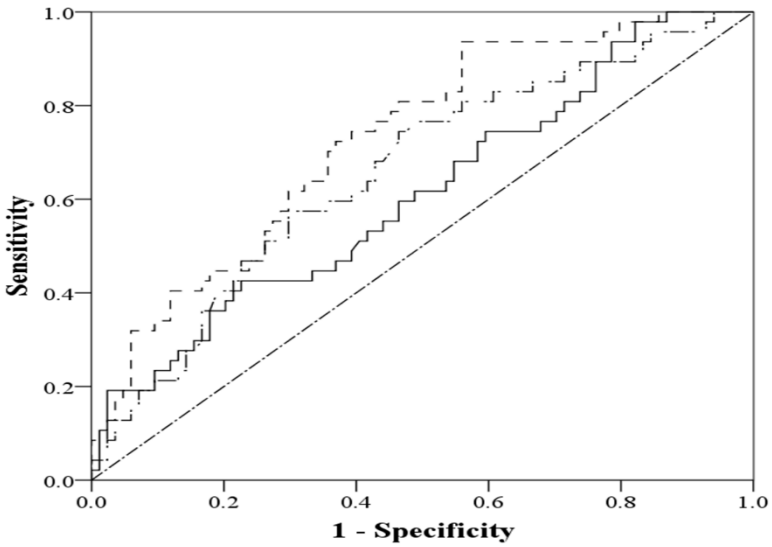

Figure 2. ROC data comparing incremental increases in diagnostic performance for the univariate models of Cu and Pb and the multivariate model comprising Cu, Pb, and patient age in women newly diagnosed with breast cancer (n=47) and benign conditions (n=84).

3.3. Correlations among urinary metals and clinicopathological factors

It was also of interest to identify potential correlations among different urinary metals to better understand exposure routes and possible pathophysiological mechanisms. Significant correlations (|Pearson r| > 0.3, *P*-value < 0.05) among different urinary metals were summarized in **Table 3**. Notably, twice as many correlations were noted in breast cancer patients compared with women with benign conditions, although this finding may result from the limited sample size of the breast cancer group. Among the benign condition group, interactions among Ba, Sr, and Ga were the strongest. Moderate correlations included those between Cu with Ni, Zn, and Cs in addition to those among trace essential metal Se with Zn and Cu, and heavy metal Sn with Co and Zn. The correlations from the breast cancer subgroup were markedly different from those observed in the benign condition group, although strong interactions among Ba, Sr, and Ga were similarly observed. Urinary Cd was correlated with several heavy metals including As, Zn, Sb, Tl, Rb, and Cu. The heavy metal Cr was also found to be correlated with Sn, Ni, Se, and Sb. Urinary Pb was correlated with Se, Ni, and Zn. Correlations among USG, patient age, Cu, and Pb also suggested Pb was weakly correlated with both USG (r = 0.201, *P*-value = 0.021) and age (r = 0.178, *P*-value = 0.042).

Prognostic immunohistochemical factors including progesterone receptor status, estrogen receptor status, Her-2/neu, and Ki67 cell proliferation marker for the 45 invasive breast cancers were compared with the 22 urinary metals to identify possible prognostic capabilities. Urinary As was found to have a weak inverse relationship with progesterone receptor status (r = -0.294, *P*-value = 0.023) and Her-2/neu (r = 0.362, *P*-value = 0.028). Other correlations included Sr and estrogen receptor status (r = 0.340, *P*-value = 0.037), Mo and Her-2/neu (r = -0.350, *P*-value = 0.034), and Cd and estrogen receptor status (r = -0.334, *P*-value = 0.04). Urinary Pb correlated moderately with estrogen receptor status (r = -0.441, *P*-value = 0.006), progesterone receptor status (r = -0.315, *P*-value = 0.044), and Ki67 (r = 0.385, *P*-value = 0.017).

Table 3. Significant correlations among USG-adjusted urinary metals.

Benign Conditions			Breast Cancer		
Correlation	**Pearson Correlation, r**	***P*-value**	**Correlation**	**Pearson Correlation, r**	***P*-value**
Sn-Zn	0.305	0.005	Zn-Cu	0.304	0.038
Sn-Co	0.307	0.004	Cd-As	0.318	0.029
Mo-Se	0.307	0.005	Zn-Ni	0.323	0.027
Cs-Sr	0.307	0.004	Cd-Zn	0.325	0.026
Cs-Rb	0.333	0.002	Pb-Se	0.327	0.025
Se-Cu	0.347	0.001	Sn-Cr	0.337	0.020
Se-Zn	0.365	0.001	Sb-Cu	0.357	0.014
Sb-Mo	0.381	<0.0005	Cs-Rb	0.359	0.014
Cu-Ni	0.427	<0.0005	Sn-Cu	0.360	0.013
Tl-Sr	0.433	<0.0005	Ni-Cr	0.371	0.010
Cs-Cu	0.470	<0.0005	Mo-Sr	0.384	0.008
Zn-Cu	0.506	<0.0005	Mo-Se	0.391	0.007
Sr-Ga	0.543	<0.0005	Ba-Se	0.394	0.006
Ba-Sr	0.666	<0.0005	Ni-Cu	0.415	0.004
Ba-Ga	0.874	<0.0005	Pb-Ni	0.415	0.004
			Sr-Ga	0.419	0.03
			Se-Cr	0.422	0.003
			Sb-Cd	0.431	0.002
			Cs-Co	0.433	0.002
			Pb-Zn	0.443	0.002
			Sb-Ni	0.457	0.001
			Tl-Cd	0.462	0.001
			Sn-Ni	0.510	<0.0005
			Sb-Cr	0.528	<0.0005
			Se-Zn	0.551	<0.0005
			Cd-Rb	0.581	<0.0005
			Ba-Sr	0.652	<0.0005
			Cd-Cu	0.656	<0.0005
			Ba-Ga	0.838	<0.0005

Finally, the correlation between Pb and Cu with cancer progression was explored using Kruskal-Wallis analyses and Dunn's multiple comparisons tests. The highly limited sample size of the DCIS subgroups warrant cautious interpretation of their results. Urinary Pb levels were arranged in the following order: Benign Condition < Grade 3 DCIS < Grade 1 DCIS < Grade 2 DCIS < Grade 1 IDC ≤ Grade 2 IDC < Grade 3 IDC. The difference between Grade 3 IDC and benign conditions was most significant (Dunn's multiple comparisons *P*-value = 0.0006). Similarly, urinary Pb was weakly correlated with cancer grade using cancer grade as a continuous variable (r = 0.265, *P*-value = 0.002). No significant differences among cancer grades were noted for urinary Cu, which was ranked in the following order: Benign conditions < Grade 2 DCIS < Grade 1 DCIS < Grade 1 IDC < Grade 3 DCIS < Grade 2 IDC < Grade 3 IDC. Weak correlations between urinary Cu and continuous variable cancer grade (r = 0.254, *P*-value = 0.003) were similarly noted.

4. DISCUSSION

We investigated 22 urinary metals in women newly diagnosed with breast cancer and benign conditions in a proof-of-concept study to determine whether urinary metallomics may serve as a useful platform for biomarker discovery. In this study, two metals, copper and lead, were encountered at significantly elevated levels in the urine of breast cancer patients. This finding is consistent with a growing body of literature concerned with *in vivo* metallomics of breast cancer tissue. For example, copper hyperaccumulation occurs in breast carcinomas [12] through dysregulated copper transport proteins [13]. Importantly, the association of urinary copper with high-grade breast cancers in particular appeared to reflect copper-based mechanisms related to cancer motility in metastatic breast carcinomas [15]. Elevated urinary lead concentrations were similarly more pronounced in high-grade breast carcinomas. Environmental lead exposure, a salient consideration for our study population, is associated with risk for developing breast cancer while recent work has suggested that lead functions as a selenium antagonist that competitively binds selenium [26]. The significant indirect correlation between lead and selenium in breast cancer patients observed in this study appears to support this proposed mechanism. While urinary cadmium weakly associated with breast cancer, no multiplicative interactions with selenium were observed (r = -0.02, *P*-value = 0.82) as

previously cited, presumably due to substantial differences in environmental cadmium exposure between our study population and that of Wei [29]. Although univariate classification models for copper and lead demonstrated limitations in breast cancer diagnostics, the multivariate model that included patient age performed remarkably well. The clinical performance of copper and particularly lead at optimized cutoff thresholds compared favorably with other breast cancer diagnostic modalities such as clinical breast examinations and mammography [37]. This diagnostic performance appears promising given the inexpensive and noninvasive character of urinary metal screening. However, the authors acknowledge the limited sample size of this study and point out that larger clinical studies are required to validate these preliminary findings.

Moreover, our results suggest that diagnostic performance may be further improved through enhanced understanding of metal and metalloid exposure routes. For example, numerous correlations among various metals in this study provided pertinent information regarding common exposure routes that include dietary intake, residential exposure, occupational exposure, and more broadly, environmental exposure. For instance, barium, strontium, and gallium, which together represented the strongest metal-metal correlations, are typically co-present in ore-bearing rock. The interaction between nickel and copper among other interactions may be similarly attributed to shared geochemical distribution [38]. Although this study was not designed to evaluate the influence of local mineralogy on urinary metal epidemiology, we feel it necessary to mention that geochemical distributions likely represent a major determinant of biometal concentrations. Supporting this claim is a comparison of previously reported urinary metal concentrations in baseline populations and those reported from our southwest Missouri cohort which demonstrates that while many essential metals obtained primarily from dietary exposure were in good agreement [39, 40], trace and toxic metals like cadmium varied considerably [29]. Differences arising from unique local geochemistry will additionally be influenced by environmental regulations. For example, the similar urinary lead values reported in our study population compared with other reported industrialized country populations likely reflects lead removal from drinking water. Further investigations into local geochemical distributions and metallomic correlations will be needed to qualify these considerations.

Finally some correlations, such as those between copper, zinc, and nickel, may additionally reflect dietary supplement use and other dietary fortifications. Hence, characterization of the exposure routes to urinary metals of interest is critical to advancing urinary metal molecular pathological epidemiology.

Finally, the correlations between urinary metals and clinicopathological factors provided new insights into disease mechanisms and clinical applicability of urinary metals in breast cancer patients. Notably, both copper and lead correlated weakly with cancer progression and peaked in high-grade IDC patients. This observation suggests copper and lead may have applicability in the detection of early stage breast cancer, an observation that merits further investigation. The ability to detect early stage breast cancer is especially critical given the potential to reduce tumor upstaging and improve patient mortality. Finally, several metals and particularly lead were shown to have potential use in prognostics. Because prognostic capability was not directly quantified, future studies should aim to quantitatively assess urinary metals for their ability to predict breast cancer outcome. Such a finding would render urinary metals a valuable supplementary technique for current immunohistochemical techniques.

5. CONCLUSIONS

In conclusion, this proof-of-concept study introduces urinary metallomics as a noninvasive platform for biomarker discovery and clinical translational research. This work provides new insights into the epidemiology of urinary metals in suspected breast cancer cases. Specifically, this work highlights the potential of urinary copper and lead as noninvasive diagnostic breast cancer biomarkers in addition to an array of urinary metals with prognostic capabilities. Future studies should aim to improve understanding of the relationship between urinary metals and source exposure, to measure the prognostic capability of individual metals in prospective clinical studies, and to evaluate copper and lead applicability to early stage breast cancer detection.

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VII. DAILY VARIATION AND EFFECT OF DIETARY FOLATE ON URINARY PTERIDINES

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ABSTRACT

Renewed interest in pteridine molecular pathology has generated new details on the emerging clinical significance of urinary pteridines as putative disease biomarkers. Central to these efforts is the need to understand the sources and extent of their non-epidemiological variation. In response, this study was designed to characterize two primary sources of urinary pteridine variance: daily variation and the effect of dietary folate. Daily variation was studied by collecting urine specimens (n = 81) three times daily for three days. The effect of dietary folate was investigated in a treatment study in which urine specimens (n = 168) were collected daily during a control week and a treatment week during which participants received dietary folate supplements. Measurements of six urinary pteridines were made using high-performance liquid chromatography – tandem mass spectrometry. Coefficients of variation were calculated to grossly characterize daily variance between and within subjects, while nearest neighbor non-parametric analyses were used to identify diurnal patterns and measure dietary folate effects. This study confirms that pteridines respond to circadian rhythms with urinary levels that typically peak in the morning. Similarly this research corroborates previous findings that dietary folate affects pteridines in a pathway dependent manner that likely impacts endogenous neopterin and biopterin biosynthesis. Together with preliminary associations between urinary pteridine levels and different activities, the findings of this study have been used to gain new insights into the pteridine biosynthetic pathway and the impact of pteridine variation on molecular epidemiology studies.

KEY WORDS

Urinary pteridines, daily variation, dietary folate, pteridine metabolism

1. INTRODUCTION

The discovery of fluorescent pteridine derivatives in urine premised new analytical methodologies for their quantitation and simultaneously renewed interest in understanding their roles in human health and disease (Stea et al. 1978). Briefly, pteridine derivatives constitute an extensive class of metabolites that are primarily involved in the biosynthetic and biodegradation pathways of vitamins and cofactors (Kośliński et al. 2011). Many pteridines additionally function as regulatory cofactors, such as tetrahydrobiopterin in aromatic acid biosynthesis and endothelial vasodilation (Thony et al. 2000; Gross and Levi 1992) and neopterin as an agent of cellular immune response (Huber et al. 1983; Reibnegger et al. 1987). Recent studies have provided new mechanistic insights into the roles of these compounds in broader pathophysiological contexts. From these studies, it has become increasingly clear that the unique physicochemical properties of pteridines, such as their remarkable capacity to generate reactive oxygen species (Lorente et al. 2011; Thomas et al. 2003; Cabrerizo et al. 2004; Petroselli et al. 2006) and even photosensitize biologically important biomolecules including DNA (Denofrio et al. 2009; Serrano et al. 2015; Serrano et al. 2012), folic acid (Dántola et al. 2010), and tryptophan (Thomas et al. 2013), carry intriguing biological implications. For these reasons, the photosensitizing aromatic pteridines derived from folic acid such as 6-carboxypterin, 6-formylpterin, 6-hydroxymethylpterin (Fukushima and Shiota 1974) and their downstream derivatives like xanthopterin and isoxanthopterin have recently come under intense investigation as putative cancer biomarkers (Ma and Burton 2013). These pteridine biomarker development efforts have primarily focused on the use of noninvasively monitored urinary pteridine panels following promising indications from early molecular epidemiological studies which have implicated these compounds in an assortment of malignancies, including lung and colon cancers (Gamagedara et al. 2011), bladder cancer (Han et al. 1999; Kośliński et al. 2014), breast cancer (Gamagedara et al. 2011; Burton et al. 2013, 2014), digestive tract cancers (Konishi et al. 1999), and brain tumors (Manjula et al. 1993).

One of the key challenges facing pteridine biomarker development is the determination of their non-epidemiological variation and the individual factors that influence endogenous pteridine biosynthesis. Daily variation of urinary pteridine levels,

which includes both diurnal fluctuations in response to circadian rhythms and day-to-day variance related to microscale changes to feeding and physical activities, is particularly salient in the context of urinary pteridine screening where spot urine specimen collection times have varied widely across studies and the differences between pathological and physiological urinary pteridine levels are often marginally significant (Burton et al. 2014; Gamagedara et al. 2011). Biological variation, such as genetic heterogeneity, disease status, environmental factors, as well as long-term lifestyle and dietary factors, has further complicated efforts to characterize pteridine metabolism and molecular epidemiology. While certain biological factors such as disease type and progression can be controlled in well-designed clinical studies, other undetermined factors remain. Dietary folate in particular has been suggested to function as a key source of biological variation (Burton et al. 2014) owing to wide discrepancies in total daily folate intake and vitamin supplementation across different demographic characteristics (Bailey et al. 2010). Finally, analytical variance related to measurement imprecision is generally marginal with well-validated methods in the modern analytical laboratory with the advent of advanced analytical techniques like tandem mass spectrometry. In this way, daily variation and the effect of dietary folate on pteridines pose the greatest uncertainty to our understanding of pteridine metabolism and molecular pathological epidemiology.

Therefore, the purpose of this study was to measure the primary sources of non-epidemiological pteridine variation: daily variation and dietary folate. Six urinary pteridines, namely neopterin, 6-biopterin, pterin, 6-carboxypterin, xanthopterin, and isoxanthopterin, were selected owing to their previously reported clinical significance to disease detection and diagnosis (Gamagedara et al. 2011; Burton et al. 2014). Daily variation was measured by collecting spot urine specimens at defined time points across several days. Meanwhile, the effect of dietary folate was measured in a treatment study in which baseline pteridine levels were determined from individuals during a control week followed by a treatment week during which participants received daily folate supplements. Daily urine specimens were collected at reproducible times during the folate study. To the best of our knowledge, systematic investigations into the non-epidemiological sources of

pteridine variation are generally lacking. The findings of this study in relation to pteridine metabolism and its broader pathophysiological implications are discussed.

2. MATERIALS AND METHODS

2.1. Study population

A total of 30 individuals were locally recruited at Missouri University of Science and Technology (Rolla, MO) to participate in this study between April and June 2015. Selection criteria included healthy adults without known or suspected medical conditions, no prior history of cancer, were not actively taking folate supplements, and were not currently pregnant or planning to become pregnant during the study duration. Participants were additionally asked to complete a questionnaire which evaluated current vitamin and supplement use, currently used medications, self-assessed physical activity levels, and participant height and weight. Study protocol was approved by the Missouri University of Science and Technology Institutional Review Board (IRB) and informed consent was obtained from all participants. Demographic composition included 50% females, 46.7% Asian Americans, 40.0% Caucasians, 6.7% African Americans, and 6.6% Asian Indians. The mean (SD) age of the participants was 27.3 (11.1) years. The mean weight (SD) of the study population was 159 (26) lbs.

2.2. Daily variation study

Nine individuals were recruited for the specific purpose of quantifying both the within-day and between-day variation of urinary pteridines. Midstream spot urine specimens were collected from these individuals thrice daily consisting of (1) second morning voids collected between 8 a.m. and 10 a.m., (2) mid-afternoon voids collected between 12 p.m. and 2 p.m., and (3) evening voids collected between 6 p.m. and 8 p.m. for a total of three days. At each collection, participants were asked to complete a daily log that detailed feeding activity and self-assessed physical activity levels since the previous collection point. Urine specimens (20-60 mL) were collected in sterile biological specimen containers and immediately stored at -80°C for approximately one month after collection. A total of 81 urine specimens were collected for this component of the study.

2.3. Dietary folate effect study

Twenty-one individuals were recruited to study the effect of dietary folate on urinary pteridine levels. This phase of the study was additionally designed to extend the results of the diurnal variation experiment by measuring between-day variation across a larger sample size for a longer period of time. In this experiment, participants were instructed to provide a single midstream spot urine specimen at reproducible times daily for two weeks (Monday – Friday). The first week served as a control to establish baseline urinary pteridine levels for each individual. During the second week, participants were given one 400 µg (100% recommended daily value) folate supplement. Urine specimens were similarly collected in sterile biological containers and immediately frozen at -80°C for approximately one month prior to analysis. Data from participants who missed two or more collection points were excluded from analysis. A total of 168 urine specimens were collected for this component of the study.

2.4. Urinary pteridine assay

Six urinary pteridines including xanthopterin (CAS #119-44-8), isoxanthopterin (CAS #529-69-1), neopterin (CAS #2009-64-5), 6-biopterin (CAS #22150-76-1), pterin (CAS #218-799-1), and 6-carboxypterin (CAS #948-60-7) were separated and quantified by a high-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) technique that has been described previously (Burton et al. 2013). Briefly, urine specimens underwent an oxidative pretreatment to oxidize residual reduced pterin species by treating a 100 µL aliquot of thawed urine prepared in 850 µL of ultra-pure water with 40 µL of 4:2% w/v potassium iodide-iodine solution (I_3^-) and 10 µL of 2 M ammonium hydroxide for 30 minutes at 4°C under dark conditions. Following incubation, the treated samples were centrifuged at 3000 g at 4°C for 20 minutes to remove bulk sediment. Centrifuged samples were filtered with a 0.22 µm nylon membrane filter and diluted 20-fold in ultra-pure water. Ammonium oxalate was prepared at a final concentration of 10 µg/L as a sodium adduct suppressant. Samples were prepared as biological duplicates (n = 2).

Urinary pteridines were separated using a Phenomenex Luna phenyl-hexyl column (3.0µm, 3.0 × 150 mm) and a Shimadzu UFLC system (Columbia, MD, USA) comprised of a degasser (DGU-30A3), two pumps (LC-20 AD XR), a temperature controlled autosampler (SIL-20AC XR), and a column oven (CTO-20A). Urinary pteridines were quantified using an AB Sciex 4000 QTrap MS/MS system (Foster City, CA, USA) that was operated under ESI-positive ionization and scheduled multiple-reaction monitoring (MRM) scan modes. The six urinary pteridines were quantified over a linear range from 0.10 µg/L to 100 µg/L with representative spiked recoveries that ranged from 80% to 112% and analytical reproducibility that ranged between 2.3% and 6.8% relative standard deviation (RSD). Urine specimens that afforded urinary pteridine levels under the corresponding detection limits were assigned a concentration of one half the limit of quantitation (LOQ/2).

2.5. Urine specific gravity adjustment

Urinalysis requires adjustments to reflect patient hydration-dilution status and time since last urination. Urine specific gravity (USG) was used for this purpose since conventionally used urinary creatinine is dependent on muscle mass and related individual characteristics (Burton et al. 2014). USG was measured refractometrically using a temperature-corrected Reichert TS 400 clinical refractometer since this method remains the most accurate (Miller et al. 2004). Freshly thawed urine specimens were allowed to equilibrate to room temperature (21-25°) whereby a 200 µL aliquot was analyzed with the refractometer. Freeze-thaw cycles were preliminarily shown to have no impact on USG (**Online Resource 1**). Urine specific gravity was measured with inter- and intra-assay RSDs of 0.12% and 0.04%, respectively. Urinary pteridine levels were adjusted to USG using the Levine-Fahy equation and a reference USG of 1.020:

$$C_{corrected} = C_{raw} \times \frac{USG_{reference} - 1}{USG_{experimental} - 1}$$

where $C_{corrected}$ is the adjusted analyte concentration, C_{raw} is the uncorrected analyte concentration, $USG_{reference}$ is a reference USG for a given population, and $USG_{experimental}$ is the experimentally determined USG.

2.6. Statistical analysis

2.6.1. Data processing

Urinary pteridine levels were normalized to USG to compensate for differences in individual hydration status and time since last urination. Overly dilute and concentrated urine specimens, defined as having a USG value less than 1.003 or greater than 1.030, respectively (Bush 2008; Alessio et al. 1985), were excluded from statistical analysis (n = 3). Similarly, urinary pteridine concentrations that fell below their respective limits of quantitation (n = 6), which have been defined elsewhere (Burton et al. 2013), were taken as one half their limit of quantitation. Anderson-Darling goodness-of-fit tests indicated pteridine distributions with substantial right skew (skew range: +0.97 – 1.88) that were most accurately modeled as lognormal distributions. Although log-transformations have been previously employed to normalize metabolite concentrations (Thompson et al. 2012), such an approach is problematic for the study of metabolite variation. Namely, transformation processes distort coefficients of variation, which serve as a primary measure in the daily variation component of the study. Secondly, the nearest neighbor ratio analyses performed in this study would lose intrinsic meaning following a log-transformation since this process inadvertently reduces resulting ratios to unity. Finally, the paired differences and ratios that formed the bases of most statistical analyses in this study were acceptably symmetric about 0 and 1, respectively (Anderson-Darling < 0.5, P-value > 0.05) for all six pteridines. For these reasons, data were not transformed.

2.6.2. Statistical methods

Coefficients of variation (%CVs) were determined from untransformed data using the method of moments defined as $\sigma/\bar{x}$ where σ is the sample standard deviation and $\bar{x}$ is the sample mean. Our study design permitted determination of two types of daily variation: (a) within-day and (b) between-day. Within-day variation was calculated as the mean %CV determined for each day for each pteridine for each individual. Between-day variation was calculated as the mean %CV for each corresponding time point (e.g. mornings, afternoons, evenings) across three days for each pteridine for each individual. The averages and %CVs of the individual daily variations were calculated as the within-individual and between-individual variance, respectively.

Nearest neighbor ratio nonparametric analysis was used to quantify diurnal patterns by taking the ratios of each successive time point for each pteridine for each individual. Ratios for major time points (e.g. morning, afternoon, and evening) were combined and subjected to a Signed Rank test with the null hypothesis that the overall ratio was equivalent to unity. The paired ratios of the case week to the control week in the folate study were similarly analyzed with a Signed Rank test. Paired equivalence tests were used to confirm equivalence where appropriate. Pearson correlations were performed to quantify the correlation between urinary pteridine values amongst one another and self-reported parameters of feeding and physical activity. Mann-Whitney *U* group comparison tests were used to measure the differences between unpaired data sets. A *P*-value below 0.05 was considered statistically significant, except in cases of multiple comparisons wherein Bonferroni corrections were made.

2.6.3. Sample size and statistical power

This study aimed to quantify the daily variation and effect of dietary folate on six urinary pteridines. The sample size for the Signed Rank tests for determining these two types of variation was determined to be 67 using a statistical power of 80%, a 5% Type I error rate, a minimum effect size of 0.15, and a standard deviation of 0.40. This standard deviation estimate was premised on the variation of serum acylcarnitines, which may similarly be considered secondary metabolites (Thompson et al. 2012), as well as derivations of previously reported clinical investigations of urinary pteridine levels (Burton et al. 2014). The observed sample sizes, 81 and 168, for the daily variation and folate experiments, respectively, enabled formative conclusions to be drawn from these analyses, although the generalizability of these findings may be extended with larger sample sizes.

3. RESULTS

3.1. Daily variation

Daily variation of urinary pteridines was assessed on within-day and between-day bases for a period of three days with three measurements taken each day at defined times for a total of nine measurements per individual (**Table 1**). Briefly, the encountered pteridine ranges reported in this study population (range: 43 nM 6-carboxypterin – 3,326

nM neopterin) were comparable to previous accounts, although considerably lower than previously reported levels in certain cancer patient populations (Gamagedara et al. 2011; Burton et al. 2013, 2014). Gamagedara and co-workers noted comparable epidemiological differences in their case-control study that examined a demographically similar control population and a substantially older case population (Gamagedara et al. 2011).

Within-day variation was studied to determine whether urinary pteridine concentrations respond to circadian rhythms. Within-subject variance was approximately 35% for all urinary pteridines with the exception of 6-carboxypterin which had substantially higher variance measured at 72.5%. High urinary 6-carboxypterin variance was generally observed across the study population as reflected by its lower 36.6% between-subject variance. The between-subject variance for the other urinary pteridines remained at ~35%. While these measures of variance provide a characterization of the total daily and biological variation, they provide little insight into the individual factors that comprise pteridine variation, such as diurnal trends in relation to circadian rhythms. To this end, nearest neighbor ratios were determined for each successive time point for each individual and composited to provide a generalized characterization of daily urinary pteridine clearance trends (**Table 2**). Specifically, 6-biopterin, pterin, neopterin, and isoxanthopterin levels in urine were significantly elevated in the morning (Signed Rank *P*-values < 0.05). Nonsignificant increases (Signed Rank *P*-values $= 0.073$) were also observed in xanthopterin and 6-carboxypterin. Morning pteridine levels were generally maintained throughout the afternoon (paired equivalence test *P*-values < 0.05) for all urinary pteridines except 6-carboxypterin, followed by substantial decreases in the evening (Signed Rank *P*-values < 0.05 for 6-biopterin, pterin, neopterin, and isoxanthopterin; *P*-value $= 0.064$ for xanthopterin; *P*-value $= 0.359$ for 6-carboxypterin).

Table 1. Daily variations (%CV) of six urinary pteridines measured at three times daily for three days in nine individuals.

		6-Biopterin	Pterin	Neopterin	Xanthopterin	6-Carboxypterin	Isoxanthopterin
	Mean Concentration (nM)	1060.0	482.5	3325.8	1743.6	43.5	514.2
	(Standard Deviation) (nM)	(580.5)	(261.9)	(1220.5)	(1127.5)	(33.6)	(255.0)
	Analytical Precision (%RSD)	3.2%	2.4%	2.3%	2.7%	6.8%	3.8%
Within-Day[1]	**Within-Individual**	35.4%	35.9%	29.5%	55.6%	72.5%	36.9%
	Between-Individual	39.6%	54.3%	29.4%	41.2%	36.6%	43.2%
Between-Day[2]	**Within-Individual**	27.5%	33.8%	23.0%	56.2%	69.2%	34.0%
	Between-Individual	25.7%	47.6%	35.7%	36.2%	33.1%	48.5%
Total Daily Variation[3]	**Within-Individual**	36.3%	40.8%	29.6%	59.2%	77.8%	41.7%
	Between-Individual	32.5%	42.5%	24.4%	35.2%	35.4%	35.1%

[1] Within-day variance was calculated as the average %CV for each individual for each pteridine for each day.

[2] Between-day variance was calculated as the average %CV for each individual for each corresponding time point for each pteridine.

[3] Total daily variation was calculated as the %CV for each pteridine across all measurements for three days.

Table 2. Diurnal patterns of six urinary pteridines as determined from nearest neighbor ratio analysis.

	6-Biopterin	Pterin	Neopterin	Xanthopterin	6-Carboxypterin	Isoxanthopterin
Morning/Evening	**1.50***	**1.14***	**1.39***	1.13	1.39	**1.28***
Afternoon/Morning	1.01	1.04	0.87	0.83	0.77	1.02
Evening/Afternoon	**0.86****	**0.85***	**0.81****	**0.75***	0.76	0.79

For each individual, the ratio of each successive time point was calculated. Corresponding ratios from each day were combined and the median values have been presented here. *P*-values were determined from a Signed Rank test under the alternative hypothesis that the overall ratio was equivalent to 1.00. *** *P*-value < 0.05, ** *P*-value < 0.01**.

Between-day variation was additionally studied to provide supplemental information about the individual sources of pteridine variation. For example, urinary pterin variance was consistently higher when measured between subjects than within subjects. A comparison of the within-day and between-day variances of urinary pterin on an individual basis revealed that the two measures were strongly correlated (Pearson $r = 0.806$, P-value = 0.029) and were considered equivalent within a 30% margin of difference (paired equivalence test P-values (lower bound, upper bound) = 0.026, 0.036) for each individual. The isomers xanthopterin and isoxanthopterin exhibited similar behavior. Specifically, comparisons between the within-day and between-day variances for xanthopterin were considered insignificant (Pearson $r = 0.706$, P-value = 0.076) and statistically equivalent within a 30% margin of difference (paired equivalence P-values = 0.010, 0.046), while those of isoxanthopterin displayed a slightly weaker correlation (Pearson $r = 0.657$, P-value = 0.109) and were not considered equivalent within a 30% margin of difference (paired equivalence P-values = 0.067, 0.029).

Total daily variation was studied by combining the nine samples collected for each pteridine from each individual over the three day period. Representative time series for xanthopterin and isoxanthopterin are shown in **Fig. 1**. Nearest neighbor ratios were tabulated for each individual and then composited to produce a generalized characterization of urinary pteridine fluctuations with respect to each successive time point (**Table 3**). This analytical approach taken together with the self-assessed descriptions of feeding and physical activities prior to each time point was used to elucidate distinct sources of pteridine variance. Specifically, individuals who had eaten calorific meals prior to overnight fasting had significantly lower 6-biopterin levels compared with individuals who had eaten smaller dinners (Mann-Whitney P-value = 0.004). High-intensity physical activities that included extended walking (>30 minutes), resistance training, and prolonged gardening tended to increase urinary neopterin levels (Mann-Whitney P-value = 0.027) up to several hours after the activity occurred, an observation that has been reported elsewhere (Sprenger et al. 1992). Exposure to sunlight was also found to substantially attenuate urinary 6-carboxypterin levels (Mann-Whitney P-value < 0.001) by up to one order of magnitude. Finally, some urine specimens appeared as elevated outliers in comparison with

the other urine specimens collected from the same individual. Reproducible times for these outlier events (e.g. evenings) on an individual basis suggested these events may coincide with certain activities that merit further investigation.

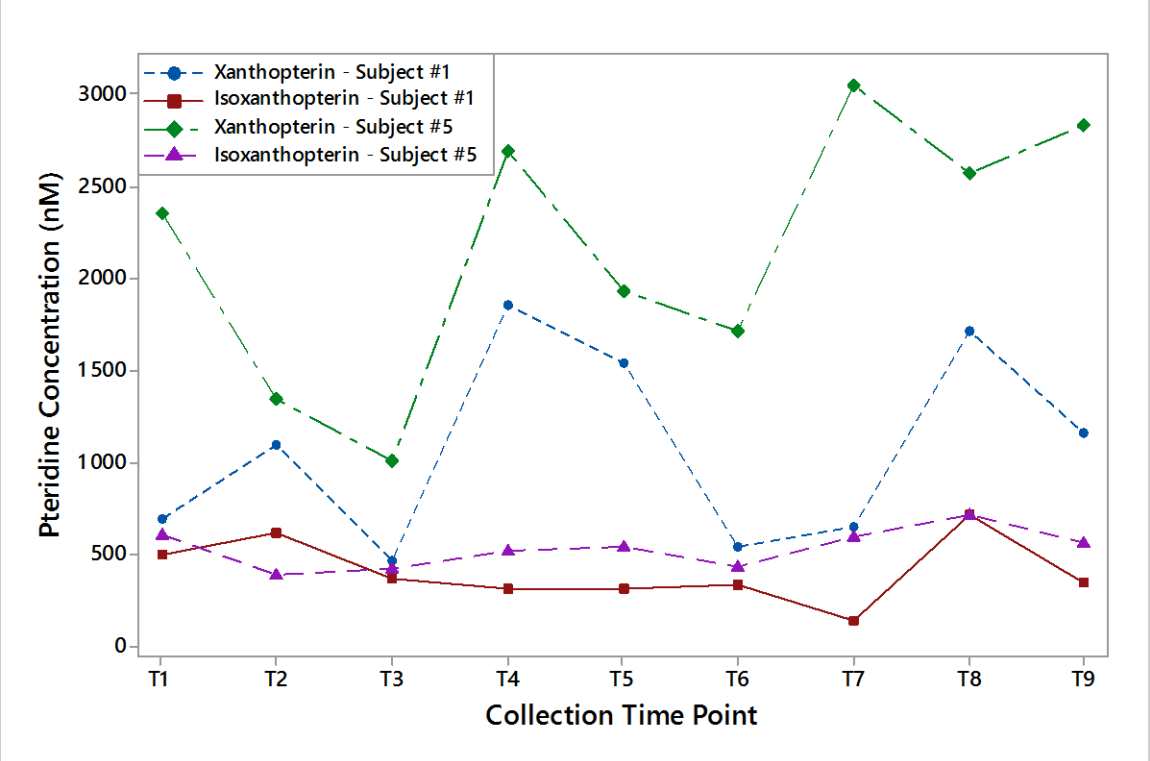

Figure 1. Representative time series for urinary xanthopterin and isoxanthopterin from nine repeat measurements taken from two subjects

Table 3. Nearest neighbor ratios for six urinary pteridines measured across nine time points from nine individuals.

Pteridine	T2 Afternoon	T3 Evening	T4 Morning	T5 Afternoon	T6 Evening	T7 Morning	T8 Afternoon	T9 Evening
6-Biopterin	1.03	0.82	1.89	0.66	0.93	1.04	1.11	0.83
Pterin	0.71	0.76	1.90	1.13	0.85	1.48	1.18	0.88
Neopterin	0.92	0.76	1.54	0.70	0.84	1.22	1.23	0.82
Xanthopterin	0.80	0.59	1.56	0.72	0.36	1.78	1.12	0.89
6-Carboxypterin	0.58	1.02	0.99	0.81	1.51	0.93	1.12	0.51
Isoxanthopterin	1.00	0.68	1.35	0.75	0.85	1.37	1.19	0.63

For each individual, the ratio of the current time point over the preceding time point was calculated from the USG-adjusted pteridine values. Consequently, T1 nearest neighbor ratios are not shown as preceding measurements were not available. Median values for the nine individuals have been presented here.

3.2. Dietary folate

Dietary folate has been suggested to serve as a key source of variation of urinary pteridine derivatives owing to its implicit role in the pteridine biosynthetic pathway as a major entry point for aromatic pteridine biosynthesis and the wide epidemiological discrepancies in total daily folate intake. Herein baseline urinary pteridine levels were established during a control week which generated between-day variations that were comparable to those shown in **Table 1** (2-sample equivalence test *P*-value = 0.024 for 10% margin of difference). Urinary pteridines were subsequently measured during the case week in which study participants were provided one folate supplement at the total daily recommended value (400 µg). The mean weekly pteridine values for each individual for each pteridine were compared by taking the ratio of the case week to the control week. Signed Rank analyses were performed to determine the effect of folate intake on urinary pteridine levels (**Table 4**).

Table 4. Effect of dietary folate supplementation on six urinary pteridine levels.

Pteridine	% Change‡	P-Value
6-Biopterin	-31	<0.0001
Pterin	+27	<0.001
Neopterin	-16	0.031
Xanthopterin	-32	0.021
6-Carboxypterin	+11	0.021
Isoxanthopterin	-5	1.000

‡Data are presented as the percent change between mean urinary pteridine levels between a control week in which no folate supplementation was given and a case week in which folate supplementation was provided at a level of 400 µg/day. P-values were calculated from Signed Rank analyses.

4. DISCUSSION

Clinical interest in pteridine derivatives as investigative cancer biomarkers has prompted efforts to study the functionality and biological significance of pteridines in human health and disease. Central to these efforts is the need to understand the sources and extent of their non-epidemiological variation. As metabolites, pteridines are subject to myriad factors capable of effecting their natural variation, which include but are not limited to circadian rhythms, genetic heterogeneity, disease status, environmental factors, diet and physical activity. This study was therefore designed to measure the extent of urinary pteridine variation with respect to daily variation and dietary folate intake, and to lesser extents, diet and physical activity, which together represent the largest sources of variation. In doing so, new insights have been gained regarding pteridine metabolism and its complicated and intersecting biosynthetic pathways.

The daily variation of urinary pteridines was approximately 35% RSD for both within-day and between-day periods for most pteridines. The extent of this daily variability is comparable to other metabolites, even highly regulated serum amino acids and acylcarnitines (Thompson et al. 2012). The clinical significance of this daily variability should be considered minimal, since pathological epidemiological differences between healthy individuals and cases typically exceed the daily variability described here. For example, Burton and co-workers demonstrated that mean urinary isoxanthopterin levels from breast cancer patients are 350% higher than those of healthy women (Burton et al. 2014). However, pteridine biomarkers with lesser effect sizes will be more acutely affected by daily variability. For this reason, clinical studies aiming to quantify pteridine biomarker performance in spot urine samples should collect at consistent time periods. Furthermore, urinary pteridine variation in this study exhibited diurnal patterns that likely reflected circadian rhythms that modulate metabolism. These patterns were generally characterized as peaking in the morning followed by a reduction in urinary pteridine levels later in the evening. These patterns corroborated similar findings that have been described for urinary neopterin, which has been more extensively studied as an inflammatory marker (Garcia-Gonzalez et al. 2006; Auzéby et al. 1988). These patterns support the hypothesis that melatonin plays a regulatory role in endogenous pteridine biosynthesis (Jang et al. 2000).

Specifically, guanosine triphosphate (GTP) is a major entry to the pteridine biosynthetic pathway and leads to the production of reduced pteridine derivatives, like tetrahydrobiopterin and dihydroneopterin, that give rise to 6-biopterin and neopterin (Huber et al. 1984). Jang and co-workers have demonstrated that melatonin modulates biopterin and neopterin biosynthesis by inhibiting GTP cyclohydrolase I, the initial enzyme in the GTP pteridine biosynthetic pathway, and downregulating its gene expression (Jang et al. 2000). Biopterin derivatives have also been shown to modulate neurotransmitters like melatonin as enzymatic cofactors to tryptophan hydroxylase, suggesting a possible feedback mechanism (Makoto et al. 1994; Wang et al. 2002). The present study supports the hypothesis that melatonin regulates endogenous pteridine biosynthesis given the sensitivity of GTP-derived pteridines, 6-biopterin, neopterin, and pterin, to circadian rhythms.

In contrast, folate-derived pteridines were not significantly affected by circadian rhythms in this study. This observation may be attributed to the distinctive biochemistry of folic acid degradation and resulting pteridine interconversion. Specifically, folic acid degradation yields 6-formylpterin that is subsequently oxidized to 6-carboxypterin, which in turn may be further converted to pterin (Off et al. 2005). Xanthine dehydrogenase catalyzes the formation of xanthopterin and isoxanthopterin from 7,8-dihydropterin and pterin, respectively (Blau et al. 1996). It has been recognized that folic acid degradation is accelerated in the presence of UV radiation (Off et al. 2005). Consequently, elevated levels of folate-derived pteridines have been measured in the skin of patients suffering from vitiligo, a depigmentation disorder (Schallreuter et al. 2001). This understanding of the folic acid photodegradation supports the observation that urinary 6-carboxypterin levels were elevated in study participants who received significant sunlight exposure, although this study was not designed to measure that effect. More importantly, the relative contributions of the GTP and folic acid entry points to shared downstream pteridine derivatives, such as pterin, isoxanthopterin, and xanthopterin, remains poorly understood. Given current understanding of the folic acid degradation pathway and the lack of diurnal behavior observed in the present study, these findings suggest that the folic acid entry point may be the dominant pathway for the formation 6-carboxypterin, xanthopterin, and

isoxanthopterin. This hypothesis is explored further in the dietary folate supplementation study.

The effect of dietary folate supplementation elicited varied responses in a seemingly pathway dependent manner. Specifically, neopterin, biopterin, and xanthopterin were appreciably downregulated while 6-carboxypterin and pterin were upregulated and no significant change was observed in isoxanthopterin. Downregulation of the primary GTP pteridines suggests a possible competitive interaction between the GTP and folic acid entry points in the pteridine biosynthetic pathway. This novel observation provides for the first time evidence that folic acid can modulate endogenous neopterin and biopterin biosynthesis. The biological significance of neopterin and biopterin derivatives in human and health and disease suggest that folate modulation may have important clinical implications. Moreover, these interactions provide new insights into the relative contributions of GTP and folic acid on pteridine production as well as into the crosstalk between the two metabolic entry points. For example, the non-specific formation of pterin from ubiquitous pteridines can occur via non-enzymatic side chain cleavage (Kośliński et al. 2011; Cañada-Cañada et al. 2009). Similarly, 7,8-dihydropteridines, including 7,8-dihydrofolate, preferentially oxidize to 7,8-dihydroxanthopterin which is subsequently oxidized to xanthopterin (Oliveros et al. 2010). The resulting crossover interactions between the two metabolic entry points has complicated the interpretation of pterin and its downstream pteridines in clinical epidemiological studies. The present work clearly demonstrates that folate supplementation augmented urinary levels of 6-carboxypterin and pterin, suggesting that folate catabolism is a major source of pterin biosynthesis. A 27% change in urinary pterin levels corresponds to approximately 20 µg folic acid when a typical 250 mL urine void is assumed. This contribution of folic acid to an individual pteridine within a spot urine sample is significant given that folic acid is primarily excreted in the form of its major metabolites, *p*-aminobenzoylglutamate (pABG) and the acetamide derivative of *p*-aminobenzoylglutamate (apABG), the latter of which lacking biological activity with excretion rates up to 20-fold higher than that of folic acid (Álvarez-Sánchez et al. 2010; Niesser et al. 2013). Moreover, the insignificant effect of dietary folate supplementation on isoxanthopterin suggests that alternatives mechanisms for

isoxanthopterin biosynthesis possibly involving xanthine dehydrogenase appear likely (Blau et al. 1996). Finally, decreases in xanthopterin levels suggest that urinary xanthopterin is primarily driven from the GTP entry point through the nonspecific oxidation of reduced GTP pteridine derivatives. With this new information, we have proposed a pteridine biosynthetic pathway that for the first time comprehensively bridges the GTP and folic acid entry points (**Fig. 2)**. Further *in vitro* work will be needed to confirm this proposed pathway.

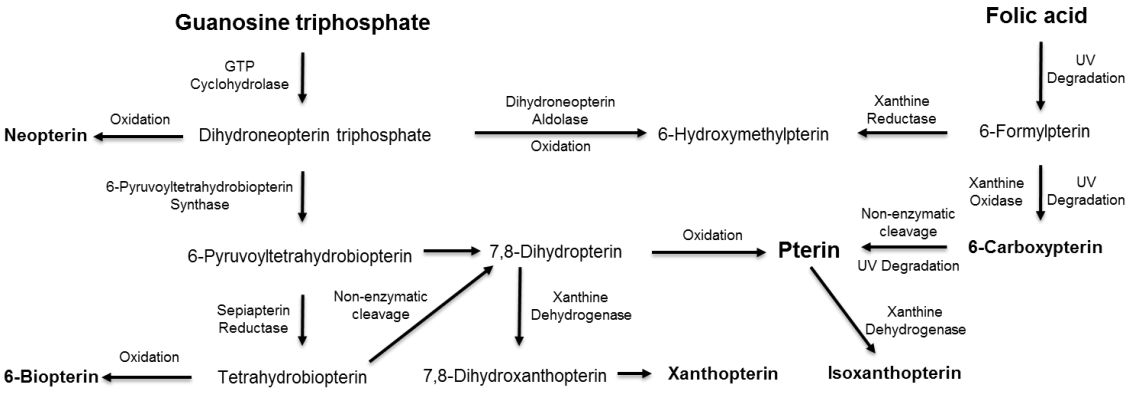

Figure 2. Simplified schematic of the proposed pteridine biosynthetic pathway. Pteridine derivatives in bold were monitored in this study

Finally, there were several limitations to the present study that may be improved upon to gain more conclusive findings. Namely, a limited number of measurements (n = 81), representing three samples collected daily for three days from nine individuals was taken to study the daily variation of urinary pteridines. Larger sample sizes and a greater number of time points may extend the generalizability of these findings across different patient populations and timespans, which may provide additional insights into the finer patterns in urinary pteridine variation. Similarly, this study was not designed to measure the effect of specific physical activities, environmental factors, and diet on urinary pteridine variation which represent useful directions on which to extend these findings.

Self-reporting of disease status additionally has intrinsic limitations, as was evidenced by the development of a bladder infection in one of the study participants which resulted in their immediate exclusion from the study. Finally, long-term studies of dietary folate supplementation would benefit from the secondary half-life of folic acid that occurs after approximately 100 days that is associated with the decay of existing body folate pools. Together these areas provide new research directions based on the findings of this study for future studies aiming to study pteridine metabolism and urinary excretion.

5. CONCLUDING REMARKS

In summary, this study aimed to quantify the daily variation and effect of dietary folate on urinary pteridine levels. Urinary pteridines were confirmed to exhibit diurnal patterns in response to circadian rhythms marked by rising levels in the morning that decrease later in the evening. Dietary folate was similarly demonstrated to have a pronounced effect on urinary pteridine levels in a pathway dependent manner. This pathway dependence suggests dietary folate may modulate endogenous neopterin and biopterin biosynthesis. A number of secondary associations between urinary pteridine levels and various feeding and physical activities were additionally made. Together these newly quantified sources of variation have provided new insights into the pteridine biosynthetic pathway and their broader effects on human metabolism, health, and disease. Better understanding of pteridine metabolism through these findings will enable more accurate interpretation of pteridine molecular pathological epidemiology and elucidate an obscure branch of metabolism with wide-reaching physiological implications.

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VIII. DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY URINARY PTERINOMICS WORKFLOW

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ABSTRACT

Pteridines have evoked considerable interest from the scientific community owing to their prominent roles in human health and disease. The availability of analytical methodologies suitable for comprehensive pteridine profiling, termed here as "pterinomics", has been limited by inconsistent sample preparation and the exclusion of lesser studied pteridine derivatives. In response, the present study describes a new pterinomics workflow using a high-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) methodology for the simultaneous analysis of 15 pteridine derivatives including five structural isomers, marking the largest quantitative pteridine panel that has been studied to-date. The validated method possessed excellent sensitivity with method detection limits (0.025 μg L^{-1} to 0.5 μg L^{-1}) that were comparable or superior to existing techniques. Spiked recovery studies demonstrated the technique was both accurate (88-112%) and precise (RSD: 0-6%). A comparative study of commonly used oxidative pretreatments, including triiodide, permanganate, and manganese dioxide, revealed that the oxidative mechanisms were inefficient, complex, and concentration dependent. Finally, 50 clinical urine specimens were examined with the new technique wherein 10 pteridine derivatives were quantified and population ranges have been given. This technique can be used to examine pteridine molecular epidemiology and biochemistry to support related research applications, and may further be readily extended to include additional pteridine derivatives and biological matrices for specific applications.

KEY WORDS

Pteridines, oxidative pretreatment, urine, HPLC-MS/MS, pterinomics

1. INTRODUCTION

Pteridines have evoked interest from the scientific community since their successful isolation from butterfly wings by Hopkins in 1889 [1]. The structural elucidation of their pyrazine [2,3-*D*]pyrimidine ring system that followed in the early 1940s prompted tremendous efforts to catalogue their biological and chemical diversity that continue to this day [2-4]. From these studies have emerged an extensive and complex family of metabolites related to the biosynthesis and biodegradation of vitamins and cofactors [5]. The key finding that 5,6,7,8-tetrahydrobiopterin is an obligatory cofactor for aromatic amino acid hydroxylases [6, 7] and nitric oxide synthase [8, 9], as well as the cytokine-inducible biosynthesis of neopterin [10, 11], has furthered research into the function of pteridine derivatives in human health and disease. In recent years, these efforts have expanded to include the aromatic pteridines derived from folic acid catabolism [12], including 6-formylpterin, 6-carboxypterin, 6-hydroxymethylpterin, pterin, and isoxanthopterin [13]. Aromatic pteridines, and to lesser extents their semi-reduced and reduced counterparts, have remarkable photocatalytic and oxidoreductive properties, including a considerable capacity to generate reactive oxygen species [14-17] and even photosensitize important biomolecules including DNA [18-20], folic acid [21], and tryptophan [22], which has led researchers to postulate that pteridines may actively participate in molecular pathology [13]. Similarly, the overexpression of the endocytic folate receptor α in solid epithelial tumors [23-25] has presented a novel pathomechanistic premise for the clinical observation of elevated levels of folate-derived pteridines in the urine of patients suffering from lung and colon cancers [26], bladder cancer [27, 28], breast cancer [26, 29, 30], digestive tract cancers [31], and brain tumors [32].

However, current understanding on the biochemistry and molecular pathology of mammalian pteridines remains fragmented [33]. As Rembold and Gyure presciently remarked over forty years ago, analytical limitations related to the general instability and trace levels of biogenic pteridines have precluded detailed studies of the prevalence and role of many lesser known derivatives [13, 34]. More recent efforts have seen an attempt to develop robust analytical methodologies using advanced instrumental platforms and oxidative pretreatments to enable sensitive detection of biologically significant pteridine

derivatives. To this end, a multitude of competent analytical techniques have emerged over the past several years using an array of sensitive instrumental platforms including capillary electrophoresis – laser-induced fluorescence (CE-LIF) [35], high-performance liquid chromatography – fluorescence detection (HPLC-FD) [28, 36-40], high-performance liquid chromatography – mass spectrometry (HPLC-MS) [41], high-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) [29, 42-44], hydrophilic interaction chromatography – tandem mass spectrometry (HILIC-MS/MS) [45], and synchronous fluorescence spectroscopy [46, 47], which have been summarized in **Table 1**.

Nevertheless, the availability of analytical methods suitable for comprehensive pteridine analysis, termed here as "pterinomics", has been limited by several notable factors. First, fragmented understanding of the pteridine biosynthetic pathway alongside the tendency to select pteridine derivatives with previously established biological significance have generally limited the inclusion of lesser known derivatives in new analytical techniques. The interconversion of 6-substituted pteridines to 7-substituted isomers via biochemical processes that are unrelated to classical pteridine biosynthetic pathways presents an additional class of pteridine derivatives with probable biological significance that have otherwise received little attention thus far [48, 49]. Secondly, the problematic practice of using a variety of oxidative and anti-oxidative pretreatments, as recently reviewed by Tomšíková and co-workers [50], has led to inconclusive or inconsistent findings. Although a number of oxidative pretreatments have been proposed and optimized for selected pteridine derivatives, comparative studies of oxidative efficiency and byproduct formation for a wide panel of pteridine derivatives are lacking. An alternative strategy to oxidative pretreatments has more recently focused on the investigation of pteridine derivatives in their native oxidative states [40-42, 51], although the relative abundance and interconversion of semi-reduced and reduced pteridine derivatives in biological fluids, and particularly urine, remains disputed [36, 52-54]. For these reasons, new methods are urgently needed to study expanded pteridine panels, including structural isomers, alongside improved knowledge on the effectiveness of oxidative pretreatments to advance pteridine research in human health and disease.

Table 1. A summary of recently developed quantitative analytical methodologies for biogenic pteridines.

Technique	Pteridines[1]	Oxidative Pretreatment	Run Time (min)[2]	Reference
CE-LIF	PTE, XAN, ISO, NEO, 6-BIO, 6-CAP, 6,7-DMP, 6-HMP	Alkaline I_3^-	20	Gibbons et al (2009)[35]
HPLC-FD	PTE, NEO, 6-BIO, XAN, ISO	None	16	De Llanos et al (2011)[39]
HPLC-FD	PTE, XAN, ISO, NEO, 6-BIO, 7-BIO, MNP, 6-CAP, 6-HMP, LUM, 6-HLUM, 7-HLUM, BLUM	Alkaline I_3^- and $KMnO_4$	39	De Llanos et al (2011)[39]
HPLC-FD	PTE, NEO, 6-BIO; XAN, ISO	None	16	Culzoni et al (2011) [40]
HPLC-MS/MS	PTE, ISO, 6-BIO, 7-BIO, NEO, MNP	Acidic MnO_2	19	Allegri et al (2012)[44]
HPLC-MS	PTE, XAN, ISO, NEO, MNP, NH2, 6-BIO, BH2, 6-HMP, 6-MP, 6-CAP	None	30	Girón et al (2012)[41]
HPLC-MS/MS	PTE, XAN, ISO, NEO, 6-BIO, 6-CAP, 6,7-DMP, 6-HMP	Alkaline I_3^-	7.5	Burton et al (2013)[29]
HPLC-FD	6-BIO, BH2, BH4, NEO, NH2	None	16	Guibal et al (2014)[38]
HPLC-FD	PTE, XAN, ISO, NEO, 6-BIO, 6-CAP, 6,7-DMP, 6-HMP	Alkaline I_3^- and $KMnO_4$	25	Kośliński et al (2014)[28]
HPLC-FD	PTE, XAN, ISO, NEO, MNP, 6-BIO, 7-BIO, 6-CAP, 6-HMP	Acidic I_3^-	29	Tornero et al (2014)[37]
HPLC-MS/MS	*Quantitative:* PTE, XAN, ISO, NEO, 6-BIO, 6-CAP, 6,7-DMP, 6-HMP	None	25	Burton et al (2015)[42]
UHPLC-MS/MS	PTE, XAN, XH2, NEO, NH2, 6-HMP, 6-HMDP, 6-FOP, 6-CAP	Mn_2O_5	8	Van Daele et al (2016)[43]
Proposed HPLC-MS/MS	PTE, XAN, XH2, ISO, NEO, MNP, 6-BIO, SEP, 6-CAP, 6,7-DMP, 6-MP, 6-HMP, LUM, 6-HLUM, 7-HLUM	None	7	--

[1] PTE: Pterin; XAN: Xanthopterin; ISO: Isoxanthopterin; NEO: Neopterin; 6-BIO: 6-Biopterin; 6-CAP: 6-Carboxypterin; 6,7-DMP: 6,7-Dimethylpterin; 6-HMP: 6-Hydroxmethylpterin; 7-BIO: 7-Biopterin; MNP: Monapterin; LUM: Lumazine; 6-HLUM: 6-Hydroxylumazine; 7-HLUM: 7-Hydroxylumazine; BLUM: Biolumazine; NH2: 7,8-Dihydroneopterin; BH2: 7,8-Dihydrobiopterin; 6-MP: 6-Methylpterin; BH4: Tetrahydrobiopterin; XH2: 7,8-Dihydroxanthopterin; PH2: Dihydropterin; PH4: Tetrahydropterin; 6,7-Dimethyltetrahydrobiopterin; NH4: Tetrahydroneopterin; 6-HMDP: 6-Hydroxymethyldihydropterin; 6-FOP: 6-Formylpterin; SEP: Sepiapterin;

[2] Run time includes pre- and post-equilibration and cleaning procedures.

In the present study, we described a new workflow for the quantitative analysis of 15 pteridine derivatives in urine using a novel HPLC-MS/MS methodology. The pteridines investigated in this study were selected primarily as folate-derived pteridines with cancer biomarker applications [55] and include five structural isomers, multiple lesser known derivatives, and two semi-reduced compounds (**Figure 1**). However, the analytical workflow has been designed with extensibility to additional pteridine derivatives and biological matrices for specific applications. This new technique was subsequently used to systematically study the in-source oxidation, interconversion, and efficiency of several commonly used oxidative pretreatments in an attempt to standardize pteridine sample preparation. The resulting standardized technique was used to profile pteridine derivatives in the urine of healthy individuals, breast cancer patients, and prostate cancer patients.

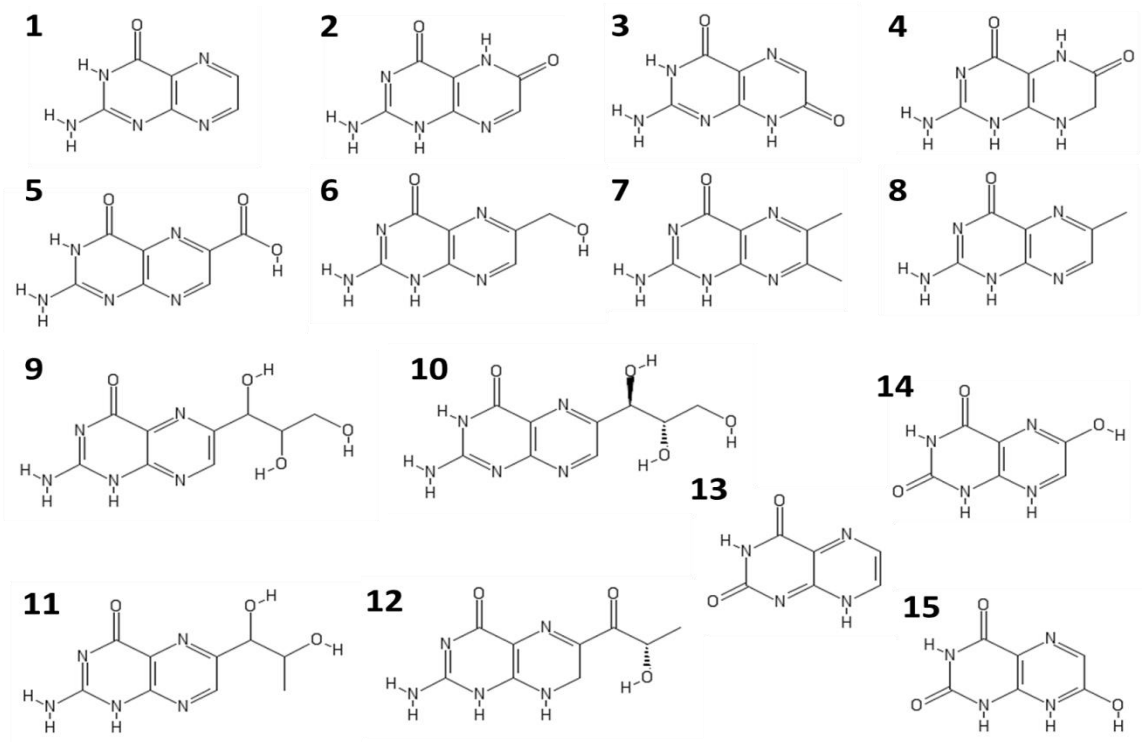

Figure 1. Chemical structures of: (1) pterin, (2) xanthopterin, (3) isoxanthopterin, (4) 7,8-dihydroxanthopterin, (5) 6-carboxypterin, (6) 6-hydroxymethylpterin, (7) 6,7-dimethylpterin, (8) 6-methylpterin, (9) neopterin, (10) L-monapterin, (11) 6-biopterin, (12) sepiapterin, (13) lumazine, (14) 6-hydroxylumazine, and (15) 7-hydroxylumazine.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials

Pterin, xanthopterin, 7,8-dihydroxanthopterin, isoxanthopterin, 6-biopterin, sepiapterin, neopterin, monapterin, 6-carboxypterin, 6-hydroxymethylpterin, 6,7-dimethylpterin, 6-methylpterin, lumazine, 6-hydroxylumazine, 7-hydroxylumazine, 6-formylpterin, leucopterin, and folic acid were purchased from Schircks Laboratory (Jona, Switzerland). LC-MS grade ammonium hydroxide and formic acid were purchased from Fisher Scientific (New Jersey, USA). Potassium iodide, iodine, ascorbic acid, and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, USA). Synthetic urine was obtained from CST Technologies Inc. (New York, USA). Ultrapure water was generated by a Milli-Q Advantage® A10 and Millipore Elix® water purification system.

2.2. Instrumentation

Pteridine derivatives were separated using a Luna phenyl-hexyl column (3.0 μm, 3.0×150 mm) and accompanying guard column (Phenomenex, Torrance, CA) in conjunction with a Shimadzu UFLC system (Columbia, MD) that included a degasser (DGU-30A3), two pumps (LC-20 AD XR), a temperature controlled autosampler (SIL-20AC XR) and a column oven (CTO-20A). Chromatography was performed at 40°C with a flow rate of 0.45 mL min^{-1} using a 50 μL injection volume. A binary gradient flow system was used to separate pteridine derivatives using the following mobile phase compositions: A: 0.025% (v/v) formic acid in 99% water / 1% acetonitrile; B: methanol. The gradient profile included an initial two-minute period in which the composition of mobile phase B was increased linearly from 7% to 20%, followed by another two-minute period in which mobile phase B was held at 20%, and rapidly returned to 7% for the final two minutes. A one-minute pre-equilibration as well as a 200 μL post-injection needle rinse procedure were added to reduce carryover effects between biological samples. The total run-to-run time was 7.0 minutes. Samples were stored in the autosampler at 4°C for a period lasting no longer than six hours.

An AB Sciex 4000 QTrap MS/MS system (Foster City, CA, USA) was used to quantify pteridine derivatives. The ion source was operated in positive-ion ESI mode with

the following flow injection parameters: ion spray voltage: +5500 V; source temperature: 600°C; curtain gas: 45 psi N_2; GS1: 25 psi N_2; GS2: 35 psi N_2; heated interface. Scheduled multiple reaction monitoring (MRM) experiments were operated with 44 second scan windows and individual target scan times of 0.7 seconds to quantify selected ions transitions. Collisionally-activated dissociation (CAD) of pteridine derivatives was achieved using 12 psi ultra-pure N_2 gas in the collision chamber.

2.3. Standard Preparation

Between 2 and 5 mg of each pteridine standard was dissolved in in 13 mL of ultrapure water and 500 µL of 2 N ammonium hydroxide in amber glass vials. Sonication was used to enhance dissolution of poorly soluble species, namely, 6,7-dimethylpterin, 6-methylpterin, pterin, and leucopterin. Dilutions from these individual standard stock solutions were used for direct infusion analysis to optimize MS/MS conditions. A stock mixture solution was similarly prepared at 10 mg/L each component from these individual stock solutions using ultra-pure water. Aliquots of the individual and stock mixture solutions were wrapped in aluminum foil and were stored at -80°C. Fresh calibration standards were prepared from these aliquots daily using serial dilutions in ultra-pure water and/or 1% synthetic urine.

2.4. Analysis of Urine Samples

Urine specimens (n = 50) were collected from consenting women 33-87 years of age (mean: 60.0 years) at Mercy Breast Center – Springfield (Springfield, MO) between October 2014 and December 2014 in a consecutive case series manner. Selection criteria included women referred to the medical center for biopsy characterization with no co-present medical conditions or previous history of cancer. Participants were drawn from a local patient population with the following demographics: 88.7% Caucasian, 3.3% Hispanic or Latino, 3.2% African American, 1.8% Asian, and 3% other or two or more races. Spot urine specimens consisting of first morning and second morning voids were collected prior to biopsy and immediately stored at -20°C for 1-6 days. Specimens were shipped to Missouri University of Science and Technology for analysis via next-day frozen ground freight. Upon arrival, specimens were thawed, aliquoted, and refrozen at -80°C

until analysis. Mercy Breast Center pathologists disclosed anonymized diagnostic reports at the conclusion of the study.

Under dark conditions, urine sample aliquots were gradually thawed to room temperature and thoroughly vortexed. The urine specific gravity (USG) was determined from 200 µL sample using a temperature-corrected Reichert TS 400 clinical refractometer. USG was used to adjust urinary pteridine concentrations to patient hydration-dilution status and time since last urination in accordance with prior work [30]. Another 100 µL sample was diluted tenfold in ultra-pure water in a yellow centrifuge tube. Diluted samples were centrifuged at 3000 g for 20 minutes at 4°C and filtered with a 0.22 µm Nylon membrane to remove sediment, proteins, and epithelial cells. Another tenfold dilution in ultra-pure water was made into a 1.5 mL amber glass vial and was subsequently vortexed and submitted to HPLC-MS/MS analysis. The total dilution factor for urine samples was 100-fold.

3. RESULTS AND DISCUSSION

3.1. MS/MS Optimization

Individual pteridine derivatives were infused at a concentration of 100 µg L^{-1} in mobile phase A (0.025% (v/v) formic acid in 99% water / 1% acetonitrile) at a flow rate of 0.2 mL $hour^{-1}$. Ion source conditions included: ion spray voltage: +5500 V; source temperature: 30°C; curtain gas: 10 psi N_2; GS1: 10 psi N_2; GS2: 10 psi N_2; heated interface. A combination of quadrupole (Q1) and linear ion trap (EMS) survey scans operating under Multiple Channel Analyzer (MCA) mode were used to study pseudomolecular ions. Sodiated and ammoniated molecular ions were present in standard stock solutions, owing to the dissolution of pteridine derivatives in ammonium hydroxide and ubiquitous sodium, respectively. The relative contributions of these adducts were consistent with previous reports [29], which may be generalized as the following: pteridines with electron withdrawing substituents favored the formation of ammoniated molecular ions whereas unsubstituted pteridines and pteridines with electron donating substituents favored the formation of sodiated molecular ions. Since molecular adducts contribute to decreased sensitivity and may require complicated ion summing procedures, infusion analysis of

pteridine standards in 1% synthetic urine was performed to study adduct formation under simulated salt concentrations. In this case, ammoniated molecular ions were not significantly observed while sodiated molecular ions remained prevalent. Sodiation was effectively controlled by applying sufficiently high declustering potentials to the orifice, which was automatically optimized by the Analyst 1.5.2 software.

In addition to pteridine ammoniation and sodiation, 6-formylpterin exhibited in-source oxidation to 6-carboxypterin (approximately 60% of the $[M+H]^+$ molecular ion) as well as significant $[M+15]^+$ and $[M+H+32]^+$ molecular ions in the presence of methanol (representing 20% and 50% of the $[M+H]^+$ molecular ion, respectively). The latter two ions were attributed to gas-phase aldol reactions between protonated aromatic aldehydes and methanol in the ion source [56]. These two species also did not appear in the linear ion trap EMS scans. Co-infusion of 100 µM ascorbic acid or dithiothreitol was found to limit its in-source oxidation to 6-carboxypterin. Furthermore, 6-formylpterin and 6,7-dimethylpterin are isobaric compounds with nearly identical fragmentation spectra with the exceptions of *m/z* 106 derived from 6,7-dimethylpterin and *m/z* 67, 92, and 119 derived from 6-formylpterin. Commonly used ion transitions for these compounds, such as 192 → 147 and 192 → 165, should be avoided since these two pteridine derivatives have similar chromatographic retention times on conventional columns [29, 43]. In addition to its negligible solubility in aqueous solutions, leucopterin suffered poor ionization efficiencies where only high declustering potentials resulted in a weak $[M+H]^+$ molecular ion and a weak $[M-36]^+$ molecular ion related to loss of water from its two ketone functionalities. For these reasons, leucopterin was not added to the current HPLC-MS/MS method. 7,8-Dihydroxanthopterin was found to have exhibited negligible in-source oxidation to xanthopterin (<5% of the $[M+H]^+$ molecular ion). The limited extent of this in-source oxidation was contradictory to recent reports by Van Daele and co-workers who suggested a thermally facilitated oxidative process [43]. However, infusion at higher temperatures (e.g. 500°C) did not meaningfully increase in-source oxidation rates; rather, in-source oxidation of 7,8-dihydroxanthopterin appeared to be solvent mediated and may be manipulated by additive selection (e.g. acetic acid). A more detailed discussion on the oxidation of 7,8-dihydroxanthopterin and its derivatives is provided in the following

sections. The optimized MRM transitions have been summarized in **Table 2**. Flow injection analysis was performed for these optimized MRM transitions to enable systematic optimization of the ion source parameters.

Table 2. Optimized ion pairs for MRM detection. Unlabeled ion pairs located directly below a labeled compound represent confirmation ion pairs.

Compound	Ion Pairs (m/z)	Declustering Potential (DP, V)	Collision Energy (CE, V)	Collision Cell Potential (CXP, V)
6-Carboxypterin	208.1/164.3	46	23	10
	208.1/190.1	46	21	12
6,7-Dimethylpterin	192.2/165.2	11	31	10
	192.2/106.2	11	39	6
6-Formylpterin	192.1/147.2	76	31	8
	192.1/149.2	76	29	8
6-Hydroxylumazine	181.1/164.2	76	23	10
	181.1/108.1	76	35	6
6-Hydroxymethylpterin	194.1/106.1	61	37	18
	194.1/176.2	61	23	12
6-Methylpterin	178.2/106.1	1	41	6
	178.2/133.2	1	31	8
7,8-Dihydroxanthopterin	182.1/126.2	71	29	22
	182.1/154.2	71	25	10
7-Hydroxylumazine	181.1/110.1	76	31	6
	181.1/55.2	76	49	8
Biopterin	238.1/220.2	66	23	14
	238.1/178.3	66	29	12
Folic Acid	442.2/176.3	56	55	10
	442.2/295.4	56	21	8
Isoxanthopterin	180.1/135.1	71	33	8
	180.1/163.2	71	27	10
Lumazine	165.1/69.1	71	55	12
	165.1/93.1	71	41	4
Monapterin	254.1/206.1	71	27	14
	254.1/236.3	71	21	16
Neopterin	254.0/206.2	76	27	12
	254.0/236.3	76	23	16
Pterin	164.1/92.1	71	43	4
	164.1/119.1	71	33	6
Sepiapterin	238.1/192.3	61	23	12
	238.1/165.3	61	33	10
XantHopterin	180.1/135.2	76	31	24
	180.1/163.1	76	25	10

3.2. HPLC Optimization

Separation parameters were initially adapted from a previously developed HPLC method using a Luna phenyl-hexyl column [29]. However, using 0.1% aqueous formic acid and acetonitrile resulted in co-elution of three of the four structural isomer pairs. Adaptation of the selective HPLC technique developed by Girón and colleagues, in which the prescribed Zorbax Eclipse XDB-C18 column was substituted with a Kinetex C18 column (2.6 μm, 3.0×150 mm), similarly failed to separate isobaric compounds and at greatly increased run times. Since acetonitrile and formic acid contain pi electrons that can suppress pi-pi interactions between analytes and phenyl bonded phases, the Luna phenyl-hexyl column was reevaluated using methanol and lower formic acid compositions. Separation of the neopterin/monapterin and 6-hydroxylumazine/7-hydroxylumazine isomer pairs were inversely affected by solvent composition, where high formic acid concentrations (>0.1% v/v) promoted neopterin/monapterin separation and high acetonitrile compositions promoted 6-hydroxylumazine/7-hydroxylumazine separation. Overall method selectivity was greatly improved using methanol and sufficient amounts of formic acid for efficient analyte ionization. Therefore, trace amounts of acetonitrile were added to the aqueous phase to enable separation of the hydroxylumazines. The optimal solvent composition that permitted separation of isobaric pairs at minimal run times was determined to be 0.025% v/v formic acid in 99:1% water:acetonitrile and pure methanol.

Furthermore, elevated column temperatures enhanced the separation of the hydroxylumazines, xanthopterin and isoxanthopterin, but partially merged neopterin and monapterin. It should be noted that 6-formylpterin and folic acid exhibited large peak widths and asymmetry on the reversed-phase Luna phenyl-hexyl column, which have been reported elsewhere [57]. The Kinetex C18 column meanwhile demonstrated superior selectivity and retention for these compounds. However, since the abundance of folic acid in urine relative to its primary degradation products, para-aminobenzoylglutamate (pABG) and para-acetamidobenzoylglutamate (apABG), is minimal [58], and 6-formylpterin is considered a reactive species that is not appreciably present in urine, both folic acid and 6-formylpterin were excluded from the validated method. Future studies aiming to quantify folic acid degradation byproducts such as 6-formylpterin should utilize alkyl- or polar-

bonded phases. An optimized overlaid extraction ion chromatogram (XIC) of the quantitative ion pairs has been provided in **Figure 2**.

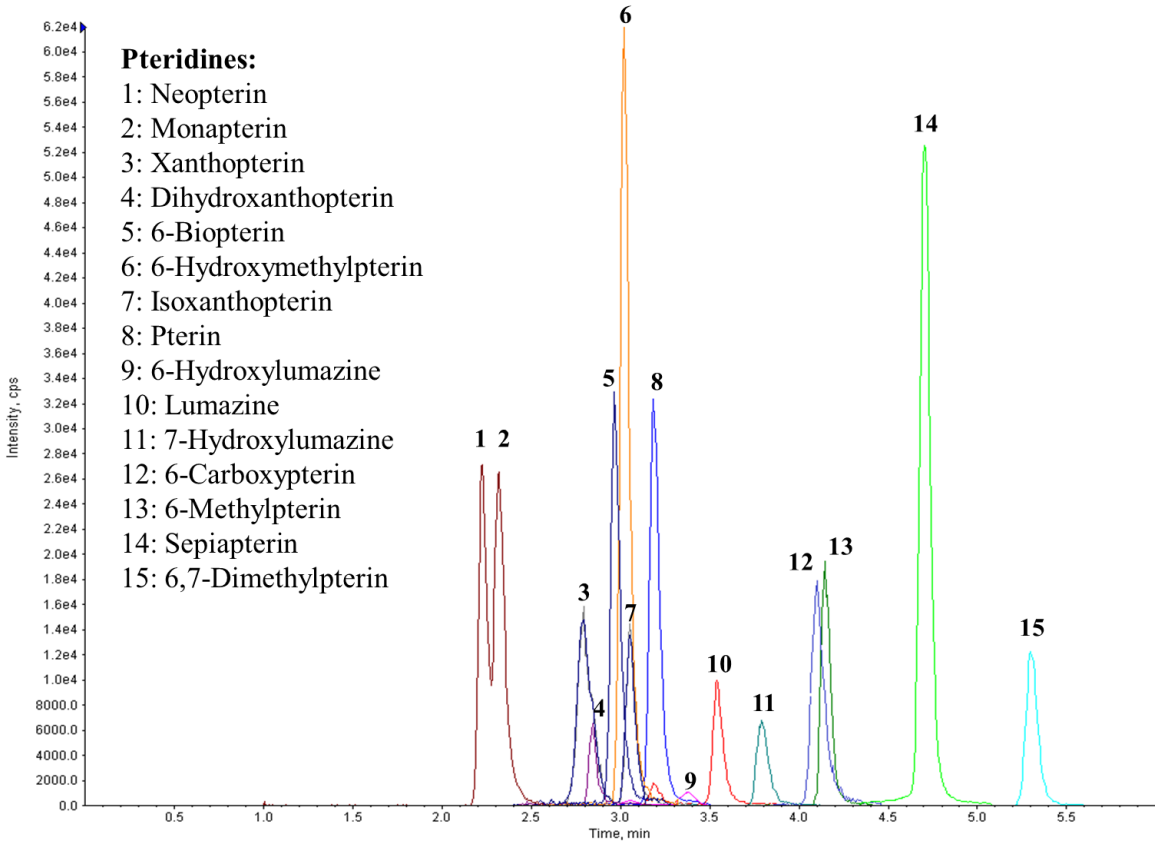

Figure 2. Overlaid extracted ion chromatograms (XIC) of the quantitative ion pairs of fifteen pteridine derivatives prepared at 10 µg/L in synthetic urine.

Since urine is a complex matrix, potential interferences with equivalent MRM transitions and precursor ions were identified using unscheduled MRM experiments and linear ion trap enhanced product ion (EPI) scans. The EPI results were used in combination with MassBank spectral databases and chemical reference standards to make tentative assignments. Special attention was given to the presence of reduced pteridine derivatives, such as 5,6,7,8-tetrahydrobiopterin, that were not included in the pteridine panel

investigated in this study. For example, 7,8-dihydrobiopterin was observed at approximately 2.85 min in untreated urine. In-source oxidation of 5,6,7,8-tetrahydrobiopterin chemical reference standard and EPI fragmentation patterns that matched those reported by Fismen et al [52] strongly suggested that this compound was 7,8-dihydrobiopterin. Similarly, a small peak at 2.1 min was observed using the MRM transition for neopterin/monapterin. Survey scans at this chromatographic time point suggested the presence of 7,8-dihydroneopterin, as evidenced by major fragmentation patterns that included 256 → 165, 178 [43]. An unidentified compound that partially eluted with pterin was found to have the following ion transitions 164 → 61, 73, 87, 101, 103, 119, 147, including the shared 164 → 119 transition. For this reason, the less abundant *m/z* 92 fragment of pterin was selected as the quantitation ion. Tentative assignments were made for 7-methylguanine (R.T. = 3.15 min) and phenylalanine (R.T. = 4.10 min) while searching for 7,8-dihydropterin (*m/z* 166). Tyrosine (R.T. = 3.1 min) was also identified at *m/z* 182 with dissimilar fragmentation to 7,8-dihydroxanthopterin. Interestingly, a significant peak was located at *m/z* 180 at 4.2 min with a fragmentation pattern analogous to xanthopterin and isoxanthopterin. Further characterization revealed major fragmentation pathways of 180 → 71, 110 and minor fragmentation pathways of 180 → 68, 80, 93, 108, 135, and 137. This pattern appeared to be indicative of pyrazine ring opening in xanthopterin, although its occurrence could not be modulated by triiodide and UV oxidative pretreatments. Further characterization will be required for structural assignment.

3.3. Comparative Study of Oxidative Pretreatments

As Tomšíková and co-workers recently reviewed, a remarkably large number of oxidative and antioxidative pretreatments have been used to quantify pteridines in biological matrices [50]. Since comparative studies of these pretreatments have been incomplete or lacking, particularly in regard to semi-reduced and folate-derived pteridines like 7,8-dihydroxanthopterin, the newly developed HPLC-MS/MS method was used to quantify the oxidative efficiency of several commonly used techniques, including Lugol's solution (I_3^-), manganese dioxide, and potassium permanganate. Specifically, Lugol's solution, comprising a 4%:2% (w/v) mixture of potassium iodide-iodine, is the most commonly used oxidative pretreatment for pteridine analysis and was evaluated herein in

both alkaline and acidic environments per previously developed protocol [27, 35, 37]. Permanganate and acidic manganese dioxide oxidative efficiency were also investigated using earlier developed methods [28, 44]. A summarized comparison of the effects of these pretreatments on pteridine standards prepared in synthetic urine has been provided in **Table 3**. From these data it becomes clear that oxidative pretreatment leads to degradation of both aromatic and reduced pteridine derivatives. Alkaline triiodide was considered the gentlest pretreatment strategy with the exceptions of pteridines with electron withdrawing substituents at the 6-position, such as xanthopterin, 6-carboxypterin, and 6-hydroxylumazine. However, the oxidation of 7,8-dihydroxanthopterin was remarkably complex with mass balance calculations indicating that the oxidative efficiency of the preferred conversion to xanthopterin was only 7%. Infusion of untreated and oxidized 7,8-dihydroxanthopterin revealed possible oxidative byproducts centered at *m/z* 175, 205, and 213, although the structures of these compounds remain undefined. An additional 5% of 7,8-dihydroxanthopterin was lost to in-source oxidation during ionization. Acidic triiodide performed similarly to alkaline triiodide with the notable absence of xanthopterin which eluted much later (R.T. = 7.2 min). The effect of triiodide concentration was also considered by comparing a typical addition of 150 µM to 15 µM where higher concentrations led to increased pteridine degradation. The possibility that this observation may be attributed to ion suppression rather than an oxidative reaction was rejected since iodide ions were found to elute at the column dead time under similar chromatographic conditions. Permanganate pretreatments resulted in substantial loss of both aromatic and semi-reduced pteridines. These permanganate findings generally agreed with those reported by Kośliński and colleagues, and were attributed to opening of the pteridine ring system [28]. Similarly, pretreatments using manganese dioxide contributed to relative losses for most pteridine derivatives.

In another experiment, we examined the effect of urine concentration-dilution on oxidative efficiency and pteridine degradation. Since synthetic urine lacks the pool of oxidizable species present in real urine matrices, it was necessary to investigate this effect using real urine samples. Alkaline triiodide was selected as the model oxidant owing to its widespread use and its relatively gentle oxidation. Urine concentration-dilution was indirectly modeled using USG, a correlate of total dissolved solids, as a surrogate [30].

Table 3. Effect of oxidative pretreatment on pteridines in synthetic urine.

Compound	15 µM alkaline I_3^-	15 µM acidic I_3^-	150 µM alkaline I_3^-	150 µM acidic I_3^-	$KMnO_4$	Acidic MnO_2
Isoxanthopterin	102%	104%	100%	100%	73%	83%
6,7-Dimethylpterin	102%	114%	106%	99%	88%	103%
Pterin	99%	106%	51%	103%	31%	95%
6-Hydroxymethylpterin	97%	110%	107%	114%	40%	83%
6-Biopterin	96%	96%	86%	92%	91%	93%
6-Methylpterin	94%	79%	78%	48%	93%	88%
Neopterin	93%	81%	87%	80%	45%	81%
7-Hydroxylumazine	91%	95%	79%	73%	37%	67%
Lumazine	91%	108%	79%	85%	35%	98%
Monapterin	85%	102%	72%	74%	41%	89%
Xanthopterin	80%	0%	63%	0%	0%	67%
6-Carboxypterin	68%	72%	76%	52%	49%	74%
6-Hydroxylumazine	62%	0%	63%	0%	26%	47%
Sepiapterin	0%	0%	0%	0%	0%	0%
7,8-Dihydroxanthopterin	0%	0%	0%	0%	0%	0%

† All values are expressed as a percentage of the untreated control group.
‡ Samples were run in triplicate at a concentration of 5 µg/L.

The percentage of pteridine derivative remaining in oxidized urine specimens, relative to corresponding untreated urine specimens, were compared with sample USG ranging from 1.005 to 1.027. Urine specimens (n = 10) were spiked with a 5 µg L^{-1} mixture of pteridine standards to ensure detection of all pteridine derivatives. Briefly, xanthopterin oxidation exhibited a strong positive correlation (Pearson's r = 0.9949) with urine concentration-dilution, ranging from 50% to 648% initial levels. Similarly, 6-biopterin oxidation positively correlated with urine concentration-dilution (Pearson's r = 0.9566) and ranged from 135% to 186% initial levels. These correlations were attributed to the oxidation of reduced derivatives of xanthopterin and 6-biopterin. However, neopterin oxidation was inversely related to urine concentration-dilution (Pearson's r = -0.9957), ranging from 72% in the 1.027 USG sample to 101% in the 1.005 USG sample. Other pteridine derivatives that exhibited correlations to urine concentration-dilution included 6-hydroxymethylpterin (Pearon's r = 0.9781), monapterin (Pearson's r = -0.9104), and pterin (Pearson's r = 0.7211). The oxidative effects on the other pteridines were generally similar to those determined in synthetic urine found in **Table 3**. These new findings are significant because previous research efforts have questioned whether triiodide ions have been provided in excess to ensure complete reduced pteridine oxidation [27, 35]. This oxidative pretreatment dependence on urine concentration-dilution marks a novel discovery and questions the validity of using oxidative pretreatments for pteridine quantitation. Moreover, the discovery of complicated oxidation mechanisms in the case of 7,8-dihydroxanthopterin casts further doubts on the utility of oxidative pretreatments. While other analytical techniques have foregone pretreatment to simplify analysis or to preserve reduced species [38, 39, 42, 43], the present work has now provided compelling and quantitative evidence that currently used oxidative pretreatments are inefficient for quantitative pterinomics.

3.4. Method Performance

Performance characteristics for the newly described HPLC-MS/MS method have been summarized in **Table 4**. Analytical sensitivity, assessed in terms of method detection limits (MDLs) and lower limits of quantitation (LLOQs), was comparable or superior to existing techniques for pteridine quantitation with MDLs ranging from 0.025 µg $L^{-1}$to 0.5

μg L^{-1}. Pteridine signal response exhibited considerable linearity ($R^2 > 0.99$) over a wide linear range that spanned several orders of magnitude. The abundance of 7,8-dihydroxanthopterin in urine specimens, which is discussed below, necessitated a higher operating range up to 1000 μg L^{-1}. Method accuracy was evaluated with spiked recoveries in a pooled urine specimen (USG = 1.015) which ranged from 88% for low concentrations of 6-carboxypterin to 112% for high concentrations of 7,8-dihydroxanthopterin. The method possessed excellent precision that remained below 6% relative standard deviation for all pteridine derivatives on both intra-daily and inter-daily bases (n = 5). This level of precision was consistent across concentrations as indicated by the spiked recovery study.

3.5. Analysis of Urine Specimens

The new pterinomics workflow was applied to 50 clinical urine specimens from women who were diagnosed with either breast cancer or a benign fibrocystic conditions. This application was selected based on prior reports on the molecular epidemiology of pteridines in breast cancer [26, 30], but also to determine whether untreated pteridine derivatives were present in urine at detectable concentrations. The latter investigation is novel because prior studies either have monitored reduced pteridine derivatives in other biological fluids or have used inferior methodologies that lack selectivity [39, 43]. At 100-fold dilution, ten of the fifteen pteridine derivatives were reliably quantified in all urine specimens (**Figure 3**). Those derivatives and their encountered concentration range were as follows: sepiapterin (22 nM – 4500 nM), isoxanthopterin (250 nM – 2010 nM), xanthopterin (320 nM – 6000 nM), 6-biopterin (0.1 μM – 9.7 μM), neopterin (0.2 μM – 26 μM), lumazine (190 nM – 1000 nM), 7-hydroxylumazine (67 nM – 465 nM), monapterin (50 nM – 2400 nM), pterin (150 nM – 2340 nM), and 7,8-dihydroxanthopterin (5 μM – 112 μM). 6-Carboxypterin was present in most urine specimens, but at levels that were not quantifiable (signal-to-noise range: 0 – 4.2), an observation that supports earlier research findings [29]. Similarly, 6-hydroxymethylpterin was observed in a majority of samples, but at unquantifiable levels (signal-to-noise range: 0 – 2.9). Incomplete separation of neopterin and monapterin coupled with large concentration differences complicated the quantitation of monapterin.

Table 4. Performance characteristics of the pterinomics workflow.

Compound	Retention Time (min)	Linear Range (µg L^{-1})	R^2	MDLs (µg L^{-1})	LLOQs (µg L^{-1})	Recovery % (1 µg L^{-1})[1]	Recovery % (10 µg L^{-1})[1]	Recovery % (100 µg L^{-1})[1]	Urinary Range (nM)
Neopterin	2.2	0.1 - 500	0.9997	0.05	0.1	96	97	102	180-26,300
Monapterin	2.3	0.1 - 500	0.9982	0.05	0.1	105	100	97	50-2400
Xantopterin	2.75	0.3 - 500	0.9995	0.2	0.3	94	100	97	320-6000
7,8-Dihydroxanthopterin	2.85	0.5 - 1000	0.9825	0.3	0.5	91	94	112	5000-112,000
6-Biopterin	2.9	0.1 - 500	0.9997	0.05	0.1	97	98	97	110-9700
6-Hydroxymethylpterin	3.0	0.05 - 500	0.9983	0.025	0.05	101	95	94	N.D.
Isoxanthopterin	3.0	0.3 - 500	0.9992	0.2	0.3	96	96	105	250-2010
Pterin	3.1	0.1 - 500	0.9992	0.05	0.1	97	103	104	150-2340
6-Hydroxylumazine	3.3	1 - 500	0.9955	0.5	1	87	99	96	N.D.
Lumazine	3.5	0.5 - 500	0.9981	0.3	0.5	94	97	107	190-1000
7-Hydroxylumazine	3.8	0.2 - 500	0.9982	0.1	0.2	94	100	100	67-465
6-Carboxypterin	4.1	0.5 - 500	0.9993	0.3	0.5	88	93	95	N.D.
6-Methylpterin	4.1	0.05 - 500	0.9992	0.05	0.1	92	98	104	N.D.
Sepiapterin	4.7	0.1 - 500	0.9995	0.05	0.1	108	100	104	22-4500
6,7-Dimethylpterin	5.2	0.1 - 500	0.9995	0.05	0.1	105	101	107	N.D.

[1] Recovery of pteridine standards spiked into a urine sample at the designated concentrations (n = 5). No oxidative pretreatment was used.

Detection of trace pteridines was improved by using lesser dilutions, such as 20-fold dilutions; however, a 100-fold dilution was preferable in order to increase longevity of the column and mass spectrometer [29]. Non-parametric Mann-Whitney *U* group comparison tests revealed that no pteridine derivatives were substantially altered between the two patient groups, although isoxanthopterin (*p*-value = 0.063) was non-significantly increased in the breast cancer group. Hence, future studies that aim to quantify urinary levels of 6,7-dimethylpterin, 6-carboxypterin, 6-methylpterin, 6-hydroxymethylpterin, and 6-hydroxylumazine may require additional preconcentration or injection at lower dilutions.

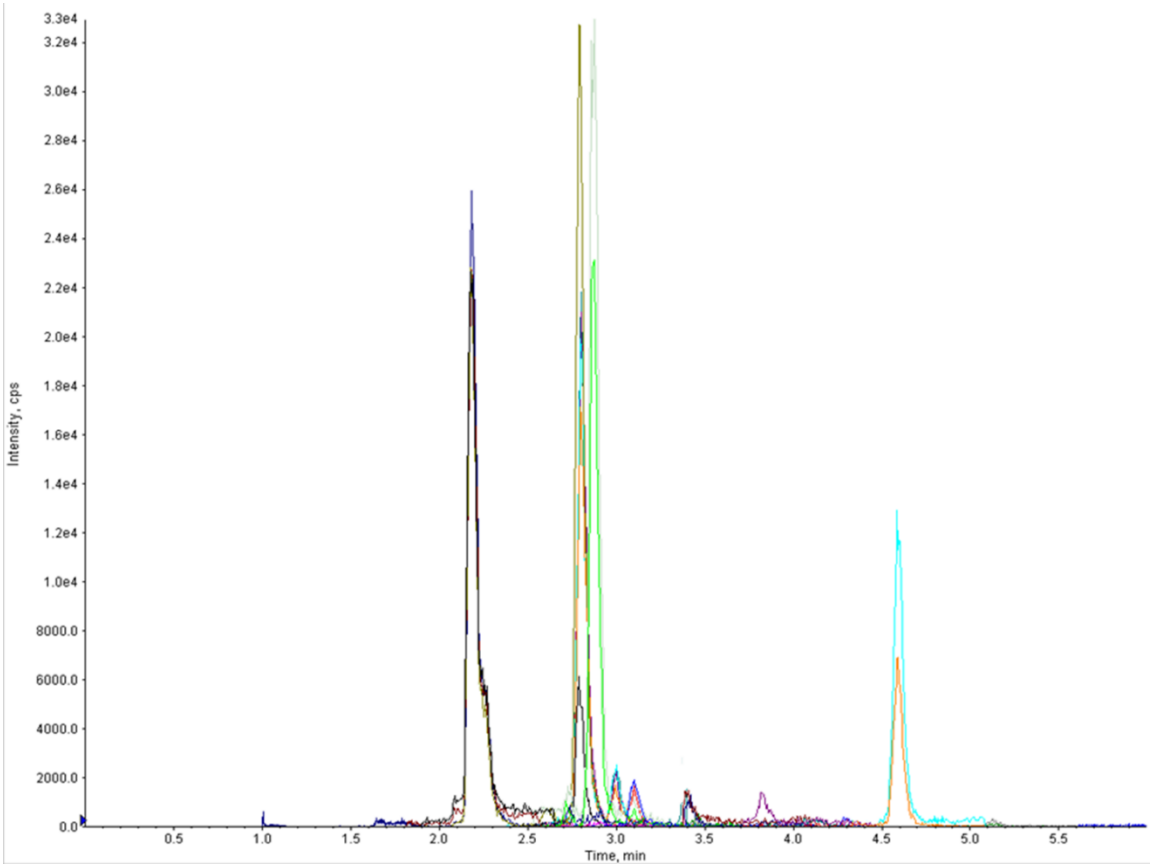

Figure 3. Overlaid extracted ion chromatograms (XIC) of the quantitative and confirmation ion pairs of native pteridine derivatives in 100-fold diluted urine with a typical USG of 1.014.

Finally, much attention has been given to the stability and interconversion of reduced and aromatic pteridine derivatives in oxygenated and aqueous environments [50]. Prior work has shown that aromatic pteridines are generally stable at low temperatures, while the stability of semi-reduced pteridines is considerably less, but still significant [59]. This led us to investigate whether the pteridine profile of fresh urine specimens significantly differed from that of aged urine specimens. Interestingly, 7,8-dihydroxanthopterin was present in fresh urine and accumulated over a period of weeks and months. This phenomenon was attributed to the non-specific oxidation and hydration of reduced pteridine derivatives to 7,8-dihydroxanthopterin [59]. For this reason, the pterinomic profile of a biological sample can be reasonably expected to change over time. For this reason, future studies should utilize either fresh urine specimens or store specimens at -80°C to prevent non-specific oxidation of reduced pteridine derivatives.

4. CONCLUSION

A new pterinomics workflow for the simultaneous determination of 15 pteridine derivatives in urine using HPLC-MS/MS has been described. The methodology expands on previous techniques by characterizing molecular adduction in ESI sources, separating five structural isomers, and investigating potential interferences present in complex urine matrices for the first time. The validated method possessed excellent sensitivity and precision that was suitable for comprehensive pteridine analysis in urine. The method was applied to a comparative study of commonly used oxidative pretreatments for pteridine analysis, revealing that oxidative mechanisms were inefficient, complex, and concentration dependent. These quantitative findings compellingly demonstrate that oxidative pretreatments are problematic for pterinomics approaches. In addition, 50 clinical urine specimens were examined with the new technique wherein 10 pteridine derivatives were quantified and population ranges have been given. The urinary levels reported in this study were generally consistent with previous work and indicated the suitability of the new technique for urinary pteridine analysis. Finally, this technique can be used to examine pteridine molecular epidemiology and biochemistry to support related research applications, and may further be readily extended to additional pteridine derivatives and biological matrices for specific applications.

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SECTION

2. CONCLUSIONS

The work presented in this dissertation explored the application of urinary metabolomics approaches to cancer biomarker discovery. The first two papers discussed novel enzymatic methods for the determination of sarcosine, a putative biomarker for prostate cancer. These studies highlighted the growing need to both identify and eliminate potential interferences from analytical methods. The next several studies presented new bioanalytical and clinical translational methods for the determination of urinary pteridines as putative biomarkers for breast cancer. These studies were embedded with several innovations, including new methods for the adjustment of urinary biomarkers to urine concentration-dilution and particularly within the context of clinical breast cancer screening, mass spectrometry-based methods to minimize undesirable adduction as a means to enhance method sensitivity, and a novel study design employing rigorous statistical workflows for the characterization of the biological variation of urinary metabolites in relation to circadian rhythms and vitamin supplementation. The pterinomics workflow presented in the final paper additionally outlines the development of a high-throughput method to support investigation of a composite panel of biomarkers for increased diagnostic accuracy. Finally, the proof-of-concept work presented in Paper VI that explores the feasibility of using urinary metals as disease biomarkers has introduced urinary metallomics as a potentially transformative new subfield of urinary metabolomics. Taken together, this body of work makes significant contributions to the applicability of urinary metabolomics as a biomarker discovery platform for early cancer detection and toward understanding the clinical and biological significance of pteridines in cancer.

VITA

Casey Franklin Burton was born in Jefferson City, MO on October 6, 1990. He received his Bachelor's of Science in Chemistry with a Minor in Biology from Missouri University of Science and Technology in May 2013. In June 2013, Casey joined Dr. Yinfa Ma's research group at Missouri University of Science and Technology for a PhD in Analytical Chemistry. Between 2013 and 2014, Casey was named a Chancellor's Graduate Research Fellow at Missouri University of Science and Technology. During the summer of 2014, Casey received a National Science Foundation East Asia and Pacific Summer Institutes Fellowship that enabled him to conduct research at the Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education at Peking University in Beijing, China. In August 2014, Casey began his tenure as a National Science Foundation Graduate Research Fellowship through the present. His research has focused primarily on the application of urinary metabolomics approach to discovery of novel biomarkers for the early detection of cancer. He was awarded a PhD in Chemistry from Missouri University of Science and Technology in May 2017.