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Normalization of urinary pteridines by urine specific gravity for early cancer detection

Casey Burton, Honglan Shi, Yinfa Ma *

Department of Chemistry and Center for Biomedical Science & Engineering, MO University of Science and Technology, Rolla, MO 65409, United States

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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 may better resist those same factors. We examined the performance of traditional creatinine adjustments relative to USG to six urinary pteridines in aggressive and benign breast cancers.
Methods: 6-Biopterin, neopterin, pterin, 6-hydroxymethylpterin, isoxanthopterin, xanthopterin, and creatinine were analyzed in 50 urine specimens with a previously developed liquid chromatography–tandem mass spectrometry technique. Creatinine and USG performance were 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, 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.

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, 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 such as 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,

* Corresponding author at: Department of Chemistry, Missouri University of Science and Technology, 400 West 11th St., Rolla, MO 65409, United States. Tel.: +1 573 341 6220; fax: +1 573 341 6033.
E-mail address: yinfa@mst.edu (Y. Ma).

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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]:

$$C_{\text{corrected}} = C_{\text{raw}} \times \frac{\text{USG}_{\text{reference}} - 1}{\text{USG}_{\text{experimental}} - 1}$$

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

2. Materials and methods

2.1. Samples and specimens

Fifty women 35–85 y (mean 61 y; SD 13 y) 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, MO. 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 2 weeks in a freezer at −20 °C at the 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.

2.2. Chemicals and materials

Isoxanthopterin, pterin, *D*-neopterin, xanthopterin, 6-biopterin, ammonium oxalate, and ammonium hydroxide were from Sigma-Aldrich. 6-Hydroxymethylpterin was from Schircks Laboratories. Potassium iodide and iodine were from Fisher Scientific. Synthetic urine was obtained from CST Technologies Inc. Ultrapure water was generated by a Milli-Q Advantage® A10 and Millipore Elix® water purification system.

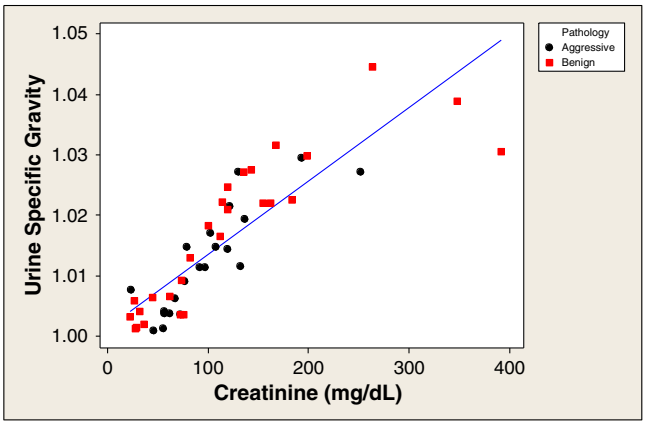

Fig. 1. Relationship between USG and creatinine in benign ($n = 27$) and aggressive breast cancers ($n = 21$).

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 (3.0 μm, 3.0 × 150 mm) and a Shimadzu UFLC system comprised of a degasser (DGU-30A3), 2 pumps (LC-20 AD XR), autosampler (SIL-20 AC XR), and a column oven (CTO-20A) operating under HPLC conditions. An API 4000Q trap MS/MS system from Applied Biosystems 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 to 1000 μg/l. Spiked recoveries in real urine samples ranged from 81% to 105% with CVs between 1% and 6% [2]. Urine specimens were centrifuged at 5000 rpm (2571 *g*) at 4 °C for 20 min and pre-treated with 2 mol/l 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 and 5000 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 was used for USG quantification. Prior to analysis, thawed, centrifuged urine specimens were equilibrated at 25 °C for 20 min. Refractive indices were converted to USG values via

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[a]	0.5000[a]	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

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