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Development of a high-performance liquid chromatography – Tandem mass spectrometry urinary pterinomics workflow

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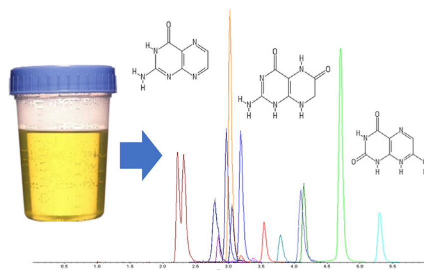
HIGHLIGHTS

- Quantitative HPLC-MS/MS methodology developed for 15 pteridine derivatives.
- Common oxidative pretreatments were investigated in a comparative study.
- Oxidative pretreatments were inefficient and should be avoided.
- The new methodology was applied to 50 benign and breast cancer urine specimens.

GRAPHICAL ABSTRACT



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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 four 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 $\mu g\ L^{-1}$ to 0.5 $\mu g\ L^{-1}$) 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.

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1. Introduction

Pteridines have evoked interest from the scientific community since their successful isolation from butterfly wings by Hopkins in

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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 certain 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 challenges. 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.

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 four structural isomers, multiple lesser known derivatives, and two semi-reduced compounds (Fig. 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.

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 min. Samples were stored in the autosampler at 4 °C for a period lasting no longer than six hours.

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