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Sample preparation and UHPLC-FD analysis of pteridines in human urine

H. Tomšíková, P. Solich, L. Nováková*

Department of Analytical Chemistry, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

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ABSTRACT

Elevated levels of pteridines can indicate the activation of cellular immune system by certain diseases. No work dealing with the simultaneous determination of urinary neopterin, biopterin and their reduced forms has been published. Therefore, a new SPE–UHPLC-FD method for the analysis of these compounds has been developed. The main emphasis was put on the stability of dihydroforms during the sample processing and storage. As a stabilizing agent, dithiothreitol, at various concentrations, and various pH values (3.8–9.8) of working solutions were tested.

Chromatographic separation was performed under HILIC isocratic conditions on BEH Amide column. The method was linear for the calibration standard solutions in the range of 10–10,000 ng/ml (dihydroforms) and 0.5–1000 ng/ml (oxidized forms), and for real samples in the range of 25–1000 ng/ml (dihydroforms) and 1–100 ng/ml (oxidized forms). The development of a new SPE sample preparation method was carried out on different types of sorbents (based on a mixed-mode cation exchange, porous graphitic carbon and a polymer comprising hydrophilic and hydrophobic components). Final validation was performed on a MCAX SPE column. Method accuracy ranged from 76.9 to 121.9%. The intra- and inter-day precision did not exceed 10.7%. The method provided high sensitivity for the use in routine clinical measurements of urine (LLOQ 1 ng/ml for oxidized forms and 25 ng/ml for dihydroforms). Average concentrations of biopterin, neopterin, and dihydrobiopterin found in urine of healthy persons were related to the mol of creatinine (66.8, 142.3, and 257.3 μmol/mol of creatinine, respectively) which corresponded to the literature data. The concentration of dihydroneopterin obtained using our method was 98.8 μmol/mol of creatinine.

1. Introduction

Pteridines can be classified as conjugated and unconjugated, as pterins and lumazines, or oxidized, di- and tetrahydroforms. Apart from conjugated pteridines, such as folic acid and riboflavin, the non-conjugated tetrahydrobiopterin (BH4) also serves as a cofactor of important enzymes. It is synthesized de novo from GTP [1]. Alternatively, it is regenerated from intermediates of BH4 metabolism including sepiapterin and dihydrobiopterin (BH2; Fig. 1).

Some pteridines, most notably neopterin (NEO) (Fig. 1), are increasingly used as markers of inflammatory processes, such as infections by intracellular bacteria and parasites, autoimmune diseases or allograft rejection, but also in chronic diseases where inflammation is involved, including atherosclerosis and, particularly, cancer [2]. Monocytes/macrophages, stimulated by interferon-γ secreted by activated T-lymphocytes, are the source of NEO and its reduced form, dihydroneopterin (NH2) (Fig. 1). Macrophages exhibiting low activity of 6-pyruvoyl-tetrahydropterin synthase are therefore not able to metabolize sufficiently NH2 triphosphate, an intermediate in BH4 synthesis. This leads to an accumulation of NH2 and NEO, and their secretion into biological fluids. The measurement of NEO is performed in plasma, cerebrospinal fluid, ascites, and especially non-invasively in urine. In addition, cancer and some other diseases were

Abbreviations: ACN, acetonitrile; BEH, ethylene bridged hybrid; BH2, dihydrobiopterin; BH4, tetrahydrobiopterin; BIO, biopterin; CREA, creatinine; DTT, dithiothreitol; EMA, European Medicines Agency; FD, fluorescence detection; HILIC, hydrophilic interaction liquid chromatography; HLOQ, high limit of quantification; HMP, 6-hydroxymethylbiopterin; HPLC, high performance liquid chromatography; LLOQ, lower limit of quantification; MCAX, mixed-mode cation exchange; MEOH, methanol; NH2, dihydroneopterin; NEO, neopterin; ONCO, oncopterin; PCA, pterin-6-carboxylate; PDA, photo diode array; PGC, porous graphitic carbon; PT, pterin; QC samples, quality control samples; SPE, solid phase extraction; UHPLC, ultra high performance liquid chromatography; ULOQ, upper limit of quantification; XP, xanthopterin.

* Corresponding author. Tel.: +420 495 067 381; fax: +420 495 518 002.
E-mail address: nol@email.cz (L. Nováková).

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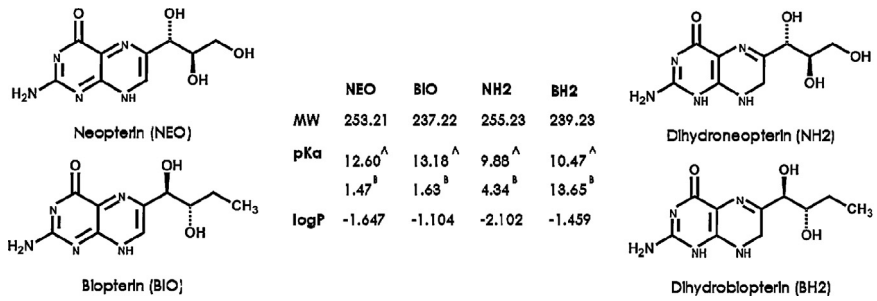

Fig. 1. Structures of pteridines studied. Explanatory notes to the figure: (SciFinder Scholar – CAS, Version 2.0) – reported values of pK_a and log P were calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994–2013 ACD/Labs); [A]most acidic (25 °C), [B]most basic (25 °C).

reported to be associated with altered levels of other BH4 or folic acid metabolites, such as pterin (PT), xanthopterin (XP), isoxanthopterin, 6-hydroxymethylbiopterin (HMP), oncopterin (ONCO), pterin-6-carboxylate (PCA), and biopterin (BIO; Fig. 1) [3–6].

Analysis of pteridines is very challenging due to the need for a special manipulation arising from their physicochemical properties (high polarity, sensitivity to light, diverse oxidation products with different sensitivity in fluorescence detection, and low solubility in water and organic solvents. They are present in biological fluids, such as serum and cerebrospinal fluid at low concentrations (nM) [2,7]. Poor solubility of pteridines in water and organic solvents complicates the preparation of standard solutions. The presence of amino, hydroxyl or thiol group decreases the solubility, while an opposite effect was achieved by the protection of N- or O-atoms of the amide functional group [8,9]. Instability and tendency of di- and tetrahydropteridines to autooxidation demand a skilful handling. The presence of oxidized analogues in the standards of dihydroforms is undesirable and there is no prevention which could avoid it. More details about the stability of pteridines are summarized in recently published review of Tomšíková et al. [3].

The retention and separation of polar pteridines in the conventional reverse phase liquid chromatography systems, using C8 and C18 columns and highly aqueous (>90% water) mobile phases, brought difficulties, such as a total loss of retention and non-reproducible retention times, gradient regeneration delays, and peak tailing [3,10,11]. Therefore, many authors have focused on different approaches including ion-pair [12,13], ion-exchange chromatography [4,14,15] or hydrophilic interaction liquid chromatography (HILIC) [16–18]. In the majority of studies fluorescence detection (FD) with high performance liquid chromatography (HPLC) was used [3]. Fluorescence was a sensitive and affordable detection tool with low limits of detection for oxidized forms of pteridines (>0.01 ng/ml) [19–21], due to their ability to strongly fluoresce. Recently, there has been an increase in the application of tandem mass spectrometry (MS/MS) for the determination of pteridines in biological samples [18, 22–25]. With this approach, a high sensitivity is achieved using selected/multiple reaction monitoring. However, the sensitivity of MS was only comparable with FD [3], which might make the latter more easily available especially when analysis cost is considered.

A pre-treatment of urine samples before pteridine analysis was in most cases accomplished by filtration or/and dilution [3]. In general, it is the easiest and fast way, how to treat biological samples. However, method selectivity can be compromised. The selectivity could be increased with combination of FD, sample pre-treatment and clean-up. Solid phase extraction (SPE) of urine samples was employed before the separation of different pteridines (NEO, BIO, HMP, PT, XP, PCA, BH4, ONCO) in several works [3, 26–29]. However, there was no work including simultaneous separation and sample preparation of NH2, BH2, BIO, and NEO in urine.

In this study an ultra-high performance liquid chromatography coupled with fluorescence detection (UHPLC-FD) and sample preparation (SPE), for the determination of urinary NEO, BIO and their reduced forms (NH2 and BH2) was developed. Furthermore, short term stability study including the influence of pH value and concentration of stabilizing agent was conducted. The selection of UHPLC-FD (in HILIC mode) and SPE sorbents was based on the fact, that pteridines are small basic polar compounds able to fluoresce.

2. Experimental

2.1. Chemicals and reagents

Reference standards of NEO (≥97.5%), BIO (≥94%), NH2 (≥97.0%), BH2 (≥94%), and creatinine (CREA; ≥98%) used in this study were obtained from Sigma Aldrich (Prague, Czech Republic). Ammonium acetate (AmAc) (≥99.99%), acetic acid, ammonium hydroxide (aqueous ammonia solution) (≥25%; w/v), all of them LC–MS grade, were purchased from Sigma Aldrich as well as HPLC gradient grade acetonitrile (ACN) (≥99.9%), methanol (MeOH) (≥99.9%) and dithiothreitol (DTT) (≥98%). HPLC grade water was obtained by a Milli-Q reverse osmosis Millipore system (Bedford, MA, USA) and met the requirements of the European Pharmacopoeia.

2.2. Preparation of standard solutions

Reference standard solutions of NEO, BIO, NH2, and BH2 were prepared fresh every day by dissolution in the mixture of ACN and 1% DTT (1:1, v/v) giving a concentration of 10 μg/ml (NEO, BIO) and 100 μg/ml (NH2 and BH2). These solutions were then 10 times diluted with the mixture of ACN/1% DTT (85:15, v/v) corresponding to the composition of the mobile phase. They were kept in the dark and cold ambient (4 °C). Calibration standard solutions were made by a sequential dilution of the reference standard solutions in the range of 10 ng/ml–10 μg/ml (for NH2 and BH2) and 0.5 ng/ml–1 μg/ml (for NEO and BIO) with the mobile phase. CREA solution (100 μg/ml) for SPE was prepared in the same manner as pteridines. The calibration curve of CREA ranged from 500 ng/ml to 100 μg/ml.

2.3. Short term stability

Reference standard solutions of NEO, BIO, NH2, and BH2 were prepared fresh for each experiment by dissolution in the mixture of ACN and DTT/or buffer (1:1, v/v) giving a concentration of 10 μg/ml (NEO, BIO) and 100 μg/ml (NH2, BH2). A short term stability study was carried out by the repeated injection (every hour, $n = 3$) of working solutions of pteridines (NEO, BIO at concentration of 1 μg/ml, and NH2, BH2 at 10 μg/ml) during 24 h. These solutions were prepared from the standard solutions, which were 10 times diluted with the mixture of ACN and DTT/or buffer (85:15, w/v). Ammonium acetate (50 mM; pH 3.8/6.8/9.8) was used as a buffer