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Differences in pteridine urinary levels in patients with malignant and benign ovarian tumors in comparison with healthy individuals

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ABSTRACT

Pteridines belong to a class of fluorescent metabolites that are excreted by humans in urine and their concentrations can reflect various pathophysiological states. We quantified the differences in urinary pteridine levels in patients with malignant and benign ovarian tumors and in healthy individuals. Urine samples were centrifuged and supernatants were oxidized by MnO_2 before analysis. Levels of neopterin, biopterin, and pterin were assessed by fluorescence analysis of human urine after HPLC separation.

We have revealed that the median neopterin levels were higher in urine samples from patients with malignant (0.226 μmol/mmol creatinine) and benign ovarian tumors (0.150 μmol/mmol creatinine) than in healthy subjects (0.056 μmol/mmol creatinine). The median neopterin levels of patients with malignant tumors were higher (1.5-times) than in patients with benign tumors. The median biopterin level in urine of patients with benign ovarian tumors (0.268 μmol/mmol creatinine) was found to be very close to the level in patients with malignant ovarian tumors (0.239 μmol/mmol creatinine), and both were higher than in healthy samples (0.096 μmol/mmol creatinine). The levels of urine pterin followed a pattern similar to neopterin levels for both ovarian tumors, but their concentrations were about three times lower than neopterin levels.

1. Introduction

Pteridines are low molecular weight substances belonging to a class of fluorescent metabolites that are excreted by humans in urine. The naturally occurring pteridine derivatives exist in three different oxidation states: tetrahydro-, dihydro-, and fully-oxidized [1]. Fully oxidized pteridines are fluorescent compounds, while the fluorescence of reduced derivatives is much weaker [2,3].

Scientific data available to-date show that in humans, pteridines are synthesized from guanosine triphosphate (GTP) by GTP-cyclohydrolase I [4]. GTP cyclohydrolase I converts GTP to 7,8-dihydroneopterin-3′-triphosphate. This intermediate is further metabolized by 6-pyruvoyl tetrahydropterin synthase to 6-pyruvoyl tetrahydropterin, which is finally converted by two NADPH-dependent reductions to tetrahydrobiopterin, the active hydroxylating compound. These NADPH-dependent hydroxylations are carried out by sepiapterin reductase [5].

5,6,7,8-Tetrahydrobiopterin has been recognized as the most important unconjugated pteridine existing in biological human fluids [1]. Biopterin and dihydrobiopterin are the oxidative products of tetrahydrobiopterin [6]. In the human organism, tetrahydrobiopterin is involved in the hydroxylation reactions of phenylalanine, tyrosine and tryptophan. It is also an essential cofactor for the biosynthesis of the neurotransmitters dopamine, noradrenaline and serotonin, and of the reactive free radical nitric oxide. In addition, tetrahydrobiopterin also enters the metabolism of lipids as a cofactor of alkylglycerol monooxygenase (also called glyceryl ether monooxygenase, GEMO). GEMO is the only enzyme known to cleave the ether bond in alkylglycerol ether [7]. Tetrahydrobiopterin provides electrons being oxidized to dihydrobiopterin, which is recycled to tetrahydrobiopterin by action of the dihydropteridine reductase.

Nearly all human cells like fibroblasts or endothelial cells produce tetrahydrobiopterin from GTP and only scarce amounts of neopterin species are formed. The only exception being monocytes/macrophages and monocyte derived dendritic cells, in which, due to the low activity of 6-pyruvoyl-tetrahydropterinsynthetase, relevant amounts of neopterin and dihydroneopterin are formed [5].

Several unconjugated pteridines, particularly neopterin, biopterin, xanthopterin, isoxanthopterin and pterin are responsible for blue autofluorescence of human urine [6]. Urinary excretion of these compounds has been found to increase as a result of several disorders such as viral

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infections, intracellular bacteria and parasites, chronic inflammatory disorders, or autoimmune diseases [8,9].

Using various fluorescence techniques, a crucial fact for diagnostics was established: the native blue fluorescence of urine pteridines in oncological patients is different from that in healthy individuals [10–12]. However, those data often came from a mixture of different tissues and tumor types [1,13,14]. Although Han et al. [14] stated that each type of tumor shows its own pattern in changes of pteridine concentrations in urine because different pteridine derivatives may play various roles in different tumor-related diseases, studies where the levels of pteridine derivatives in urine from patients with exclusively one type of tumor are assessed are scarce [15].

Ovarian cancer is the third most common gynecological cancer [16], but there is still lack of studies dealing with the quantification of pteridine levels in patients with ovarian malignancies [17–20], and urine pteridine studies focused on distinguishing between malignant and benign ovarian tumors are completely missing. The quantitative evaluation of pteridines is very important not only for a potential early diagnostics of the disease, but it also helps to explain the differences in metabolism of pteridines in various types of tumors and to tell apart malignant and benign tumors.

In this study, we used high performance liquid chromatography (HPLC) analysis with fluorescence detection for quantitative evaluation of three pteridines (neopterin, biopterin, pterin) in urine of patients with ovarian tumors to establish differences between patients with malignant and benign ovarian tumors and healthy individuals.

2. Materials and Methods

2.1. Chemicals

6-Biopterin, D-erythro-neopterin, pterin, creatinine, methanol and water (HPLC grade) were purchased from Sigma-Aldrich Co. Sodium hydroxide, hydrochloric acid, monopotassium phosphate monobasic and manganese dioxide were obtained from Slavus Ltd. (Bratislava, Slovakia).

2.2. Buffer Preparation

A 10 mmol/l aqueous solution of KH_2PO_4 sample buffer was prepared by diluting 10 ml of 1 mol/l KH_2PO_4 with water and by adding 30 ml of methanol. The buffer was filled with water to a final volume of 1 l and adjusted to pH 4.5 with HCl.

2.3. Standard Preparation

Pteridine stock solutions (0.01% w/v) were prepared by dissolving separately 1 mg of neopterin, biopterin, or pterin in 1 ml 0.1 mol/l NaOH. We kept solutions in an ultrasound bath for 30 s, then we added 9 ml 0.1 mol/l HCl, and kept it in an ultrasound bath for another 30 s. Pteridine working solutions (0.001% w/v) were prepared by diluting 1 ml of each 0.01% pteridine stock solution with 9 ml 0.05 mol/l HCl. Urine standard mixture was prepared by pipetting 324 μl resp. 2530 μl (pterin resp. neopterin and biopterin) of 0.001% pteridine working solutions in a 20-ml flask and filling with 0.05 mol/l HCl to the mark. A dilution series of this mixture were used to generate calibration curves (1, 2, 3, 4 and 5 μmol/l for neopterin and biopterin, respectively; 0.2, 0.4, 0.6, 0.8 and 1 μmol/l for pterin).

Standard creatinine stock solution was prepared by dissolving 45 mg creatinine in 10 ml 0.05 mol/l solution HCl to make the creatinine concentration of 40 mmol/l. This standard stock solution was diluted to an appropriate concentration with 0.05 mol/l solution of HCl (for calibration 5, 10, 20, 30 and 40 mmol/l). The standard solutions were stored at −35 °C.

2.4. Urine Sample Preparation

This study has been cleared by The National Cancer Institute (Slovakia) Ethics Review Board for human study and patients have signed an informed consent. 32 morning urine samples from fasting normal volunteers and 75 patients were used in this study. All of them were analyzed for pH, protein, glucose, bilirubin, nitrate, hemoglobin, ketones, acetone, and urobilinogen. The presence of red blood cells, white blood cells, casts, epithelial cells and crystals was also tested in these samples at the Department of Clinical Biochemistry, National Cancer Institute, Bratislava, Slovakia. Urine samples were taken from patients who did not undergo chemical or radiation therapy, they were taken before the start of anticancer or antibiotic therapy. The control group volunteers did not take any medications including vitamin supplements. The age distribution was 24–77 years for patients and 23–64 years for healthy subjects. The group of oncological patients was represented by 36 patients with confirmed ovarian malignant tumor (ICD 10 code C56) and 39 patients with ovarian benign tumor (ICD 10 code D27). Urine samples were centrifuged at 3000 rpm for 10 min at room temperature (22 ± 1 °C) and undiluted supernatants were used for analysis. All samples were stored in a freezer at −35 °C. Prior to analysis, the samples were removed from the freezer and brought to room temperature.

Human urine samples were subjected to HPLC analysis after MnO_2 oxidation. We pipetted 1 ml of fresh random urine into a centrifugation vial and adjusted pH to 1.0–1.5 with 40 μl of 6 mol/l HCl. After that we added 20 mg of MnO_2 and shook for 5 min at room temperature. Then, we centrifuged for 5 min at 3000 ×*g* and immediately transferred clear supernatant into a vial. The vial was wrapped in aluminium foil to protect its content from light.

2.5. High Performance Liquid Chromatography System

The chromatographic studies were performed on a modular HPLC system Prominence 20A (Shimadzu Co.), equipped with degasser (DGU-20A5), solvent delivery unit with quaternary pump (LC-20AD), autosampler with a sample cooler (SIL-20AC), column oven (CTO-20AC), UV–VIS absorption detector (SPD-20A), fluorescence detector (RF-10AXL), system controller (CBM-20A) and the LC solutions software (1.25 SP1) to control the instrument, data acquisition and data analysis. The analytical columns used were Nucleosil® C18, 150 mm × 3.2 mm, 5 μm particle size (Supelco Analytical).

2.6. High Performance Liquid Chromatography Analysis

Oxidized samples of urine were eluted isocratically with buffer solution (10 mmol/l aqueous solution of KH_2PO_4, 3% methanol) at a flow of 0.6 ml/min. The injected volume sample was 5 μl. After 20 min, when pteridines were eluted, the next sample could be injected. After 5 injections of biological samples, the columns were rinsed with aqueous solution of methanol (1:1) followed by buffer solution, to condition them for the next series of samples. The system temperature was adjusted to 25 °C. Detection was performed with photometric detector at 250 nm for creatinine analysis, and fluorimetric detector at 450 nm (excitation at 350 nm) for pteridine analysis, connected in serial.

2.7. Statistical Analysis

Pteridine concentrations were determined by linear regression against experimentally generated calibration curves by LC solutions software (Shimadzu Co.). The collected data were graphically and statistically processed by the R software. The Mann–Whitney U test was used to compare medians of pteridine urine levels from healthy persons and patients.