Analysis for Fully Oxidized Neopterin in Serum by High-Performance Liquid Chromatography

J. J. Rippin

Fully oxidized d-neopterin in serum can be measured by HPLC. Serum samples were preincubated with ferric nitrate/EDTA solution to remove any dihydroneopterin, which is unstable and may give spuriously high results because of its conversion to d-neopterin. Pterins were extracted onto solid-phase propylbenzensulfonic acid minicolumns and eluted with a 1:5 (by vol) mixture of ammonia solution (308 g/L) in acetonitrile. Extracts were evaporated and then reconstituted in mobile phase (50 mL of methanol per liter of 50 mmol/L phosphate buffer, pH 6.2) before injection. Separation was performed with a 25-cm ODS2 column (particle size, 5 μm) at 32 °C with fluorescence detection (λex 360 nm, λem 440 nm). The between-batch CV was 7.1% and 5.6% for neopterin concentrations of 21.7 and 67.3 nmol/L, respectively. The limit of detection was 0.75 nmol/L, and the mean recovery of the extraction procedure was 90% for neopterin and internal standard. Correlation with a radioimmunoassay (x) gave y = 0.99x + 0.64 (r = 0.970, Sxy = 2.75). The method allows daily analysis of serum d-neopterin in small batches and is currently used to monitor patients undergoing bone-marrow transplant.

Additional Keyphrases: pterins • bone-marrow transplant • monitoring therapy • chromatography, reversed-phase

D-Neopterin belongs to a group of pteridine compounds containing a 2-amino-4-oxo-pyrazine–pyrimidine (pterin) ring. Derived from guanosine triphosphate via a magnesium-dependent cyclohydrolase (1), it is an intermediate in the synthesis of biopterin, an essential cofactor in the synthesis of neurotransmitters (2). Synthesis by macrophages in vivo is stimulated by γ-interferon, and increased serum concentrations are associated with viral and intracellular bacterial infections (3). Increased concentrations of neopterin in serum and urine in transplant patients have been observed to herald episodes of graft rejection (4–7), and daily measurement of neopterin is often used in monitoring such patients. Measurement is commonly used as an early indication of graft-vs-host disease in bone-marrow transplant patients. Determination of neopterin concentrations has also been useful in the management of patients infected with human immunodeficiency virus (8).

Radioimmunoassays for neopterin are expensive and therefore prohibit daily analysis of small batches of samples. Pterins may also be measured by their native fluorescence, and several methods have been described for their determination by HPLC (9–11). Most methods include an acid deproteinization step, which may lead to the degradation of an indeterminate quantity of neopterin; in addition, the increase in fluid volume incurred decreases the assay sensitivity. During the acid precipitation, the relatively unstable dihydroneopterin is converted to the fully oxidized aromatic form; therefore, these methods measure total pterins. Because dihydroneopterin is unstable, its contribution to the total concentrations will be variable; determination of the native stable d-neopterin is therefore preferable. Only one HPLC method purports to measure the fully oxidized species of neopterin, and it requires use of an expensive cartridge module (12).

The following HPLC method for determining fully oxidized neopterin involves a simple low-cost solid-phase extraction of pterins from 100 μL of serum. The method is relatively inexpensive and is suitable for the daily analysis of small batches of pediatric samples. It is currently being used for monitoring patients undergoing bone-marrow transplantation and to assess other forms of immunological activation.

Materials and Methods

Apparatus

Analyses were performed by HPLC with an isocratic PU4100 HPLC pump, a programmable PU4027 fluorescence detector, and PC Chromate Data System (all from Phillips Scientific, Cambridge, UK), and a 250 × 4.5 mm (5-μm particle size) S5ODS2-6616 (fully capped) Excel octadecylsilil reversed-phase column, protected by an ODS2 cartridge guard column (Hichrom Ltd., Theale, Reading, UK). The column was incubated in a Shimadzu column oven and samples were loaded by a Shimadzu SIL-9A automatic sampler (Dyson Instruments, Houghton-le-Spring, UK). Analytchem SCX (propylbenzenesulfonic acid silica sorbent) 1-mL BondElut tubes were obtained from Jones Chromatography (Hengoed, Mid-Glamorgan, UK). Cellulose nitrate filters were obtained from Whatman (Maidstone, Kent, UK).

Reagents

Neopterin and 6-methylpterin were from Sigma Ltd. (Poole, Dorset, UK). All other pterins were obtained from Schirks Labs. (Jona, Switzerland). Radioimmunoassay kits for d-neopterin (comparison assay) were from Henning (Berlin, FRG). I used Analar-grade analytical reagents from BDH Ltd. (Poole, Dorset, UK). All water was distilled, de-ionized, and purified by a Liquipure system (Jencons Ltd., Leighton Buzzard, UK).
**Buffer and mobile phase.** Buffer was 50 nmol/L potassium phosphate, pH 6.0: 4.03 g of potassium dihydrogen orthophosphate, 0.58 g of anhydrous disodium hydrogen orthophosphate, and 0.5 g of sodium azide were dissolved in 500 mL of de-ionized water. The mobile phase—methanol, 50 mL/L of buffer (minus azide)—was de-gassed and filtered under suction through 47-mm, 0.2-μm cellulose nitrate filters and was prepared fresh each day.

**Neopterin standards.** Neopterin, 30 mg, was dissolved in 1000 mL of buffer to give a stock solution of 118.6 μmol/L. The stock standard solution was diluted 100-fold, then threefold, to give 396 nmol/L. Serial twofold dilutions of this standard with buffer yielded neopterin standards covering the range 3–396 nmol/L. Fresh standards were made weekly.

**Other reagents.** For Fe-EDTA solution, I mixed 4.0 g of Fe(NO₃)₃·9H₂O and 3.7 g of EDTA in 100 mL of de-ionized water. The eluent consisted of 200 mL of ammonia solution (308 g/L) per liter of acetonitrile. The internal standard was 6-methylpterin, 70 nmol/L in buffer.

**Procedures**

**Sample treatment.** Pterins are light-sensitive; therefore, standards, controls, and patients' serum samples were kept in the dark and stored at −70 °C. Dihydropteropterin in samples is converted to the fully oxidized form during the eluting step of the extraction and must be removed before the extraction. I added 10 μL of the Fe-EDTA solution to 100 μL of serum and incubated at room temperature for 20 min to eliminate reduced neopterin (12).

**Extraction procedure.** I added 0.1 mL of internal standard to the Fe(NO₃)₃·EDTA-treated preparation, then added 0.8 mL of 0.18 mol/L hydrochloric acid solution.

The SCX Bond-Elut columns were prepared as follows. To clean the columns, I passed 1 mL of methanol through them, under pressure, followed by 1 mL of 0.18 mol/L hydrochloric acid solution to acidify the column in preparation for the sample. I placed 0.95 mL of the treated sample onto the Bond-Elut column and let the sample pass through under gravity. After flushing out any residual sample by applying pressure, I flushed the column with 1 mL of methanol to remove any adherent protein. The pterins were eluted with 1 mL of eluent; the eluate was collected into 3-mL glass tubes and evaporated under air. The sample was reconstituted with 0.1 mL of mobile phase and mixed thoroughly; 20 μL of this solution was injected into the HPLC.

**HPLC.** Mobile phase was pumped isocratically at a rate of 1.0 mL/min, column temperature was maintained at 32 °C, and the system pressure did not exceed 13.0 MPa. Neopterin and 6-methylpterin were measured by their native fluorescence (excitation wavelength 360 nm, emission wavelength 440 nm). Neopterin and 6-methylpterin were eluted with retention times of 3.22 and 12.51 min, respectively. A cycle time of 20 min was allowed between injections.

**Results**

Neopterin and 6-methylpterin were extracted quantitatively from serum on the propylbenzenesulfonic acid silica sorbent, with 95–96% recovery.

Chromatographic peak height and area were calculated by the PC Chromate System and are given in arbitrary units. Figure 1 shows a typical chromatogram of neopterin-supplemented serum. Neopterin concentrations were calculated by the PC Chromate from a standard curve of $N_1/I.S._a$ vs $N_2$, where $N_1$ is the area of neopterin standard peak, $I.S._a$ is the area of internal standard peak, and $N_2$ is the neopterin standard value (nmol/L). Neopterin unknown values were given by $N_u = [(N_u/I.S._a) - N_2]N_u$, where $N_u$ is the neopterin unknown value (nmol/L), $N_u$ is the area of neopterin unknown peak, and $N_2$ is the abscissa intercept of the standard curve, and $N_u$ is the slope of the standard curve.

**Linearity.** Extraction and assay of eight aqueous samples in the range 700–3.6 nmol/L (double dilutions) yielded a dilution curve with $r = 0.9999$.

**Precision.** Daily analyses for 42 days gave between-batch imprecision data (calculated according to recommendations of the U.S. National Committee for Clinical Laboratory Standards (13)) of 7.1% at 21.7 nmol/L and 5.6% at 67.3 nmol/L.

**Correlation study.** Serum neopterin results obtained by HPLC (y) were compared with results obtained with an RIA method (x) for 46 patients' serum samples having values in the physiological and pathological range (5–60 nmol/L). The equation for the regression line was $y = 0.99x + 0.64$ ($S_y = 2.75$).

**Analytical recovery.** A serum with a measured neopterin value of 17 nmol/L was supplemented with neopterin to give values of 52, 66, 84, and 109 nmol/L and measured by HPLC. Recovery of added neopterin by this method was 94%, 91%, 96%, and 92%, respectively.

**Limit of detection.** The minimum signal distinguishable from baseline noise by the PC Chromate System.
corresponded to a neopterin concentration (extracted sample) of 0.75 nmol/L.

Reference range. Blood samples from 22 laboratory workers who had no apparent viral or bacterial infections were analyzed by this method and gave a mean value of 5.3 (SD 2.2) nmol/L.

Interference. Patients undergoing bone-marrow transplant are treated with various pterin-related drugs, including methotrexate and folinic acid. On extraction of a patient's serum, these drugs and their metabolites could become fully oxidized and thus interfere with the assay. I therefore added to serum samples with zero concentrations of neopterin the following metabolites and analyzed for possible interference: \(N^{10}\)-methylfolate, methotrexate (4-amino-\(N^{10}\)-methylpteroylglutamic acid), folinic acid, diaminomethylpterocarboxylic acid, and 7-hydroxymethotrexate. None of these compounds interfered with the assay.

Discussion

The extraction procedure gave a satisfactory yield of neopterin, which extracted proportionally to the internal standard. Use of the mobile phase to reconstitute the extracts minimized solvent-front peaks. The reconstitution volume used afforded minimum change in original sample concentration, thus giving maximum sensitivity. There was no significant difference between the recovery of neopterin and the internal standard in aqueous solution and serum, thus allowing aqueous standards to be used for the assay. The assay settings used gave good resolution of neopterin and monopterin, but poorly resolved biotin and isoxanthopterin. However, serum concentrations of the latter two analytes are not of interest in monitoring bone-marrow patients. Thus I optimized the assay variables to allow a satisfactory daily throughput of samples. Although 6-methylpterin has a relatively long retention time, it is not present in body fluids and was thus deemed suitable as an internal standard.

The assay showed good linearity over a wide analytical range, thus allowing a minimal number of standards to be used. Thermostatic control of column temperature was required to stabilize peak retention times, so that peak data could be processed by the PC Chromat System and used to calculate neopterin concentrations. Serum concentrations of neopterin in healthy subjects were <10 nmol/L. The concentrations in bone-marrow transplant patients several weeks post-transplant are usually 10–20 nmol/L in the absence of graft vs host disease or infection. The assay gives a satisfactory limit of detection, and sensitivity may be improved by applying a larger volume of sample to the column.

Dihydronopterin is unstable in solution, being rapidly degraded to 7,8-dihydroxanthopterin by loss of the aliphatic side-chain (I4). D-Neopterin is very stable and is thus a better analyte for analysis.

In summary: this method represents a low-cost, convenient assay for daily assays of D-neopterin and is suitable for monitoring patients. The extraction does not require the acquisition of an expensive module. By modification of the mobile phase and flow-rate, the method should be adaptable to give a full pterin profile.

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References