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Highly Sensitive, Specific Enzyme-Linked Immunosorbent Assay of Neopterin and Biopterin in Biological Samples

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An enzyme immunosorbent assay of neopterin and biopterin on a polystyrene microtiter plate has been developed. A conjugate of neopterin or biopterin to bovine serum albumin was used to raise a specific antiserum against neopterin or biopterin in rabbits. An incubation mixture of the antiserum and samples prepared from human serum underwent another antigen–antibody reaction with the hapten fixed on the microtiter plate. The amount of antibody bound to the fixed hapten, which is inverse to the amount of hapten in the sample, was determined by using anti-rabbit IgG–horseradish peroxidase conjugate in a usual manner by measuring absorbance at 490 nm after reaction with *o*-phenylenediamine and hydrogen peroxide. The minimal detectable amounts of neopterin and biopterin were ~0.1 pmol. The specificity of the assay was so high that the assay system for neopterin completely distinguished it from biopterin, as judged from the cross-reaction of 0.002%, and *vice versa*. The amounts of neopterin and biopterin in human serum determined by the present method agreed well with those determined by high-performance liquid chromatography. We used the present method to determine the concentrations of neopterin in serum from healthy control subjects and patients with cancers and systemic lupus erythematosus; the results were consistent with literature data.

Additional Keyphrases: *cancer* · *systemic lupus erythematosus*

Neopterin (D-*erythro*-neopterin; 1 in Figure 1) and biopterin (L-*erythro*-biopterin; 3) are biosynthesized from guanosine triphosphate via 7,8-dihydroneopterin triphosphate; their concentrations in body fluids and tissues reflect various physiological conditions (*1, 2*). Biopterin is a metabolite of (6*R*)-5,6,7,8-tetrahydrobiopterin, which is required as the essential cofactor in biosynthesis of the neurotransmitters catecholamine and indoleamine in the central nervous system. There is a close relationship between a deficiency of biopterin cofactor and neurological disturbances such as atypical phenylketonuria (*3, 4*) and Parkinson disease (*5, 6*).

Neopterin is secreted from macrophages on activation with interferon-γ released from T cells (*7*). Thus, neopterin is recognized as a biochemical marker of the activation state of the cell-mediated immune system (*8*) and is useful in the follow-up of cancer, acquired immunodeficiency syndrome, allograft rejection, and other such disorders (*1*).

Determination of neopterin and biopterin is an essential part of biochemical and clinical studies of these diseases. Two popular assay methods, high-performance liquid chromatography (HPLC) and radioimmunoassay, have some drawbacks for these studies. HPLC is sensitive but is not suitable for analyzing many samples at once. Radioimmunoassay has the advantage of being able to analyze many samples simultaneously but involves handling radioactive ligands. Here we report a highly sensitive and specific enzyme-linked immunosorbent assay suitable for determining neopterin and biopterin in human serum; the method is simple and overcomes the above-mentioned problems. A preliminary part of this study appeared elsewhere (*9*).

Materials and Methods

Materials

Specific antisera against neopterin and biopterin. The specific antisera against neopterin and biopterin were prepared in rabbits by using neopterinylcaproyl– or biopterinylcaproyl–bovine serum albumin conjugate as the antigen, as previously reported (*10, 11*). The antisera raised were lyophilized in 20-μL portions and stored at −20 °C. Before use, one portion of lyophilized antiserum was dissolved in 20 mL (2000-fold dilution) of 0.01 mol/L phosphate-buffered saline, pH 7.4, containing normal sheep serum, 100 mL/L; stirred well with kaolin (15 mg); and centrifuged at 900 × *g* for 10 min. The supernate was used for the immunoassay.

Other materials. The N^2-(3-aminopropyl) derivatives of neopterin and biopterin [2, 4] were synthesized as described by Sawada et al. (*12*). The following pterin ligands were also synthesized in our laboratory according to known methods: neopterin and its stereoisomers (*13*) and biopterin and its stereoisomers (*14*).

The 96-well microtiter plate (Falcon 3075) was purchased from Becton Dickinson & Co. (Oxnard, CA); goat anti-rabbit IgG–horseradish peroxidase conjugate from

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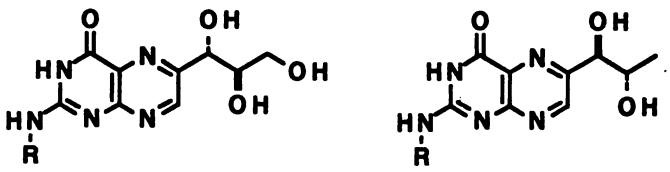

1: R = H

2: R = $(CH_2)_3NH_2$

3: R = H

4: R = $(CH_2)_3NH_2$

Fig. 1. Structures of neopterin [1], biopterin [3], and their N^2-(3-aminopropyl) derivatives [2, 4]

Bio-Rad Co. (Richmond, CA); normal sheep serum from Funakoshi Co. (Tokyo, Japan); poly-L-lysine hydrobromide (M_r 250 000) from Sigma Chemical Co. (St. Louis, MO); and glutaraldehyde, Tween 20, and kaolin from Nakarai Co. (Kyoto, Japan). Dowex 50W-×4 (Bio-Rad Co.) was packed in our laboratory to prepare a 0.5 cm (i.d.) × 3 cm column.

Serum samples. One volume of human serum (0.2–1.6 mL) was mixed with one-fourth volume of 2 mol/L trichloroacetic acid and centrifuged at 900 × *g* for 10 min. The supernate was passed through a 0.5 cm (i.d.) × 3 cm Dowex 50W-×4 column and washed with 10 mL of water. The eluate, obtained by elution with 2 mL of 1 mol/L ammonia, was lyophilized and the residue was dissolved in phosphate-buffered saline (20 μL) before assay.

Procedures

Binding the hapten to the microtiter plate. Each well of a microtiter plate was rinsed three times with 200 μL of 0.01 mol/L phosphate-buffered saline containing Tween 20 (0.5 mL/L) after each step of the following binding and immunoassay processes. The entire procedures were carried out at 24 °C unless otherwise indicated.

To each well of a microtiter plate we added 150 μL of 0.1 g/L poly-L-lysine solution and kept this at 4 °C for 10 h. The plate was incubated with 100 g/L glutaraldehyde reagent (200 μL/well) for 1 h; with the N^2-(3-aminopropyl) derivative of neopterin [2], 10 μmol/L (100 μL), for 1 h; with 10 g/L sodium borohydride reagent (200 μL) for 0.5 h; and finally with 100 mL/L normal sheep serum (200 μL) for 1 h. The plate can be stored at 4 °C for several weeks without changing its characteristics.

The microtiter plate for the immunoassay of biopterin was similarly prepared by using the N^2-(3-aminopropyl) derivative [4] of biopterin.

Immunoassay of neopterin and biopterin. To assay neopterin in serum, we first reacted the sample with a fixed amount of anti-neopterin antiserum. The amount of excess antiserum was determined with the prepared microtiter plate as described below.

We incubated 150 μL of 2000-fold-diluted anti-neopterin antiserum and 20 μL of the sample solution [or 0.01 mol/L phosphate-buffered saline, pH 7.4, containing neopterin standards (from 0 to 100 pmol)] for 1 h. We then transferred 150 μL of the incubated mixture to a well on the plate and let this stand for 1 h. After washing the well (see above), we added 150 μL of goat anti-rabbit IgG–horseradish peroxidase conjugate diluted 2000-fold with 0.01 mol/L phosphate-buffered saline, pH 7.4, and incubated for 1 h. We then added 200 μL of 5 g/L *o*-phenylenediamine solution containing 80 μL of 10 mol/L H_2O_2. After 10 min, we stopped the reaction by adding 50 μL of 6 mol/L H_2SO_4 to the well, and measured the absorbance at 490 nm with an NJ-2000 immuno reader (Japan Intermed Co., Tokyo, Japan).

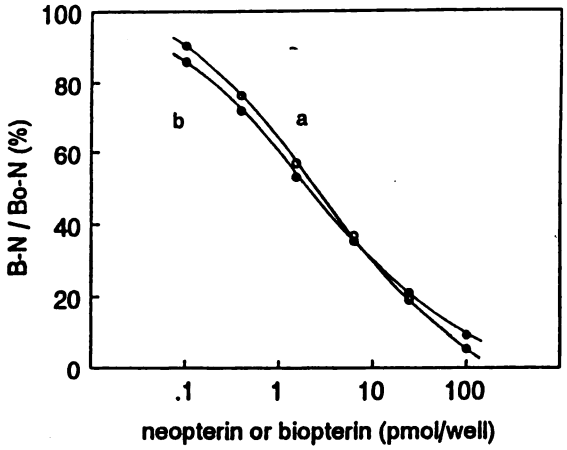

Fig. 2. The standard curves for neopterin (*a*) and biopterin (*b*) by enzyme-linked immunosorbent assay

B, absorbance at 490 nm in the assay system when the specific antiserum was incubated with samples containing various amounts of the hapten; B_0, absorbance at 490 nm in the absence of the free hapten, which corresponds to the maximal amount of the antibody bound to the fixed hapten on the plate; N, absorbance at 490 nm in the absence of free hapten and fixed hapten, owing to nonspecific binding of the anti-neopterin or anti-biopterin antiserum and anti-rabbit IgG–horseradish peroxidase conjugate

The determination of biopterin was carried out similarly with a microtiter plate containing bound biopterin derivative [4], and with use of the anti-biopterin antiserum and biopterin standards (from 0 to 100 pmol).

HPLC. Analytical HPLC to determine neopterin and biopterin in human serum was carried out with a JASCO 880-PU (Japan Spectroscopic Co., Tokyo, Japan), a JASCO FP-210 fluorescence detector (excitation, 355 nm; emission, 450 nm), an SIC Chromatocorder 12 (from GL Science Co., Tokyo, Japan), a 4.6 mm (i.d.) × 250 mm Develosil reversed-phase column (Nomura Chemical Co., Aichi, Japan), and ammonium phosphate buffer (30 mmol/L, pH 3.5) as the elution solvent at a flow rate of 1 mL/min.

Results

Sensitivity and specificity. The measurable amounts of neopterin and biopterin in the present immunoassay ranged from ~0.1 pmol (30 pg) to 100 pmol (3 ng) per well for each, as shown in the standard curves (Figure 2). The sensitivity (detection limit) of the present assay was almost equivalent to those of previously reported radioimmunoassays (*10, 11*). Addition of normal sheep serum to the dilution media of the antisera and treatment with kaolin effectively suppressed the nonspecific binding of the antibodies to the plate. In a typical experiment, the B_0 value, which corresponds to the amount of antiserum bound to the fixed hapten on the

Table 1. Cross-Reaction of Pterin Ligands with Antisera against Neopterin and Biopterin

Ligand	Cross-reactivity, %	
	Anti-neopterin antiserum	Anti-biopterin antiserum
D-*erythro*-Neopterin	100	0.002
L-*erythro*-Neopterin	0.02	0.13
D-*threo*-Neopterin	0.2	0.02
L-*erythro*-Biopterin	0.002	100
D-*erythro*-Biopterin	3	0.003
L-*threo*-Biopterin	0.002	0.3
D-*threo*-Biopterin	0.02	4
Pterin-6-carboxylic acid	0.0006	0.0007
Pterin	0.0001	0.003

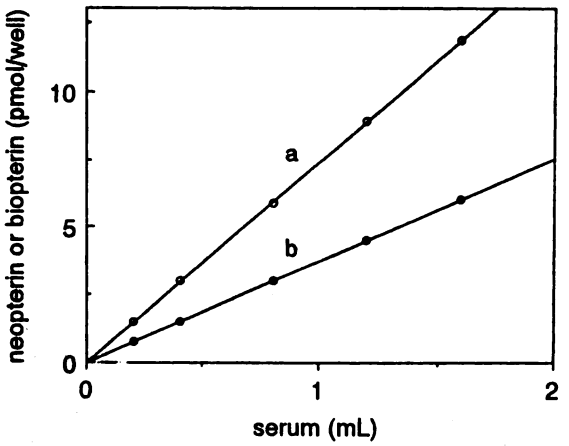

Fig. 3. Linearity studies on the enzyme-linked immunosorbent assay of neopterin (*a*) and biopterin (*b*) in human serum

Appropriate dilution of the lyophilized residue was made so that 20 μL of each sample solution contained serum at the volume shown in the figure

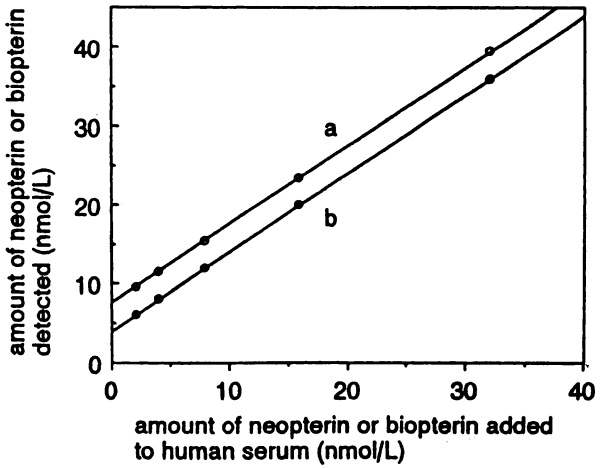

Fig. 4. Analytical recovery studies with the enzyme-linked immunosorbent assay of neopterin (*a*) and biopterin (*b*) added to human serum

Various amounts of neopterin or biopterin were added to human serum, after which the total amount of neopterin or biopterin was measured

Table 2. Concentrations of Neopterin and Biopterin in Human Serum Determined by the Present Immunoassay and HPLC

Conc., nmol/L			
Neopterin		Biopterin	
Immunoassay	HPLC	Immunoassay	HPLC
8.2	8.1	3.5	3.7
6.1	6.5	3.2	3.6
9.5	9.7	2.7	3.1
8.0	7.6	4.9	4.8
8.9	9.0	5.0	4.8
8.5	8.2	4.9	5.0
7.5	7.3	4.0	3.9

Each value is an average of two experiments (SD <0.3).

plate after the antiserum was incubated in the absence of free hapten, was 1.70 at 490 nm. The N value, corresponding to the nonspecific binding of the antiserum, was 0.16.

The percent cross-reaction of various pterin ligands to the anti-neopterin antiserum and anti-biopterin antiserum (Table 1) indicates a high specificity of the present immunoassay. Neopterin and biopterin were almost completely distinguished. This characteristic is important for accurate measurement of the two pterins, because neopterin and biopterin are often found together in many biological samples, body fluids, and tissues. The stereoisomers, including enantiomers, and other pteridines occurring in nature were also well distinguished, as judged from the very low percent cross-reactions.

Linearity and recovery. The amount of neopterin or biopterin was completely proportional to the volume of serum sample assayed, up to 1.6 mL (Figure 3). When a known amount of neopterin or biopterin was added to serum, the analytical recovery was nearly quantitative (Figure 4). The intra-assay and interassay results for determinations of neopterin in a serum sample were 7.3 (SD 0.2) and 7.6 (SD 0.3) nmol/L, respectively (n = 7 each). The intra-assay and interassay results for biopterin were 3.8 (SD 0.4) and 3.5 (SD 0.4) nmol/L, respectively (n = 7 each).

Physiological data and reliability. To prove the reliability of the present method, we also measured the concentrations of neopterin and biopterin in human serum by HPLC–fluorometry. The values determined by the present immunoassay agreed well with the values obtained by HPLC-fluorometry (Table 2).

We used the present method to determine the concentrations of neopterin in serum from healthy control subjects and patients with cancers and systemic lupus erythematosus. The mean (SD) values for the concentration of neopterin in serum (Figure 5) were as follows (nmol/L): control subjects, 6.4 (1.8; n = 18); myeloma, 32.1 (33.6; n = 10); prostatic cancer, 27.8 (20.5; n = 4); hepatoma, 27.4 (21.8; n = 9); lymphoma, 25.8 (26.0; n = 6); lung cancer, 25.8 (11.3; n = 9); ovarian cancer, 16.2 (16.4; n = 6); acute myelocytic leukemia, 15.5 (7.2; n = 4); bladder cancer, 11.1 (6.3; n = 7); acute lymphocytic leukemia, 6.7 (n = 1); and systemic lupus erythematosus, 12.2 (5.1; n = 5).

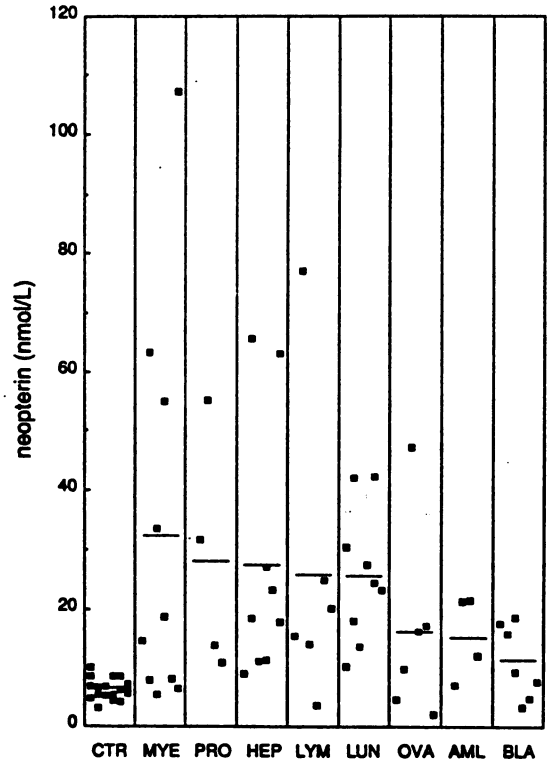

Fig. 5. Concentrations of neopterin in human serum of noncancer control subjects and cancer patients determined by the present enzyme-linked immunosorbent assay

CTR, control; MYE, myeloma; PRO, prostatic cancer; HEP, hepatoma; LYM, lymphoma; LUN, lung cancer; OVA, ovarian cancer; AML, acute myelocytic leukemia; BLA, bladder cancer. The *horizontal bar* indicates the mean value of each column

Discussion

The quantitative analysis of neopterin and biopterin has been tried by various methods (*15*). The classic methods, i.e., gas chromatography–mass spectrometry on silylated biopterin and neopterin (*16*), bioassay with *Crithidia fasciculata* (*17*), and a radioenzymatic assay based on the cofactor activity of the tetrahydro-derivative for phenylalanine hydroxylase (*18*) have largely been replaced by HPLC or radioimmunoassay (e.g., *19, 20*). The main reasons for this are as follows: gas chromatography–mass spectrometry is hardly applicable for the assay of the two pterins in biological samples, and bioassay and radioenzymatic assay are both time consuming and do not exclude other pterins having a polyhydroxyalkyl side chain with the same configuration as biopterin.

HPLC–fluorometry is sensitive and suitable for the analysis of a small number of samples. On the other hand, radioimmunoassay (*10, 11, 21*) can analyze many samples at once with a very high sensitivity and specificity. The serious drawback of radioimmunoassay is the handling of radioactive substances. Polarization fluoroimmunoassay (*12*) can also handle many samples. However, this method is suitable to analyze only biological samples, such as urine, having a high concentration of the pterins, because of the rather low assay sensitivity.

The present enzyme-linked immunosorbent assay for biopterin and neopterin is highly sensitive and can determine the two pterins in concentrations as low as 0.1 pmol/well (~30 pg/well), the same as the concentrations measurable with our radioimmunoassay (*10, 11*). The specificity of the assay is great enough to distinguish D-*erythro*-neopterin or L-*erythro*-biopterin from their stereoisomers, as judged from the percent cross-reactivity of these stereoisomers, shown in Table 1. Also, the anti-neopterin antiserum distinguishes neopterin from biopterin, which cross-reacts by just 0.002%. Similarly, the anti-biopterin antiserum distinguishes biopterin from neopterin with 0.002% cross-reactivity. Because these two pterins are present in various tissues and body fluids in almost equivalent amounts, the utility of the present assay with such high specificity should be particularly emphasized. Many samples (96/plate) can be measured in the same assay with this method, which should be particularly useful when screening many samples in clinical chemistry.

Increased concentrations of neopterin in urine and other body fluids have been reported in various diseases (*1, 15*). Because the assessments of native neopterin and total neopterin (neopterin and reduced derivatives) are of almost equal diagnostic potential (*22, 23*), only the native neopterin was measured in the present study, to make the procedure less laborious. As shown in Figure 5, the neopterin concentrations in serum from myeloma, prostatic cancer, hepatoma, lymphoma, and lung cancer patients were four- to fivefold higher than the control value ($P < 0.005$). Patients with other cancers such as ovarian cancer, acute myelocytic leukemia, and bladder cancer all exhibited lower values: only 1.5- to 2.5-fold that of the control value; nonetheless, the P-values were <0.01. Patients with systemic lupus erythematosus also exhibited significantly high ($P < 0.005$) neopterin concentrations in serum, as reported elsewhere (*20*).

In a separate experiment, we used the present method to measure the total neopterin (native neopterin plus its reduced derivatives) in serum of healthy control subjects and patients with various types of cancer (data not shown). In contrast to the observations previously reported and also to our present observation, we saw no significant correlation between the serum concentrations of neopterin and these forms of cancer (control value: 14.5 nmol/L). Perhaps the patients with the malignant diseases we studied have low concentrations of reduced derivatives of neopterin in serum.

We conclude that the present method is very useful for determining serum neopterin concentrations, which is difficult by other methods because of the low concentrations involved. However, the procedure for sample preparation is still cumbersome and needs to be improved.

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