

## ENZYME-LINKED IMMUNOSORBENT ASSAY OF NEOPTERIN USING PENICILLINASE AS LABEL

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### ABSTRACT

An enzyme-linked immunosorbent assay for neopterin using penicillinase as marker enzyme is reported here by polyclonal antibodies against neopterin conjugated to bovine serum albumin which were raised in rabbits. Immunoglobulin fractions were purified and coated on wells of microtiter plates. A chain heterology was introduced in neopterin derivative and conjugated to penicillinase. The assay is completed within 4 hr. The limit of detection was 10 pg of neopterin with a sensitivity range between 15-10000 pg. A low level of cross-reactivity with other pteridines was noted (biopterin <0.05%, pteric acid <0.05%, and pterin <5%). The sensitivity and selectivity observed in the assay may be attributable to the selection of penicillinase as the enzyme marker and the element of conformation (heterology between the antigen-linked and enzyme-conjugated hapten).

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### INTRODUCTION

Neopterin is a pyrazino-compound derived from GTP. It represents an intermediate product in the synthetic pathway of biopterin. Neopterin is formed by human monocytes/macrophages upon stimulation by interferon gamma which acts on the enzyme cyclohydrolase I.<sup>1</sup> Hence the measurement of neopterin concentration provides an immediate, sensitive and reliable method to monitor activation of T-cells and macrophages.<sup>2,3</sup> Recent studies have also emphasized the importance of serum neopterin measurement in blood transfusion/blood screening.<sup>4</sup> The clinical study of neopterin involved measurement of urinary neopterin content by high performance liquid chromatography (HPLC)<sup>5</sup>, radioimmunoassay (RIA),<sup>6,7</sup> and enzyme immunoassay

(EIA).<sup>8-11</sup> In developing a rapid and practical assay using enzyme as label, one factor is the stability of enzyme and color development. Penicillinase ( $\beta$ -lactamase; EC: 3.5.2.6) was found to be a suitable enzyme for this purpose.<sup>12</sup> The use of penicillinase as a marker enzyme in EIAs has been reported for several steroids and protein hormones.<sup>13-15</sup> So far, however, this enzyme has not been used for measuring the concentration of neopterin. We describe here a competitive, fast and sensitive solid phase heterologous enzyme immunoassay for neopterin measurement in human plasma, utilizing penicillinase as a marker enzyme.

### MATERIAL AND METHODS

Penicillinase ( $\beta$ -lactamase; EC 3.5.2.6.), neopterin, bovine serum albumin (BSA), pterin, pteric acid, biopterin, folic acid, penicillin V, 4-(N-maleimidomethyl)-

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cyclohexane 4-carboxylic acid, N-hydroxy-succinimide ester (MCH-NHS), dichloroethane (DCE),  $\text{NaBH}_4$ , glutaraldehyde (pure), Sephadex G-25, starch and iodine were obtained from Sigma Chemical Company, St. Louis, MO.

### Preparation of neopterin-BSA conjugate

Neopterin was conjugated to BSA following the procedure explained by Kitagawa et al.<sup>16</sup> with a minor modification as follows. Neopterin (2.5 mg, 50  $\mu\text{mol}$ ) was dissolved in 500  $\mu\text{L}$  of bicarbonate buffer (50 mmol, pH= 10). MCH-NHS (3.4 mg, 50  $\mu\text{mol}$ ) was dissolved in 100  $\mu\text{L}$  of THF. The above two solutions were mixed together and stirred at 30°C for 2 hr. Solvent was removed by nitrogen gas flushing and unreacted MCH-NHS was removed by extracting with DCE (3 mL  $\times$  3 times). Aqueous phase was used for the next steps (product I).

Ten milligrams of BSA was dissolved in 0.5 mL of bicarbonate buffer (50 mmol, pH= 10). Urea was added to the above mixture such that a 6 molar solution is obtained with final volume adjusted to 1 mL. A molar concentration of EDTA was added to the above mixture to obtain a 0.1 mol solution. Ten milligrams of  $\text{NaBH}_4$  and 0.2 mL of normal butanol was added subsequently and slowly. The reaction mixture was stirred at room temperature for 1 hr. To stop the reaction 0.5 mL of sodium monophosphate (0.1 mol) and 0.2 mL acetone was added (product II).

Finally the two products were mixed together and

Table I. Cross-reaction of anti-neopterin antibody with structurally related pteridines.

SN	Compound	% Cross-reaction
1	Neopterin	100
2	Biopterin	<0.05
3	Pteric acid	<0.5
4	Pterin	<5
5	Folic acid	<0.05

SN: sample number

stirred for 3 hr at room temperature and then incubated overnight at 4°C. At the end of the incubation period the products (Fig. 1) were dialyzed against three changes of phosphate buffer (10 mmol, pH=7.4) in eight hour intervals, freeze-dried and stored at 4°C until use.

### Antibody production and purification

Polyclonal antibodies were produced against neopterin-BSA obtained in the previous reaction, following the method explained by Vaitukaitis et al.<sup>17</sup> The antisera obtained were characterized for titer, specificity and affinity as explained elsewhere.<sup>18</sup> Immunoglobulin fractions were purified following the procedure of Levey and Sober.<sup>19</sup>

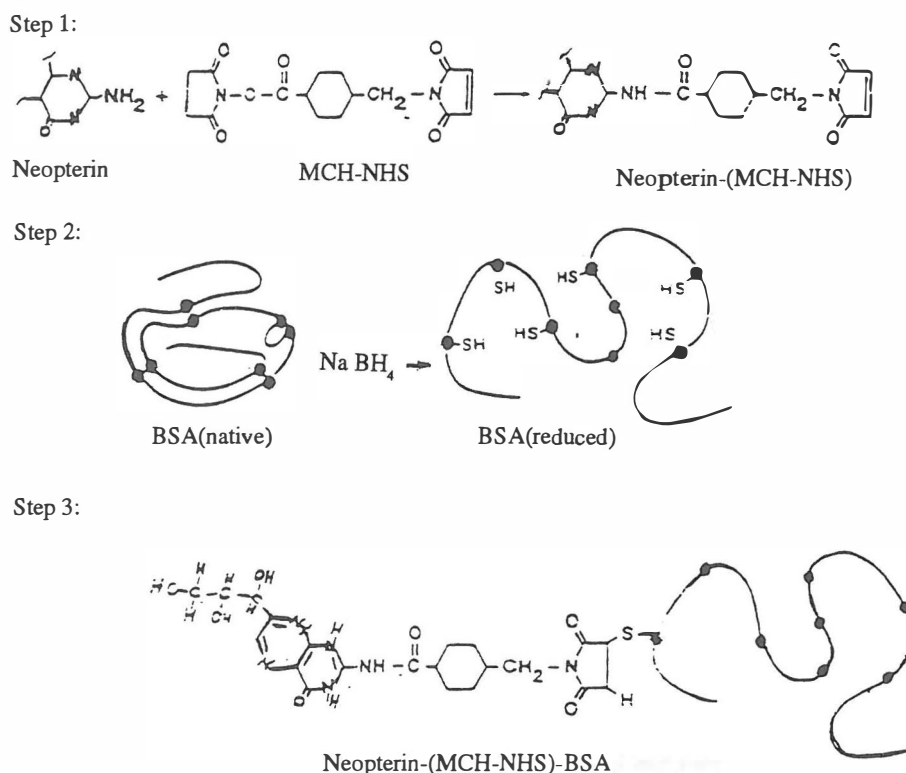


Fig. 1. Preparation of Neo-BSA using MCH-NHS as linker.

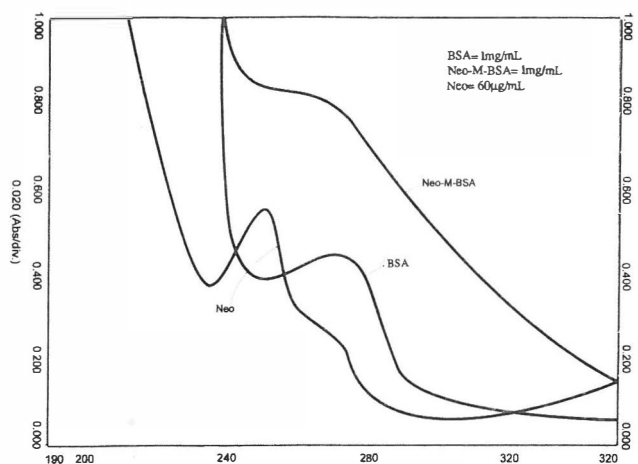


Fig. 2. Spectrophotometric spectrum of neopterin, NEO-M-BSA and BSA in the range of 200-350 nm.

**Preparation of neopterin-penicillinase conjugate**

To introduce a chain heterology, penicillinase was conjugated to neopterin following a one-step glutaraldehyde procedure, briefly as follows: neopterin (60 µg, 10 mmol) was dissolved in 1 mL of bicarbonate

buffer (50 mmol, pH= 10), and 200 µg of penicillinase type II was added to this solution and stirred slowly in the dark until a clear solution was obtained. Finally 50 µL of a 1% glutaraldehyde solution was slowly added to the reaction mixture and incubated at 30°C for 2 hr. The product was dialyzed at 4°C overnight against PBS (10 mmol, pH= 7.4), or chromatographed on Sephadex G-25 as described previously.<sup>18</sup>

**Enzyme-linked immunosorbent assay**

**A) Checkerboard titration assay**

To obtain the optimum titer of antibody and enzyme conjugate, different concentrations of purified anti-neopterin IgG were prepared in PBS (10 mmol, pH= 7.4, containing 0.1% NaN<sub>3</sub>), and coated onto the wells of microtiter plates, leaving a sufficient number of wells for corresponding antibody concentrations and non-specific binding (NSB), which were coated with normal rabbit serum. Plates were incubated at 37°C overnight until the antibody solution was completely dried. Wells were washed 4 times with 10 mmol PBS, pH= 7.4 and tapped. Remaining sites were blocked with a 0.3% solution of gelatin, washed and kept until use. Different dilutions of enzyme-neopterin conjugate were prepared in PBS containing 0.1% gelatin

**Table II. Analytical recovery of neopterin added to immunostripped serum using Neo-G-Pen. Results of 6 experiments for each concentration.**

CV%	High (10 ng/well)	CV%	Medium (1 ng/well)	CV%	Low (0.01 ng/well)
9.5	105±10	73	95±7	7.5	98±1.2

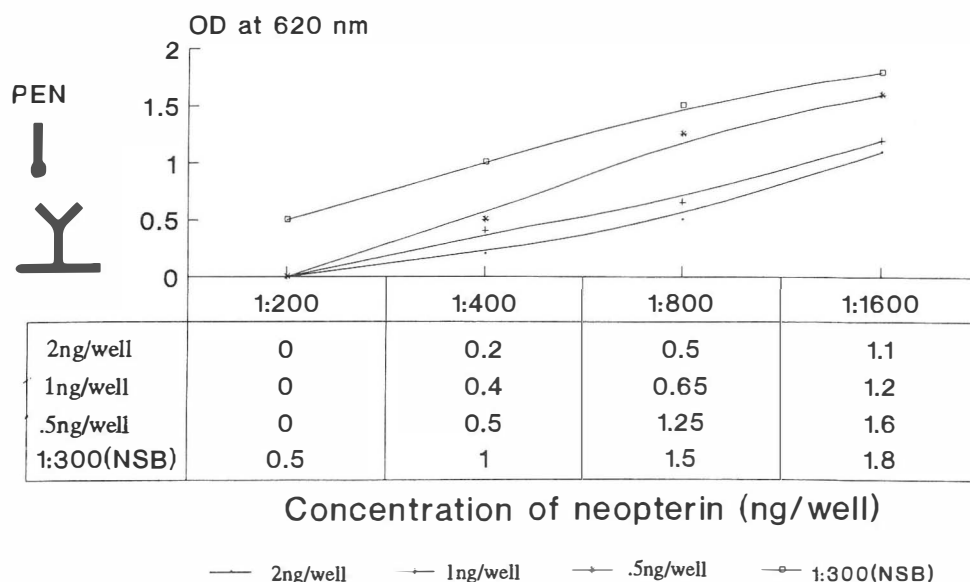


Fig. 3. Titration assay of rabbit anti-NEO-M-BSA using NEO- G-PEN in serum.

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Table III. Precision profile

CV%	Pool C Mean±SD (10 ng/well)	CV%	Pool B Mean±SD (1 ng/well)	CV%	Pool A Mean±SD (0.01 ng/well)	N	Exp N
<b>intra-assay</b>							
1	5	0.34±0.03	8	0.66±0.03	4.5	0.76±0.06	7.8
1	5	0.31±0.02	6.4	0.63±0.07	11	0.72±0.07	9.8
2	5	0.36±0.05	13.8	0.67±0.06	8.9	0.77±0.05	6.5
3	5	0.35±0.03	8.5	0.65±0.03	4.6	0.79±0.4	5
4	5	0.33±0.02	6	0.7±0.05	7.1	0.78±0.06	7.6
5	5	0.37±0.025	6.7	0.63±0.04	6.3	0.75±0.07	9.3
<b>inter-assay</b>							
1	5	0.34±0.029	8.28	0.65±0.045	7	0.76±0.08	7.5

N= number of times each sample was analyzed for inter-assay variation.

Table IV. Neopterin concentration as measured by ELISA developed in our laboratory.

SN	Male nmol/L	Female nmol/L	Hepatitis B nmol/L	Cirrhosis nmol/L
10	4.2-7.2	-	-	-
10	-	4.1-7.5	-	-
15	-	-	8-12	-
10	-	-	-	7-8

SN= Sample number

(10 mmol, pH= 7.4) and added to each well except the two wells representing total optical density. Plates were incubated at 37°C for 2 hr, decanted, washed, and substrate solution (2.8 mg/10 mL of 0.2 mol PB, pH= 7.2) was added to each well and incubated for 1 hr at 37°C.

At the end of incubation time, color reagent<sup>18</sup> was added and incubated for 10-20 min. The reaction was stopped by adding 50 µL of a 5N HCl solution. The color was read at 620 nm on an ELISA reader (Titertec. MS220).

### B) Standard assay

Appropriate amounts of antibody were coated onto each well of the microtiter plate as discussed before. Different concentrations of neopterin were prepared in PBS (10 mmol, pH= 7.4 containing 0.1% gelatin), added to pre-coated wells, and incubated for 1 hr. A suitable amount of neopterin-enzyme conjugate was added,

incubated at 37°C for 2 hr, washed, tapped, and treated as explained in the previous section.

### Validation Assay

#### A) Cross-reactivity of the antibody

Structurally related pteridines were run in parallel with standard assay and percent cross-reactions were calculated as explained before.<sup>18</sup>

#### B) Recovery

Serum samples were stripped off endogenous neopterin by immuno-stripping procedure and supplemented with three ranges of neopterin (low, medium and high concentrations); the amount of neopterin content of these samples was determined in replicates and number of occasions.

#### C) Inter- and intra-assay variations

Samples from recovery experiments were performed in various ways in a number of occasions.

### Sample measurements

Samples from apparently normal subjects were collected and measured for neopterin content. In addition, 15 samples from prediagnosed hepatitis patients and 10 from cirrhotic patients were examined.

## RESULTS

Figure 2 shows the spectrophotometric analysis of the immunogen prepared in this study. The results indicated

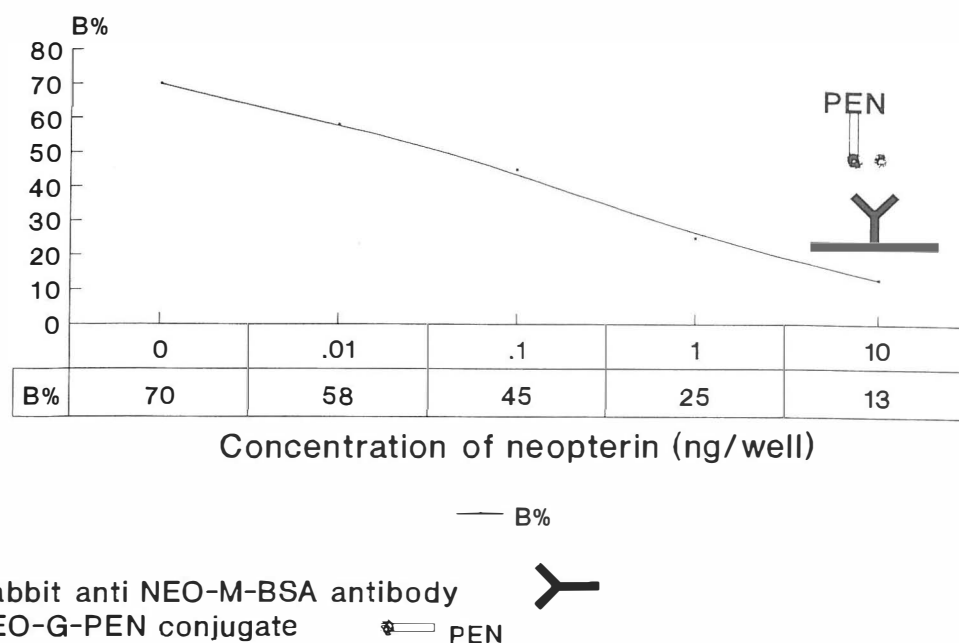


Fig. 4. Competitive standard curve of anti-NEO-M-BSA and NEO-G-PEN by ELISA in buffer.

that around 16-18 molecules of neopterin were conjugated to each BSA molecule. These were calculated as explained before.<sup>18</sup>

Figure 3 is the result obtained from titer assay experiments (checker-board). In these experiments the optimum concentration of antibody was found to be 1  $\mu\text{g}/\text{well}$  and the dilution of enzyme conjugate 1: 800.

Figure 4 is the result of several experiments performed in various occasions. The coefficient of variation between each point was calculated and found to be within the acceptable range. The affinity of the antibody toward neopterin was calculated to be  $K_a = 0.5 \times 10^8 \text{ L/mol}$ .

Table I represents our results obtained for antibody cross-reaction with structurally related pteridines. These results show a minor cross-reaction of antibodies obtained in these experiments with the pterin, and no considerable cross-reaction with other important pteridins.

Table II represents our results in recovery experiments in which 95-100% of exogenously added neopterin was recovered.

In Table III results of inter- and intra-assay variation are being shown in which each concentration was tested in five replicates and repeated six times on different occasions. The coefficient of variation in these experiments was calculated to be within the acceptable range of statistical permission (6.3 to 10).

Finally, in Table IV samples from normal, established hepatitis, and cirrhotic patients were measured for neopterin. The results indicated that the normal range of neopterin is 4.2-7.2 nmol/L and does not differ in male or female samples, while in cases with established hepatitis and cirrhosis, values were higher than normal subjects.

## DISCUSSION

The biological significance of neopterin measurement is the subject of many recent investigations.<sup>20</sup> The quantitative analysis of neopterin has been attempted by various methods such as gas chromatography mass spectrometry,<sup>21</sup> radio enzymatic assay based on the cofactor activity of the tetrahydro derivative for phenylalanine hydroxylase,<sup>22</sup> bioassay with *Crithidia fasciculata*,<sup>23</sup> and HPLC.<sup>5</sup> However, these methods have largely been replaced with immunoassay, having the capability of easy use in clinical laboratories with large samples to be measured in every occasion. The first immunoassay represented a form of RIA,<sup>6</sup> with considerable sensitivity and specificity. However, due to the hazard encountered in the use of radioactive material, non-radiometric immunoassays such as fluoroimmunoassay (FIA)<sup>24</sup> and EIA<sup>8-11</sup> came to use. Although FIA replaced RIA, the sensitivity of this assay is not sufficient to measure the neopterin content of serum samples. Therefore a suitable alternative method would be EIA, which held the same sensitivity and specificity as represented in RIA.

In this report we developed a simple and sensitive enzyme-linked immunosorbent assay using a heterologous system in which immunogen (neopterin-protein conjugate) is prepared by a novel method using MCH-NHS as cross-linker, while for the enzyme conjugate glutaraldehyde was used as cross-linker. Although it was suggested by Matsuura et al.<sup>25</sup> that glutaraldehyde could not be used as a cross-linker for neopterin-protein conjugation, we were able to perform this experiment repeatedly and successfully. We observed that this heterology is essential

for the assay to be sensitive (10 pg/well) and specific (less than 5% cross-reaction with related pteridins). As we mentioned earlier, this is the first time that penicillinase has been used as a method for neopterin measurement. We observed the suitability of this enzyme in our experiments indicating that penicillinase may be used in the commercial preparation of test kits for various molecules such as neopterin.

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