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Original Research

Comparison of two different HPLC methods and ELISA method for measurement of serum neopterin

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Abstract

Neopterin is a pyrazinopyrimidine compound, which has been used as a biomarker of cellular immune system. It is secreted as a result of activation of cellular immune system, primarily by monocytes, macrophages and dendrytic cells with the stimulation of interferon gamma. Various chromatographic methods have been used for measurement of neopterin. HPLC is widely used instrument for this purpose. In this study, we compared the serum neopterin levels with two HPLC method and commercial neopterin ELISA kit. HPLC method using potassium phosphate buffer (pH: 6,4) as mobile phase and trichloroacetic acid for deproteinization was named as "method 1"; and the method which we have recently developed using water/acetonitrile (99/1, v:v) as a mobile phase and acetonitrile for deproteinization, was named as "method 2". Neopterin retention time was 6,4 min. for method 1 and 2,4 min. for method 2. The linearity of method 1 and method 2 was quite good (r2: 0.987 and r2: 0.998, respectively). Repeatability of method 2 was higher than method 1 and commercial kit. In conclusion, our developed HPLC method has better analytical performance and shorter analysis time and easier to apply with respect to the HPLC method 1.

INTRODUCTION

Neopterin is a biomarker derived from pteridine which has not a specific receptor and is secreted from human monocytes and macrophages with the effect of interferon gamma (IFN-γ) [1, 2]. Neopterin is formulated as 2-amino-4-oxo-6 (1',2',3'-hydroxypropyl). Its chemical formula is stated as C9H11N5O4 and molecular weight is 253 Dalton [3]. It has 4 isomers (D-eritro, L-eritro, D-treo, L-treo) and biologically synthesized from guanosine triphosphate. Neopterin is considered to be a biological marker for macrophage and monocyte activation [4].

Serum neopterin levels increase in some conditions such as various infections, sepsis, autoimmune disorders, malignancies, allograft rejection, sarcoidosis, tuberculosis, activation of multiple sclerosis, coronary artery disease, myocardial infarction, viral infections (human immunodeficiency virus (HIV) infection, hepatitis C), central nervous system disorders of children, kidney, liver, heart, pancreas and bone marrow transplant rejections [5-9]. Neopterin is considered to be a biochemical marker of activation of cellular immune system [10].

Neopterin measurement is performed by various methods such as high performance liquid chromatography (HPLC), radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), electrophoresis, thin layer chromatography, paper chromatography. Today, HPLC is considered to be the gold standard method all over the world [11, 12].

In this study, we aimed to compare the performance of two different HPLC methods and neopterin ELISA kit.

MATERIALS AND METHODS

Chemicals

Neopterin and sodium hydroxide (NaOH) were purchased from Sigma (St.Louis, MO, USA). Potassium dihydrogen phosphate (KH2PO4), dipotassium hydrogen phosphate (K2HPO4), methanol, isopropanol, acetonitrile, trichloroacetic acid (TCA) and phosphoric acid (H3PO4) were purchased from Merck (Darmstadt, Germany).

Equipment

The HPLC System was an Agilent Model 1200 consisting of a quadratic pump, a degasser, thermostat and an auto sampler. The fluorescence detector was an Agilent Model 1200 operating at an excitation wavelength of 353 nm and emission wavelength of 438 nm. The data were analyzed with the Chemstation program for Windows XP operating system.

METHOD

Blood samples were collected by venipuncture, and immediately centrifuged, at 4000 g for 10 min at 4°C, for serum separation. Then, the samples were stored at −80°C until analysis. Samples were protected from light during these steps.

Two different sample preparation methods were employed for neopterin measurement with HPLC. First method was described by Cayci et al. [13] was called as method 1. Briefly, for acid precipitation of protein by adding 500 µL of serum, 100 µL of 2 M TCA followed by vortexing then centrifugation (4 ◦C and 10,000 × g for 10 min); 100 µL of the acid supernatant was then placed in an autosampler vial for HPLC analysis. Second method which we developed was called as method 2. We employed acetonitrile to precipitate the protein. 100 µL of serum was combined with 100 µL of 100% acetonitrile, vortexed and centrifuged (4 ◦C and 10,000 × g for 10 min). 100 µL of the supernatant was then transferred to an autosampler vial for HPLC analysis.

For method 1, neopterin standards were diluted with distillated water. For method 2, these standards were diluted with acetonitrile.

For the comparison of the results obtained by HPLC, serum neopterin concentrations were assayed in the same samples, by an ELISA kit (DRG, Germany) according to the manufacturer's test procedure.

Liquid chromatography

Column

Samples performed on an ODS-3 C18 analytical column (150 X 4.6 mm, 5 µm particle-size, Hichrom), protected with a Phenomenex C18, 5 µm guard column.

Mobile phases

For method 1 the mobile phase was potassium phosphate buffer, pH 6.4, at a flow rate 0.8 mL/min. For method 2 the mobile phase was water/acetonitrile (99/1, v/v) at a flow rate 1 mL/min. The fluorescence at 353 nm of excitation and 438 nm of emission was detected.

Comparison of methods

For method comparison, 100 serum samples in different neopterin concentrations (2.79 - 54.2 nmol/L) were used. Neopterin levels of the serum samples were determined by three methods. Kruskal-Wallis one-way ANOVA t-test for paired samples was used for the comparison of serum neopterin levels measured by HPLC and ELISA. The statistical package SPSS for Windows, version 15.0 (Chicago, IL, USA), Microsoft Excel and EP Evaluator Release 8 (Canada, USA) was used for statistical procedures.

RESULT AND DISCUSSION

The representative chromatograms for serum neopterin assay from a serum sample and neopterin standard material containing 30.1 nmol/L neopterin for 2 methods are shown in figure 1 and figure 2.

For method 1, the retention times in minutes were 6.38 for serum sample and 6.42 for neopterin standard sample. For method 2, retention times were 2.21 for serum sample and 2.28 for neopterin standard sample.

The accuracy of method 2 was assessed by comparison with reference HPLC method. We thought method 1 as reference HPLC method in this study. Serum samples from 100 volunteers were analyzed and neopterin concentrations were determined. Passing–Bablok regression analysis figure 4(A) showed a linear relationship between the two methods, $y= 0.9572x + 1.1353$, $r^2= 0.972$. The Bland–Altman test shown in figure 4(B) was obtained by plotting the difference in neopterin concentration measured by our and by the reference HPLC method, against the average of the two values.

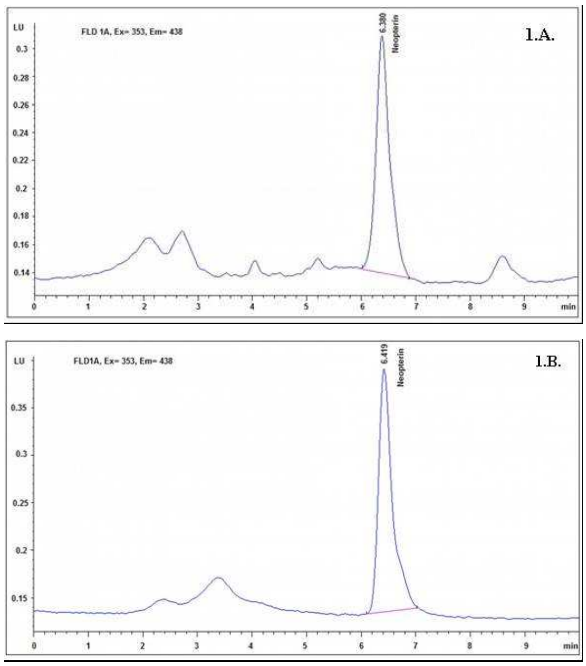

Figure 1. Chromatograms obtained by method 1. **A.** Chromatogram of serum containing 21.5 nmol/L neopterin (RT: 6.38 min.) **B.** Chromatogram of neopterin standard material containing 30.1 nmol/L neopterin (RT: 6.42 min.). Chromatograms were obtained by method 1. RT: retention time

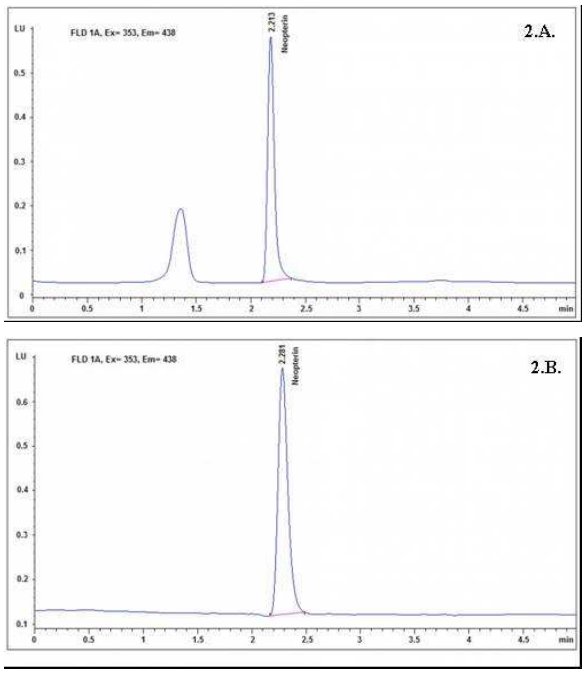

Figure 2. Chromatograms obtained by method 2. **A.** Chromatogram of serum containing 21.5 nmol/L neopterin (RT: 2.21 min.) **B.** Chromatogram of neopterin standard material containing 30.1 nmol/L neopterin (RT: 2.28 min.). Chromatograms were obtained by method 1. RT: retention time

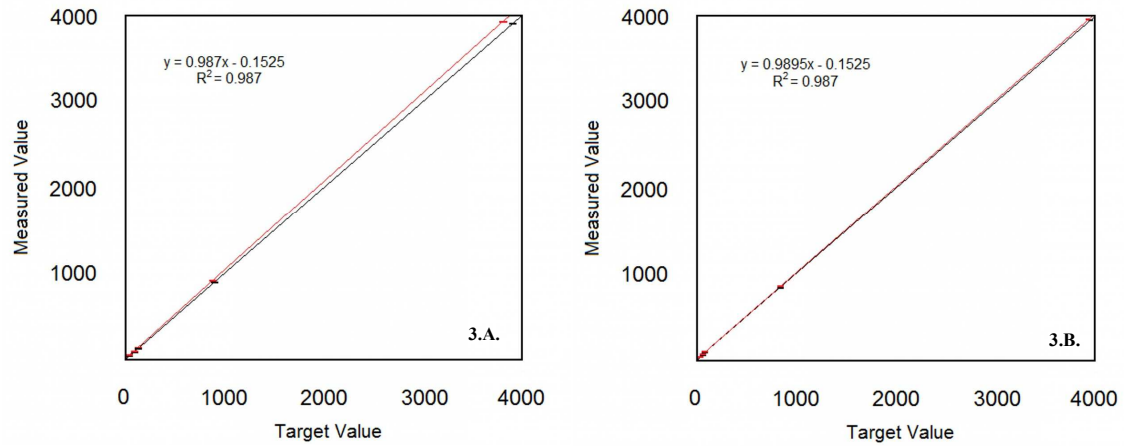

Figure 3. Linearity curves **A.** Method 1 and **B.** Method 2

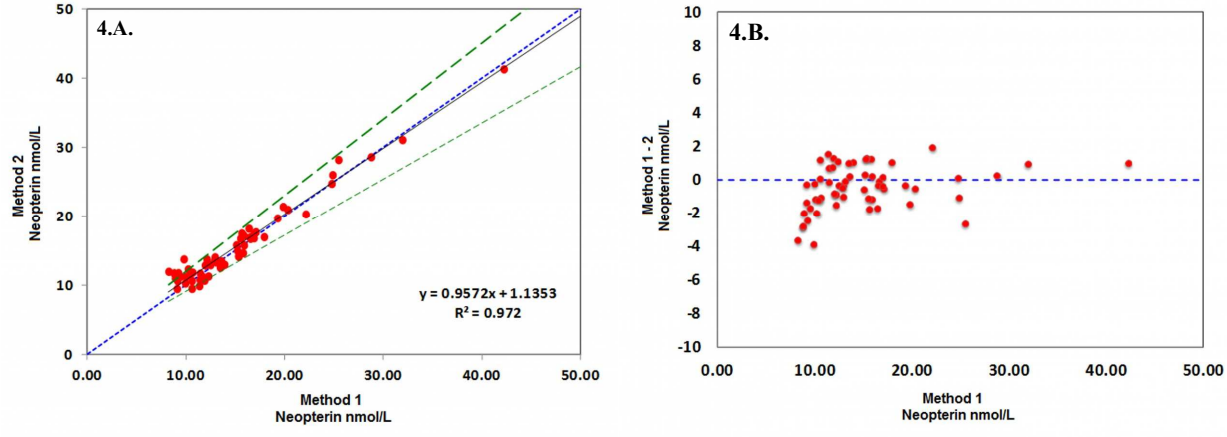

Figure 4. The comparison of method 1 and method 2. **A.** Passing-Bablok regression analysis **B.** Bland-Altman test.

Two HPLC methods and ELISA method also were compared with EP Evaluator software according to multiple instrument comparison (Figure 5). Total allowable error (TEa) was accepted as 20% and error index is measured. Error index is the ratio of difference (results - target) to TEa. The error index is measured for each specimen for each instrument. An index is greater than 1.00 or less than -1.00 is unacceptable – it means the difference between the instrument and the target exceeds TEa. According to this analysis, there were some values which belong to method 1 and ELISA method, exceeded allowable error.

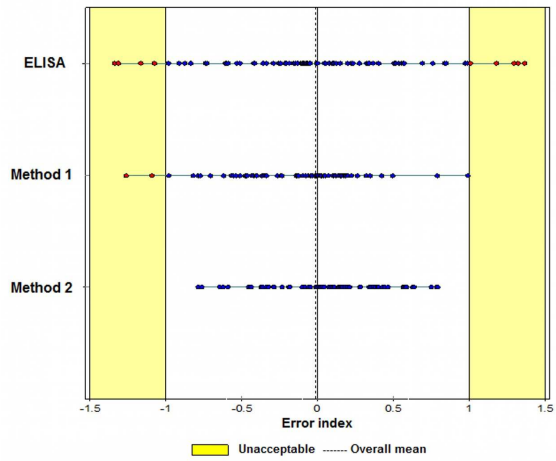

Figure 5. Error index by instrument graphic for method 1, method 2 and Elisa method. In method 1 and Elisa method, there are unacceptable values.

Several analytical methods including HPLC, RIA, and ELISA have been reported for the measurement of neopterin and such compounds in serum. Among these HPLC with fluorometric detection is the most widely used technique.

The methods described in our study are well suited for analysis of neopterin in serum. Our estimates of intra-assay and inter-assay reproducibility of three methods are acceptable at both apparently healthy subjects and high neopterin concentrations. Although, assay reproducibility and linearity of three methods are acceptable, method 2 had the best method validation parameters among all.

The use of acetonitrile for removing serum proteins, markedly improved the signal to noise ratio seen on chromatogram compared to TCA treatment (Figure 1, 2). In parallel of our findings Flavall et al. [14] showed the same results. The neopterin peak identity was confirmed by spiking serum samples with neopterin standard sample. Peak areas of neopterin were approximately the same in acetonitrile and TCA treatment methods while Flavall et al. thought TCA method were higher. This might be because of removal of unknown amounts of neopterin together with the proteins while removal of serum proteins by an acid precipitation step [15].

In method 2, the neopterin peak obtained with water/acetonitrile (99:1, v/v) as eluent which was used also by Carru et al. [16] was completely resolved from impurities in less than 5 min as shown in Figure 2. Besides, with potassium phosphate mobile phase, retention time was about 6.4 min as shown in Figure 1. This provided shorter analyze time as 5 min. vs. 12 min. for method 1. Moreover the preparation of mobile phase is simpler because pH adjustment and buffer filtration were not required.

The best intra-assay and inter-assay replicate sample measurements among the three methods were consistent with method 2. Also method 2 showed the best performance when applying three method comparison tests with EP Evaluator software.

Neopterin limit of detection was 0.2 nmol/L was consistent with other studies [17, 18].

In conclusion, we develop a new fast and easy method for serum neopterin measurement. The use of acetonitrile rather than acidic conditions to precipitate and remove serum proteins provided a more accurate neopterin result in samples. Water/acetonitrile mobile phase provided shorter analyze time and better chromatogram according to peak shape and baseline noise. Also, analytical method validation performance of our new method was better than the other methods.

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