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CHEMICAL SCIENCES

Determination of neopterin in urine of industrial workers by HPLC

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Abstract: This study aimed to determine neopterin levels in the urine of industrial workers by the high-performance liquid chromatography method. Intra- and inter-day precision values for neopterin in urine were less than 3.14, and accuracy (relative error) was better than 3.00%. The limits of detection and quantification of neopterin were 0.3 and 1.0 ng/mL, respectively. Also, the developed method was applied to real samples to determine the neopterin levels in the urines of industrial workers, who have been exposed to various chemicals such as formaldehyde, heavy metals and thinners. Urine neopterin levels of industrial workers including auto painters, bodywork and furniture workers were statistically compared with healthy volunteers. The highest and lowest values of urinary neopterin for industrial workers were obtained 908.96 and 119.86 μmol/mol, respectively. Our investigation demonstrates that there is a meaningful difference in urinary neopterin levels between the workers and the control groups (P<0.05). Workers in the auto paint, body and furniture business may have been exposed to a toxic environmental exposure in their occupation. As a result, an increase in the concentration of neopterin in the urine may be important in the diagnosis and treatment of various diseases.

Key words: Neopterin, biomarker, urine, HPLC.

INTRODUCTION

Neopterin (Figure 1) is a pyrazino-pyrimidine compound that is a member of the pteridine group. It is a well-established biochemical marker that provides information about the activation of the cellular immune system (Hamerlinck 1999). With more investigations in recent years, the biological roles of neopterin and its derivatives have come to be well known. In addition, oxygen, which is used as an electron acceptor by many enzymes, causes the formation of unpaired electron-carrying reactive oxygen compounds (ROC), such as hydroxyl radical or superoxide ion. ROC rapidly attacks cellular macromolecules, causing cell damage. Therefore, to control excessive oxidant activity, cells apply a series of antioxidant defense mechanisms consisting of effective antioxidants such as glutathione, ascorbic acid or α-tocopherol, or enzymes such as catalase and superoxide dismutase, which detoxify oxidizing agents. When these antioxidant systems cannot cope with the increased production of ROC, oxidative stress develops. Especially in the activation of the immune system, large amounts of ROCs are produced by the immune cells together with neopterin (Widner et al. 2002). The amount of neopterin produced by monocytes/macrophages and their capacity to produce ROC were found to be highly correlated. (Schroecksnadel et al. 2004). Additionally, interferon-gamma and similar cytokines strongly stimulate burst reactions in phagocytes. That's why, oxidative stress occurs

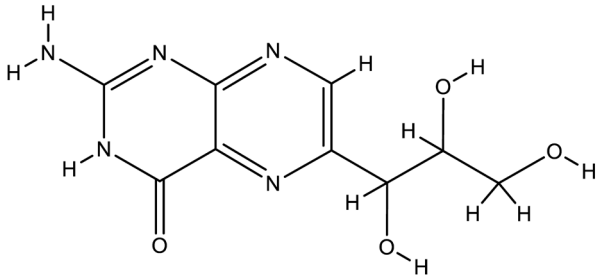

Figure 1. Chemical structure of neopterin.

in inflammation, immune system activation and diseases that cause endogenous interferon-gamma production. In parallel, it was found that the concentration of antioxidant substances decreased in immune system activation and inflammatory diseases (Widner et al. 2002).

According to studies, increased neopterin levels in biologic fluids are connected with various pathological conditions such as viral infections (Al-Kuraishy et al. 2021), autoimmune diseases (Nasonov et al. 2000), inflammatory diseases (Gieseg et al. 2018), neurological (Yoshida et al. 1999) and cardiovascular diseases (Pacileo et al. 2007).

Several methods have been reported for the determination of neopterin including ELISA (Westermann et al. 2000) or HPLC (Werener et al. 1987, Fukushima et al. 1980, Andondonskaja-Renz & Zeitler 1983) in biological fluids. HPLC-based analytical techniques are the most preferred methods in neopterin analysis due to their reproducibility and appropriate sensitivity. As the amount of components in the urine sample differs depending on the concentration, urine neopterin levels are expressed by using the neopterin/creatinine ratio (Krcmova et al. 2011).

To date, it's well known that cell-mediated immunity has a close relationship with environmental factors (Veldhoen et al. 2008). As the neopterin level serves as a biomarker for cell-mediated immune activity, the effects of vocational diseases related to environmental conditions on neopterin levels have become a popular research area (Pingle et al. 2008). Previous studies reveal that there is a remarkable increase in neopterin levels of individuals who work in toxic environments (Sarac et al. 2013, Ozdemir et al. 2006, Ulker et al. 2007, Altindag et al. 2003). Thus,neopterin may be an effective biomarker in measuring toxic exposures of industrial workers.

To the best of our knowledge, no method has been reported for the determination of urine neopterin levels of industrial workers including auto painters, bodywork and furniture workers in Turkey. Therefore, this study was designed for the determination of neopterin in the urine of industrial workers with an HPLC-fluorescence detection method. Besides, the present method has several advantages, including a straightforward and single-step extraction procedure that uses low-cost chemicals and a short run time. The developed method was applied to real samples to determine the neopterin levels in the urines of industrial workers, who have been exposed to various chemicals such as formaldehyde, heavy metals and thinners. Urine neopterin levels of industrial workers including auto painters, bodywork and furniture workers were statistically compared with healthy volunteers.

MATERIALS AND METHODS

Chemicals and reagents

Neopterin (purity ≥97.5%), creatinine (purity ≥98.0%), dihydrogen potassium orthophosphate and methanol were purchased from Sigma (St. Louis, MO, USA). All chemicals used in the study were of quality analytical grade. Distilled water was prepared by an ultra-water purification system.

HPLC system and chromatographic conditions

The high-performance liquid chromatography system consisted of a fluorescence detector and Total Chrom Chromatography Data System software (Shimadzu, Duisburg, Germany). Also, the HPLC system is equipped with a pump LC-10AD VP, an autosampler SIL-10A and a UV-Vis detector. Separation was performed on the Ace C_{18} column (250 x 4.6 mm i.d., 5 µm). Also, it used with was a guard column (4 mm × 3 mm i.d.).
The mobile phase consisted of a 15 mM phosphate buffer (pH 7) containing 2.5% methanol. The mobile phase flow rate was 1.0 mL/min in the isocratic elution mode. Neopterin was monitored at excitation wavelength 353 nm and emission 438 nm by fluorescence detection. Creatinine was monitored at a wavelength of 235 nm by UV detection.

Preparation of stock, standard and quality control solutions

Neopterin stock solution was prepared at 1.0 mg/mL concentration in 0.1 M NaOH solution. Neopterin working solution was diluted with 0.1 M NaOH. Standard calibration samples were prepared at 1.0-1000 ng/mL. Creatinine stock solution was prepared at 1.0 mg/mL concentration in water. Standard calibration samples were prepared at 0.5-500 µg/mL. All the standard solutions were stored at 4 °C.

The quality control (QC) solutions of neopterin were prepared at 150, 450 and 750 ng/mL. Also, the QC solutions of creatinine were prepared at 75, 250 and 450 µg/mL. The six replicates of QC samples were analyzed on two consecutive days six times.

Collection of samples

The clinical protocol was approved by Ataturk University Ethics Committee prior to the study (2018/307). All volunteers were informed according to the principles of the Declaration of Helsinki in the study. Workers in the industry were generally healthy and worked in auto painters, bodywork, and furniture. 33 volunteers were selected and medically examined in this project. The worker group included 33 men aged 20 to 57 years (mean age 35.2 ±11.9), while the control group consisted of 17 male healthy subjects their ages ranged from 21 to 46 (mean age 33 ±7.5). When their smoking habits were questioned, 63.6% of the workers and 41.7% of the healthy individuals were smoking. Urine was collected once in the morning, as 5 ml, from the volunteers. Then, urine samples were put in a 10 ml tube. All urine samples were stored at -20 °C. Neopterin is more soluble in water than organic solvents. For this reason, extraction with organic solvents does not yield effective results (Wachter et al. 2004). Therefore, no effort was made to extract neopterin from the urine. The urine was diluted with distilled water at a ratio of 1/100 and it was analyzed 2 days after urine collection using HPLC. The results were expressed as neopterin/creatinine ratio (µmol neopterin/mol creatinine).

Statistical analysis

All statistical tests were performed using the "Statistical Package for the Social Sciences" version 15.0 (SPSS V.15.0) computer program. Regression analysis was used in the preparation of neopterin and creatinine standard lines and in calculations. Groups comparison of means between students was completed using Student's t-test. For statistical significance, $P<0.05$ was accepted and the results were presented as mean ± standard deviation.

RESULTS

Method development and optimization

While the method is being developed, it was focused on chromatographic separation,

optimization of column selection, sample preparation, symmetric peaks shape and short run time. Neopterin and creatinine can be satisfactorily measured by a reversed-phase column (C_{18}).

Neopterin wavelength was measured at excitation 353 nm and emission 438 nm. Also, creatinine wavelength was measured at 235 nm. Various mobile phase compositions were performed for neopterin and creatinine. The results were obtained with a mobile phase consisting of 15 mM phosphate buffer (pH 7) containing 2.5% methanol. The retention times of neopterin and creatinine were 6.2 and 5.4 min, respectively. A representative chromatogram of neopterin and creatinine was given in Figure 2.

Method validation

Method validation was tested by selectivity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), stability, dilution integrity and matrix effect according to ICH guidance (Ulker et al. 2007).

Selectivity

The selectivity was checked by comparing the chromatograms of blank urine with the spiked urine. Neopterin and creatinine retention times were approximately 6.2 and 5.4 min (Figure 3). Also, the blank urine sample was analyzed. Endogenous interferences were not observed.

Linearity

Standards calibration curves were drawn according to peak area (y) of neopterin and creatinine versus drug concentration. It was found to be linear over the concentration range 1.0-1000 ng/mL and 0.5-500 µg/mL for neopterin and creatinine, respectively. The linearity properties of the calibration curves of neopterin and creatinine were shown in Table I. The correlation coefficients of standard calibration curves were greater than 0.99.

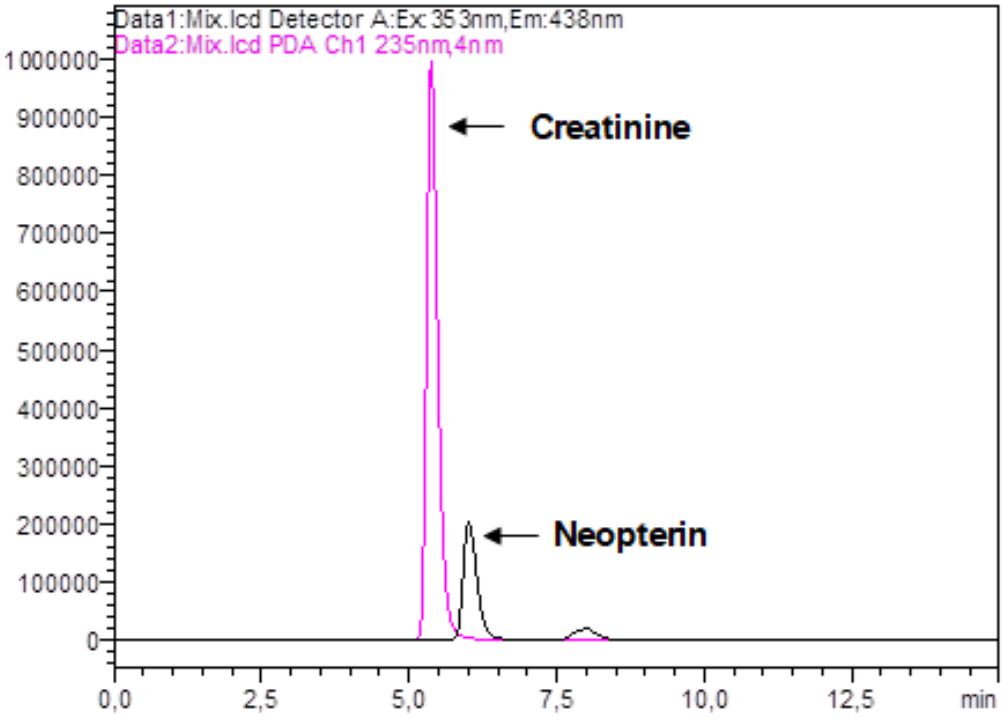

Figure 2. Representative chromatograms of neopterin and creatinine.

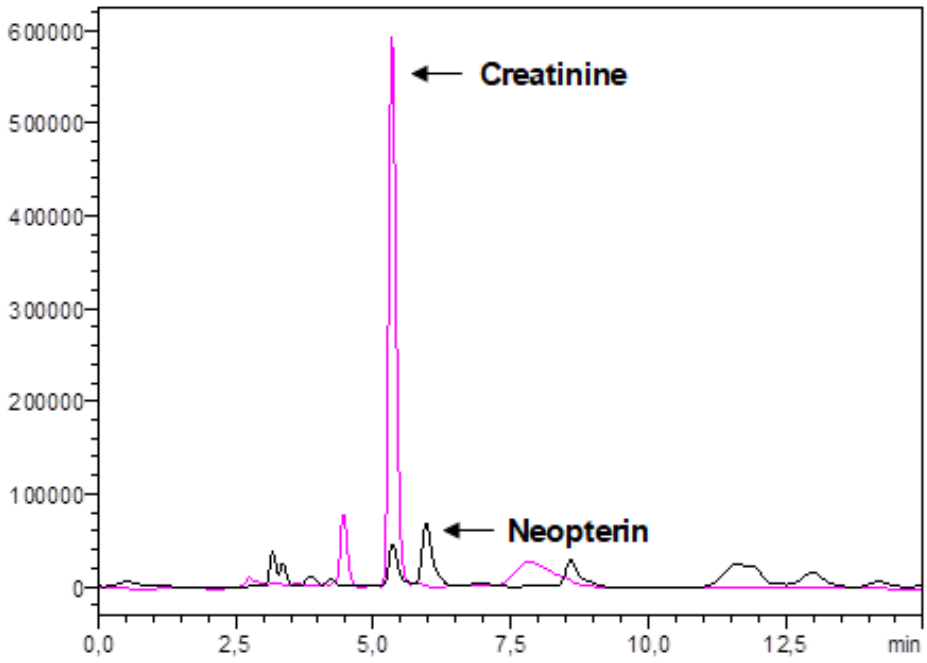

Figure 3. Representative chromatogram of urine of an industrial worker.

Table I. Linearity of neopterin and creatinine in human urine.

Parameter	Neopterin (ng/mL)	Creatinine (µg/mL)
Linearity	1 -1000	0.5 - 500
Regression equation	y=506.09x+595.74	y=3647.3x + 1671
Standard deviation of slope	3.56	17.3
Standard deviation of intercept	2.86	4.24
Correlation coefficient	0.998	0.998
Limit of detection	0.30	0.15
Limit of quantification	1.00	0.50

Precision and accuracy

The analytical method precision was determined by intra-day and intermediate precision. The intra-day precision was measured by analyzing six replicates for each of three different concentrations. The intermediate precision was measured by analyzing the same urine samples for two successive days.

The intra-day and intermediate precision were assessed as the percent relative standard deviation (RSD%). Furthermore, the accuracy of the method was assessed as the percentage relative error.

Intra- and inter-day precision and accuracy for neopterin and creatinine from urine samples data are shown in Table II. The results indicated very good precision and accuracy.

Table II. Precision and accuracy of neopterin and creatinine in human urine.

Added	INTRA-DAY			INTER-DAY		
	Found ± SD	Precision % RSD	Accuracy	Found ± SD	Precision % RSD	Accuracy
Neopterin (ng/mL)						
3.00	2.94 ± 0.08	2.72	-2.00	3.04 ± 0.07	2.30	1.33
450	438 ± 9.82	2.24	2.67	443 ± 13.9	3.14	1.56
750	754 ± 7.87	1.05	0.53	767 ± 17.9	2.33	2.27
Creatinine (µg/mL)						
1.50	1.46 ± 0.04	2.74	2.67	1.57 ± 0.05	3.18	4.67
250	241 ± 16.4	6.80	-3.60	255 ± 20.2	7.90	2.24
450	461 ± 19.22	4.17	2.44	458 ± 16.8	3.66	2.13

LOD and LOQ

The LOD was determined as the lowest concentration, which gives a signal-to-noise ratio of 3 for neopterin or creatinine. The LOQ value was evaluated as the lowest concentration of the urine spiked with neopterin and creatinine. The LOD values were 0.30 ng/mL and 0.15 µg/mL for neopterin and creatinine, respectively. Also, the LOQ values were 1.0 ng/mL and 0.50 µg/mL neopterin and creatinine, respectively. The precisions of the limit of quantification for neopterin and creatinine were satisfactory with RSD less than 7.0% and accuracy with relative error within ± 11.0%

Stability

The stability of neopterin and creatinine stock solution were evaluated for at least 24 hours at room temperature. Also, the stabilities of neopterin and creatinine in human urine were studied under a variety of storage. Then, the stability was tested by comparing the instrument response with freshly prepared neopterin and creatinine solutions. The short-term temperature stability was assessed by analyzing three aliquots of each of the low and high concentration samples. The samples were thawed at room temperature and kept at this temperature for 8 h. Freeze-thaw stability was checked through three cycles (-20 °C in urine). When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw cycles were repeated three times and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low and high concentrations stored at -20 °C for 2 days. The stability results were given in Table III. No significant degradation of neopterin was observed under the tested conditions.

Dilution integrity

The dilution integrity was performed to test for higher analyte concentrations above the upper LOQ because it may be encountered during real sample analysis. The accuracy and precision of neopterin for $1/5^{th}$ and $1/10^{th}$ dilution ranged from 98.3 to 101.7 and 2.14 to 4.17%.

Table III. Stability of neopterin and creatinine in human urine under various storage conditions (*n* = 3).

	Storage conditions	Concentration	Calculated concentration	% RSD	% Relative error
Neopterin (ng/mL)	Room temperature for 8 h	50	48	2.8	-4.0
		150	143	4.4	-4.7
	Three freze-thaw cycles	50	49	3.1	-2.0
		150	141	5.9	-6.0
	2 day at -20 °C	50	51	3.4	2.0
		150	144	5.8	-4.0
Creatinine (µg/mL)	Room temperature for 8 h	150	145	5.8	-3.3
		300	313	4.4	4.3
	Three freze-thaw cycles	150	143	7.4	-4.7
		300	316	6.5	5.3
	2 day at -20 °C	150	141	5.6	-6.0
		300	319	6.7	6.3

Ruggedness

A ruggedness test was performed as the mobile phase, pH of the mobile phase, temperature and concentration in the method validation. Retention time and peak area of neopterin and creatinine were determined. The retention time and peak area of neopterin and creatinine were not affected by the variation of mobile phase concentration. Temperature changes influence neopterin and creatinine concentration in standard solutions in the range of 98-102%.

System suitability

A system suitability test was performed before each validation. Therefore, five replicate injections of neopterin and creatinine were analyzed. The %RSD of peak area, tailing factor and efficiency of neopterin and creatinine for the five suitability injections were determined. In all of the samples, the tailing factor was ≤ 1.02, efficiency ≥ 3957 and %RSD ≤2.0%. The % RSD of retention time and peak area for neopterin and creatinine were within 2%.

DISCUSSION

Biomarkers are always used in the evaluation of early diagnosis and treatment activities in the field of health. Especially, neopterin is a biomarker that can be used directly for early diagnosis in the clinic. Also, neopterin level can be used to identify the right time for clinical operations. Additionally, it contributes to supporting the analyzed results and it can be beneficial in confirming clinical observations.

Neopterin production increases in various diseases and infections. However, the biopterin level changes little or not at all. (Schroecksnadel et al. 2004). Diseases associated with oxidative stress are often accompanied by high neopterin concentrations (Widner et al. 2002). Thus, it is declared that neopterin derivatives can be an indicator of the oxidative stress induced by the immune system.

Neopterin can be easily analyzed in biological fluids. Also, it has been reported that neopterin can be used as a sensitive biomarker in the diagnosis and prognosis of many diseases in which T-cells or macrophages are activated

such as autoimmune, malignant, infection, solid organ transplantation, renal pathologies, down syndrome, schizophrenia, atypical phenylketonuria (Kus et al. 2004, Melichar et al. 2006).

High neopterin concentrations were found in 90% of hematological neoplasms, 80% of ovarian cancers, 70% of pancreatic carcinomas, 58% of lung cancers and 55% of cervical carcinomas (Murr et al. 2002).

Moreover, it has been indicated that not only the type of tumor, but also the stage of the tumor affects the increase in neopterin concentration. Neopterin levels are generally found to be higher in advanced stages than in the initial stages of cancers (Murr et al. 2002, Reibnegger et al. 1991).

On the other hand, in many cases, the decrease or normalization of the neopterin level is associated with the fact that the treatment is successful, or neoplasia decreased. A correlation has been indicated between estimated tumor mass and urinary neopterin concentration. Neopterin level has been proven to be an important and independent indicator of prognosis in malign diseases. That's why, the increase in neopterin level in serum or urine indicates that the disease has a bad trend (Reibnegger et al. 1991).

Especially in terms of employee health, it is important to determine the neopterin level in the urine of the people working in the industry. Neopterin analysis can be done by radioenzymatic methods. However, radioenzymatic methods take a lot of time. Therefore, HPLC or immunoassay methods are preferred instead (Ogiwara et al. 1992). HPLC is a sensitive and convenient method for analyzing few numbers of samples (Hansen et al. 1982, Fuchs et al. 1992). Therefore, in this study, an HPLC method has been developed for the determination of neopterin in the urine of the workers who are exposed to environmental factors. Neopterin is detected at 353 nm excitation and 438 nm emission wavelength by utilizing the natural fluorescence property of neopterin. A very small amount of urine sample is sufficient for measurement. It has been reported that it can be stored for 2 days provided that it is protected from light (Wachter et al. 2004). If the urine samples are not analyzed the same day, they can be stored at +4 °C. Otherwise, it is a safer method to store them frozen.

Additionally, neopterin concentrations in urine are given in proportion to creatinine to eliminate the effects of the differences in urine concentration (Schroecksnadel et al. 2004). Creatinine concentrations are approximately 12 mmol/L. Changes in creatinine clearance affected by age and gender are reflected in neopterin excretion. For example, creatinine level, decreasing with age, causes an increase in neopterin concentration in urine. Neopterin level is 101-133 µmol/mol in men. Women have a greater neopterin level in their urine, ranging from 124 to156 µmol/mol (Schroecksnadel et al. 2004).

In this work, the highest and lowest values of urinary neopterin were obtained for industrial workers as 908.96 and 119.86 µmol/mol, respectively. The highest and lowest values of urinary neopterin were obtained for the control group as 480.54 and 164.84 µmol/mol, respectively (Table IV). Neopterin levels were found to be higher in industrial workers than in the healthy controls (P<0.05). Our investigation demonstrates that there is a meaningful

Table IV. Mean neopterin levels in urine samples (mmol neopterin/mol creatinine).

Groups	Workers (n=33)	Control (n=17)
Mean ± SD[a]	491.92 ± 206.95	324.87 ± 93.48
Min-Max levels	119.86 – 908.96	164.84 – 480.54

difference in urinary neopterin levels between the workers and the control groups (P<0.05). Workers in the auto paint, body and furniture business may have been exposed to a toxic environmental exposure in their occupation. Neopterin may be an early critical marker inthe early stages. The increase in neopterin concentrations in patient urine is an important marker in the diagnosis and treatment of various diseases. Therefore, neopterin measurements can be useful not only for cellular immune neopterin levels, but also for determining the extent of disease and evaluating therapeutic response. In order to obtain healthier results, it is recommended to continue the studies by increasing the population sample.

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Author contributions

HY collected urine samples from the volunteers. This study is also the subject of HY's doctoral thesis. BY is also HY's PhD thesis advisor. All authors analyzed neopterin and creatinine levels in urine samples using HPLC. At the same time, all authors contributed to the paper writing.