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DETERMINATION OF NEOPTERINE IN HUMAN URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Since the analysis of urinary neopterine is important in diagnosing malignancy, a method has been developed for its rapid and sensitive separation and quantitation using high-performance liquid chromatography (HPLC) on reversed phase. Eluted neopterine is monitored by fluorescence at an excitation wavelength of 353 nm and measured at 438 nm. Separation was optimized by elution with 15 mmol/l potassium phosphate (pH 6.4) at a flow-rate of 0.8 ml/min.

Urinary neopterine was related to creatinine with the aim of reducing variations due to fluctuating urinary concentrations. The proposed method has good performance characteristics and is not influenced by the presence of reduced neopterine in urine.

Using this HPLC method, urinary neopterine related to creatinine was determined for 148 healthy male adults (mean neopterine/creatinine 113 µmol/mol), 146 healthy female adults (mean neopterine/creatinine 140 µmol/mol) and 60 healthy children (mean neopterine/creatinine 163 µmol/mol). The neopterine levels for four healthy individuals were measured daily over a period of one month.

INTRODUCTION

Determination of pteridines in biological fluids is considered to be important, since there are reports showing a correlation between high pteridine excretion rates and increased growth or proliferation. Previous investigations conducted in our laboratory [1,2] have revealed a connection between urinary excretion of a fluorescent compound and malignant growth. These preliminary observations were backed up by experiments on mice with Ehrlich ascites tumor, which showed a significant increase of a urinary fluorescent metabolite in response to the tumor [3]. We identified this fluorescent metabolite as 6-hydroxylumazine originating during analysis from non-fluorescent 7,8-

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dihydro-6-hydroxylumazine by autoxidation [4] and the metabolite from human urine as neopterine [5]. Recently, it was shown that in patients with haematological neoplasmias the urinary neopterine levels correlated well to clinical features and to tumor staging [6]. Moreover, the pteridine excretion is generally altered when there is cell proliferation or growth [7—11].

The reports demonstrate that a need exists for a simple and rapid method of measurement of pteridines in biological fluids, particularly of urinary neopterine. Published methods for determining pteridines by ion-exchange separation [12,13] have proved to be tedious and time-consuming. This paper, therefore, proposes a simple and rapid technique for a specific and sensitive measurement of urinary neopterine by high-performance liquid chromatography (HPLC) on reversed phase. The method is well-suited for automation and routine clinical laboratory use together with a quantification of creatinine and uric acid levels [14,15].

This report also describes an application of the method to quantify neopterine excreted in the urine of healthy subjects as a function of sex and age.

EXPERIMENTAL

Chemicals

Acetonitrile (chromatography grade), dipotassium hydrogen phosphate and potassium dihydrogen phosphate (analytical grade) were purchased from Merck (Darmstadt, G.F.R.). Neopterine was obtained from Fluka (Basle, Switzerland).

Apparatus

Chromatography was performed on a Varian Model LC 5000 instrument (Varian Assoc., Palo Alto, CA, U.S.A.) equipped with an automatic sample injection system (Auto Sampler 8000, Varian) in conjunction with a 10-μl injection valve (Valco Instruments, Houston, TX, U.S.A.). The effluent was monitored with a spectrofluorometer Model SFM 23 (Kontron, Eching/Munich, G.F.R.) and with a Model PM 2 DLC variable-wavelength UV absorption detector (Zeiss, Oberkochen, G.F.R.). Peak areas were measured with a Model CDS 111 chromatography data system (Varian).

Column

Ready-to-use columns (stainless steel, 300 $\times$ 3.9 mm I.D.), packed with 10-μm Bondapak C_{18}, were purchased from Waters Assoc., Milford, MA, U.S.A.

Liquid chromatography

Chromatographic elution is performed with an aqueous 15 mmol/l potassium phosphate buffer (pH 6.4, column temperature 30°C). The buffer is degassed under vacuum just prior to use. During a period corresponding to the first 7 min, a flow-rate of 0.8 ml/min is suitable for separation of urinary pteridines. During the ensuing period, from 7—8 min, a linear flow gradient between 0.8 and 1.4 ml/min and a linear elution gradient between 0 and 1.218 mol/l acetonitrile can be used to cleanse the column. In the next period, from 8—12.5 min, the flow and composition remain constant. From 12.5—13.5 min the starting

conditions are restored. The effluent is monitored by setting the fluorescence detector for excitation at 353 nm and emission at 438 nm. The absorption detector is set at 235 nm. Chromatograms are quantified by means of a chromatography data system employing the external standard method.

Collection of specimens

The first morning urine was collected from 148 healthy male and 146 female volunteers and from 60 healthy children. Samples were either analysed immediately, or stored at −20°C in the dark. When the urine samples were kept in a frozen state we added 100 mg of disodium ethylenediamine tetraacetate to 10 ml of urine.

To protect neopterine from photooxidation, all operations were conducted in dim light.

Sample preparation

To avoid column contamination we prepurified the urinary samples through commercially available Sep-Pak C_{18} cartridges (Waters Assoc.); 1.0-ml aliquots of urine were pressed through the cartridge using a 10-ml syringe with Luer end fitting. The high-polarity components such as neopterine, creatinine and uric acid were eluted with 9 ml of water. The 1.0-ml and the 9-ml elution were mixed and a 10-µl aliquot corresponding to 1 µl of urine is injected on to the column.

Sediment formation

To take the possibilities of error due to urinary sediments into account, the following sediments were precipitated in the presence of neopterine and creatinine contained either in standard solutions or urinary sample: calcium phosphate, calcium oxalate, calcium carbonate, sodium urate and uric acid [16]. After centrifugation the precipitates as well as the supernatants were tested for neopterine and creatinine. Urine, or standard solutions containing 5.92 µmol/l neopterine, were irradiated with light of a wavelength of either 254 nm or 366 nm, using a Model Uvis mercury lamp (Desaga, Heidelberg, G.F.R.). The distance from the lamp was 12 cm. Assays for the oxidation of neopterine (5.92 µmol/l) of 7,8-dihydroneopterine (14.9 µmol/l) and of 5,6,7,8-tetrahydroneopterine (10.89 µmol/l) were carried out by stirring with manganese dioxide and hydrogen chloride, by digesting with hydrogen peroxide, or by blowing in atmospheric oxygen.

RESULTS

Development of separation

The experimental conditions were optimized to permit quantitation of polar components such as neopterine and creatinine. The mobile phase was varied by altering the concentration and pH of aqueous potassium phosphate buffer and by adding acetonitrile, methanol or tetrahydrofuran. The best separation was obtained using the buffer alone. Although a slightly superior resolution resulted with lower phosphate concentrations, we chose 15 mmol/l phosphate. Thus better reproducibility of retention times was achieved due to higher

buffering capacity. Separation decreased in the presence of various concentrations of acetonitrile, methanol or tetrahydrofuran. It was found that retention times were mostly sensitive to changes in pH. A pH optimum for the separation of neopterine, creatinine and uric acid was reached at a value of 6.4. At this pH, superior resolution resulted, especially in the first part of the chromatogram.

A flow-rate of 0.8 ml/min was found to be suitable for obtaining high resolution in the interesting part of the chromatogram. After elution of neopterine, a flow and concentration program was instituted by adding acetonitrile. This permitted rapid purification of the column, thus reducing analysis time. Fig. 1 shows the separation of a urine sample chromatographed under the described conditions.

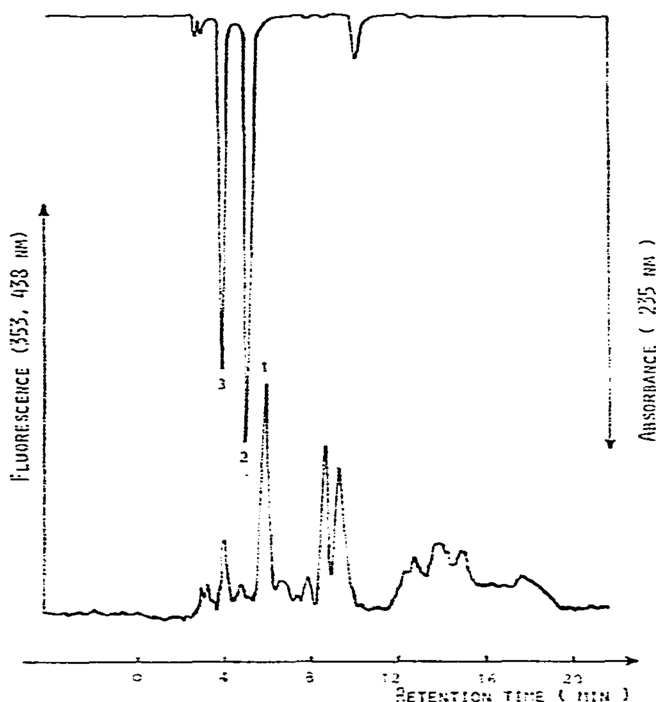

Fig. 1. Chromatogram of neopterine (1.5 nmol), creatinine (13 μmol) and uric acid (2.0 μmol) in a 1-ml urine sample. Upper chromatogram was obtained with the UV detector (0.64 a.u.f.s. at 235 nm). Lower chromatogram was obtained with the fluorescence detector (353 nm excitation and 438 nm measuring). Separation of urine was on a 10-μm Bondapak C_{18} column (300 × 3.9 mm I.D.). Solvent, 15 mmol/l potassium phosphate buffer (pH 6.4); flow-rate, 0.8 ml/min; column temperature, 30°C. Peaks: 1 = neopterine, 2 = creatinine, 3 = uric acid.

Elution through Sep-Pak

Urinary components more non-polar than xanthine were removed by sample preparation with Sep-Pak C_{18} cartridges. By this prepurification we avoided column contamination by solid or non-polar urinary components. Further, the dilution obtained has the advantage of remaining in the linear range for creatinine estimation. Although the precision of absolute neopterine and

creatinine values was diminished slightly by this sample prepurification, the neopterine/creatinine ratio is much less affected and has an acceptable level of precision. Using standard solutions, 99.7% creatinine and 99.7% neopterine were eluted with 9 ml of water. In this sample preparation the neopterine/creatinine ratio did not appear to be affected.

Relation of urinary neopterine to creatinine

Because of physiologically variable concentrations of urine, it is useful to relate urinary neopterine to creatinine. Table I shows that the neopterine/creatinine ratio of three typical individuals remains relatively constant during a 24-h period. On the other hand, the neopterine and creatinine levels show distinct variations. Use of such a neopterine/creatinine relation decreases the physiological variation in the urinary concentration as well as eliminating possible errors due to sample preparation or sample injection.

Urinary sediments

Urinary sediments composed of calcium phosphate, calcium oxalate or calcium carbonate did not contain neopterine or creatinine. Here, all the neopterine and creatinine were recovered in the supernatant. In sediments of uric acid or sodium urate using standard solutions or urine, the precipitate contained neopterine and creatinine. In the supernatant the neopterine levels were 16% lower and the creatinine levels 10% lower than the starting values. From this result it is to be concluded that, when the urine samples are kept frozen, sediment formation has to be prevented, for example through the addition of 100 mg of disodium ethylenediamine tetraacetate to 10 ml of urine.

Stability of neopterine and of its reduced forms

Neopterine is not oxidized by air within 7 h at pH 6.4 and pH 1.0 in standard solution or urine, respectively. Since neopterine is found in human urine in reduced as well as in oxidized form, the measurement of both is possibly of interest. In assaying the oxidation of 7,8-dihydroneopterine and 5,6,7,8-tetrahydroneopterine, best results were obtained using manganese dioxide and hydrogen chloride for 15 min. In standard solutions, 7,8-dihydroneopterine produced only 60—70% neopterine. Using a urine sample or tetrahydroneopterine the yield varied even more. Thus, we found that the oxidation of reduced neopterines by these procedures appeared not to give consistent and comparable results.

Upon irradiation at 254 nm and 366 nm, neopterine photooxidizes markedly within 5 min, showing the importance of always working in a dim light.

Performance characteristics

Linearity and sensitivity. Excellent linearity was noted in the relationship of the peak area of standard solutions to neopterine concentration, which ranged from 200 nmol/l to at least 40 μmol/l. The following regression equation was obtained: $y = 1.006\,x - 2.94$; $r = 0.9999$.

Sensitivity and selectivity were increased by measuring fluorescence at an excitation wavelength of 353 nm and an emission wavelength of 438 nm. The

TABLE I

LEVELS OF URINARY CREATININE, NEOPTERINE AND NEOPTERINE IN RELATION TO CREATININE OF THREE INDIVIDUALS DETERMINED DURING A 24-h PERIOD

	Individual I (37 years, female)			Individual II (26 years, male)			Individual III (51 years, female)		
	Creatinine	Neopterine	Neopterine/creatinine	Creatinine	Neopterine	Neopterine/creatinine	Creatinine	Neopterine	Neopterine/creatinine
n	7	7	7	6	6	6	6	6	6
$\bar{x}$	10.4 mmol/l	1.53 µmol/l	151 µmol/mol	11.5 mmol/l	1.05 µmol/l	92 µmol/mol	19.0 mmol/l	2.06 µmol/l	109 µmol/mol
± S.D.	5.0 mmol/l	0.70 µmol/l	26 µmol/mol	2.4 mmol/l	0.22 µmol/l	14 µmol/mol	5.4 mmol/l	0.58 µmol/l	16 µmol/mol
C.V. (%)	48.1	45.8	17.2	20.9	21.0	15.2	28.4	28.2	14.7

sensitivity at a peak-to-noise ratio of 5 : 1 was determined to be 120 nmol/l of undiluted sample, or 120 fmol per injection.

Precision and accuracy. Within-run precision was obtained by assaying 25 aliquots of three urine samples containing different concentrations of neopterine and creatinine. In the same way, day-to-day precision was observed for 15 days, using three urine samples. Table II shows the reproducibility within-day and between-day for neopterine in relation to creatinine. Recovery experiments with six spiked urine samples yielded a mean value of 102.2% for neopterine and of 99.3% for neopterine in relation to creatinine.

TABLE II

PRECISION OF URINARY NEOPTERINE IN RELATION TO CREATININE DETERMINED BY HPLC ON REVERSED PHASE

	Sample No.					
	1	2	3	4	5	6
	Within-run			Day-to-day		
n	20	24	24	15	15	15
$\bar{x}$ (μmol/mol)	95	214	244	183	260	229
± S.D. (μmol/mol)	6.2	8.1	9.3	13.6	7.3	16.3
C.V. (%)	6.5	3.8	3.8	7.4	2.8	7.1

Specificity and interferences. Urine contains only a few components having similar polarity or similar fluorescence characteristics; most are pteridines. The following pteridines did not interfere because of their different elution times: 6-pterincarboxylate, monapterine, biopterine, xanthopterine, isoxanthopterine, pterine and 6-hydroxymethylpterine. 7,8-Dihydroneopterine and 5,6,7,8-tetrahydroneopterine eluted with the same retention time as neopterine. Because of the very low fluorescence of 7,8-dihydroneopterine at the fluorescence wavelengths used and because tetrahydroneopterine did not fluoresce, any positive bias in measuring neopterine remains negligible.

A comparison of fluorescence spectra for standard and eluted urine samples provides further information on possible interferences. The two fluorescence maxima were identical for both samples (353 and 438 nm). Additionally, the fluorescence maxima of 7,8-dihydroneopterine (362 and 408 nm) were detected in urine, indicating that this compound is eluted together with neopterine. This result corroborates the specificity of the urinary neopterine estimation.

Practicability. Analysis of one urine sample, including preparation and column regeneration, takes 20 min.

Application

The technique was applied to the study of neopterine related to creatinine in healthy subjects as a function of sex and age. Table III shows the results for 148 male and 146 female individuals with mean age of 40.3 and 42.2 years, respectively. The mean urinary neopterine level related to creatinine is lower in males than in females. The mean values vary only a little between different age groups.

TABLE III

URINARY NEOPTERINE IN RELATION TO CREATININE AS A FUNCTION OF SEX AND AGE

Sex	Age (years)	Number of individuals	Neopterine/creatinine (μmol/mol)		C.V. (%)
			$\bar{x}$	± S.D.	
Male	3—18	35	168	64.8	38.6
	19—25	39	118	29.9	23.7
	26—35	25	97	32.6	33.6
	36—45	28	113	29.5	26.1
	46—55	23	103	36.2	35.2
	56—65	18	115	44.2	38.4
	66—80	15	120	39.8	33.2
	19—80	148	113	35.3	31.2
Female	3—16	25	155	62.5	40.3
	17—25	38	128	34.4	25.9
	26—35	21	124	31.3	25.2
	36—45	26	142	40.2	28.3
	46—55	21	147	29.9	20.3
	56—65	20	156	34.4	22.1
	66—85	20	146	37.1	25.4
	17—85	146	140	35.7	25.5
Children					
Male	3—18	35 } 60	163	65.1	39.9
Female	3—16	25			

In the children's group notably higher mean urinary neopterine values were observed than in the adult group. Similarly, the mean value is slightly higher in females above the age of 35 years. Within a particular age group the level of neopterine related to creatinine shows a very low standard deviation and coefficient of variation, indicating a relatively constant excretion of neopterine. In addition, the urinary neopterine levels were controlled daily in four normal individuals over a one-month period. Table IV shows that the level of neopterine related to creatinine varies within the same range as is found to be typical for people of the same sex and age group.

TABLE IV

NEOPTERINE IN RELATION TO CREATININE DETERMINED OVER A PERIOD OF ONE MONTH FOR FOUR INDIVIDUALS USING THE FIRST MORNING URINE

	Individual			
	1	2	3	4
Sex	Female	Female	Female	Male
Age (years)	36	37	26	26
n	31	31	31	28
$\bar{x}$ (μmol/mol)	154	111	115	105
± S.D. (μmol/mol)	43	30	36	30
C.V. (%)	27.9	32.5	31.9	28.6

DISCUSSION

Analytical considerations

The method described permits very rapid and sensitive measurement of urinary neopterine, creatinine and uric acid in a single chromatographic run. Quantification of these urine components inclusive of sample prepurification can be accomplished in 20 min. The technique is suitable for routine clinical laboratory use.

Application to healthy individuals

The higher mean neopterine value for children indicates fast growth behaviour. Interpretation of the slightly increased mean neopterine values of females over 35 years of age is the subject of further investigations.

Quantitation of neopterine present in oxidized or reduced form

Since it is well known that neopterine originates through biosynthesis from guanosine triphosphate via reduced forms [17], estimation of neopterine in its reduced forms should also be of interest. Measurement is restricted to urinary neopterine in the oxidized form, because oxidation of reduced forms is not yet possible with the desired precision and accuracy. Reduced forms of neopterine do not fluoresce or only slightly. Thus the neopterine present in oxidized form alone can be determined with a high level of sensitivity. Further, under dim light, neopterine as well as its reduced forms remain sufficiently stable for hours when exposed to atmospheric oxygen. This method, therefore, gives good precision and is not subject to distortion through the appearance of reduced forms of neopterine or through the disappearance of the latter as the result of oxidation. The fact that measurement of urinary neopterine without determination of reduced forms showed slight biological variations among healthy individuals, as well as good correlations to diagnosis in cases of malignancy, indicates that this method of estimation can be useful in the diagnosis and monitoring treatment of neoplastic diseases.

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