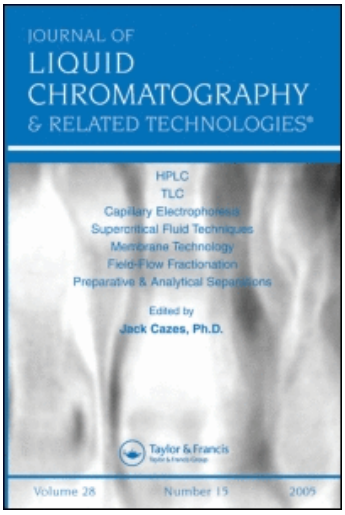

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THE RAPID DETERMINATION OF NEOPTERINE IN HUMAN URINE BY ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An isocratic high-performance liquid chromatography method is described for the determination of Neopterine eliminated in human urine, using a μ-Bondapak C_{18} column (300 x 3.9 mm I.D.) and a strongly polar phosphate buffer (pH 6.2) for elution. This analysis requires only 15 minutes and allows very good reproductibility of retention times. This method is well-suited for automation and routine clinical laboratory in order to quantify human urinary Neopterine in healthy subjects and in subjects with malignant disorders.

INTRODUCTION

Neopterine (2-Amino 4-hydroxy 6-trihydroxypropyl-pteridine) (Fig. 1) is produced from guanosine-5'-triphosphate by proliferating cells (1) or activated T-lymphocytes (2) and eliminated in the urine.

This elimination is increased in several diseases : viral diseases (2), atypical phenylketonuria (3), allograft rejections (2), and in a large number of neoplasic diseases (4) such as haematological neoplasias (5) and genital cancer (6). Furthermore, urinary Neopterine values are in connection with the severity of the disease and a rapid determination offers a great clinical interest.

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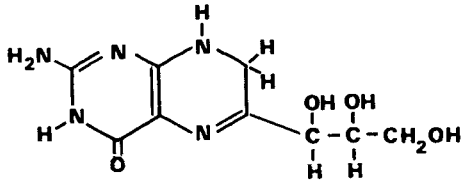

FIGURE 1. Neopterine structure.

The reversed phase liquid-chromatographic methods proposed to date, have used a flow and concentration program by adding acetonitrile (7) or methanol (8) to a phosphate buffer (15 mmol/l). This sophisticated program permits a good separation but does not afford a good reproductibility of retention times. Most of these methods propose a sample pretreatment (7)(9) under dim light which decreases the precision of the measure since pteridines are light sensitive. Niederwieser et al (10) report an automated liquid-chromatographic system for pteridines, thus eliminating sample pretreatment, but their method requires special equipment for automatic switching.

All these methods are complex and we propose here a simple isocratic HPLC method for the determination of urinary Neopterine using a strongly polar phosphate buffer and involving neither sample pretreatment nor special equipment. As an application of the method, variations of Neopterine eliminated according to sex and age were determined.

EXPERIMENTAL

High Performance Liquid Chromatography

The HPLC system used consists of a Beckman 112 solvent delivery system, coupled with a Kontron SFM 23 fluorescence spectrometer. Sample injection is via a Rheodyne 7125 injector fitted with a 50 µl sample loop. A (300 x 3.9 mm I.D.) µ-Bondapak C_{18} column was used for the analysis. The mobile phase was a phosphate buffer pH = 6.20 ± 0.10 : KH_2PO_4 (7 mmol/l) and $Na_2HPO_4 \cdot 12\ H_2O$

(33 mmol/l), thoroughly degassed before use. The flow rate was 1.5 ml/min. The detector was set at an excitation wavelength of 353 nm and an emission wavenlength of 438 nm.

Neopterine Standard and Reagents

KH_2PO_4 and $Na_2HPO_4 \cdot 12\ H_2O$ are from Merck (Darmstadt ; Germany). Neopterine purchased from Fluka (Buchs ; Switzerland) is dissolved in NaOH 0.5 N for a final concentration of 0.40 µg/ml. This stock solution was stored at -20°C in the dark.

Calibration Curve

The calibration curve (Fig. 2) was obtained from aqueous solutions by diluting the stock solution with NaOH 0.5 N in order to obtain 0.40, 0.20, 0.10 µg/ml of Neopterine and is linear within this range. Each calibration point was run in duplicate.

HPLC Determination

50 µl of the sample thoroughly diluted with NaOH 0.5 N (1:2 v/v) were injected (Fig. 3). The concentration of Neopterine was calculated by comparing peak heights with those of Neopterine standard solutions. The final result was expressed in µmoles of Neopterine per mole of creatinine. Urinary creatinine was quantified by kinetic Jaffé reaction. It is useful to relate urinary Neopterine to creatinine, because of the physiologically variable concentrations of urine.

RESULTS AND DISCUSSION

Many liquid-chromatographic methods for Neopterine have been developed and are considered to be efficient means of determining Neopterine in human urine. Fukushima and Nixon,(1979)(8) proposed an isocratic elution with methanol-water (1:19). According to our experience, acetonitrile or methanol in the mobile phase decreases the quality of the separation : the best separation is obtained using the phosphate buffer alone. Moreover, the absence of a buffer is prejudicial to the reproductibility of the retention time

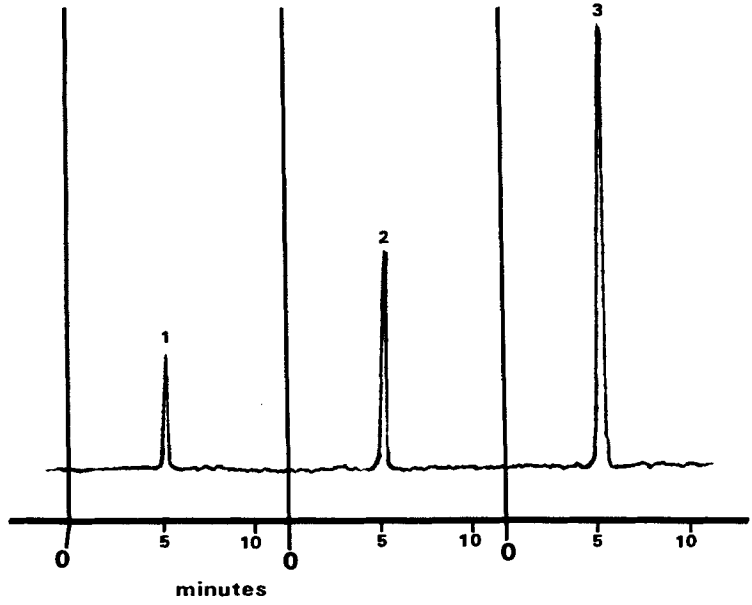

FIGURE 2. Calibration curve
1 : 0.10 µg/ml 2 : 0.20 µg/ml 3 : 0.40 µg/ml

of Neopterine which is very sensitive to changes in pH. Hausen et al (1982) (7) used a flow and concentration program by adding acetonitrile in order to reduce the analysis time. Because of the time required for returning to initial conditions, total analysis time was not really reduced.

The flow-rate of 1.5 ml/mn was found to be suitable to obtain high resolution and to complete the analysis in 15 minutes. Moreover, this constant flow-rate coupled with a high buffering capacity of the mobile phase produces a C.V. of 0.65 % for retention times of Neopterine (n = 20). Within-run precision of the method was evaluated by repeated analyses of a urine sample containing 140 µmoles/mole : the C.V. was 3.2 % (n = 15). We also investigated the influence of pH upon separation : it was optimized at a pH 6.20.

The use of fluorescent detection makes the method both specific and sensitive. Matsubara et al (1984) (3) proposed 338 nm for

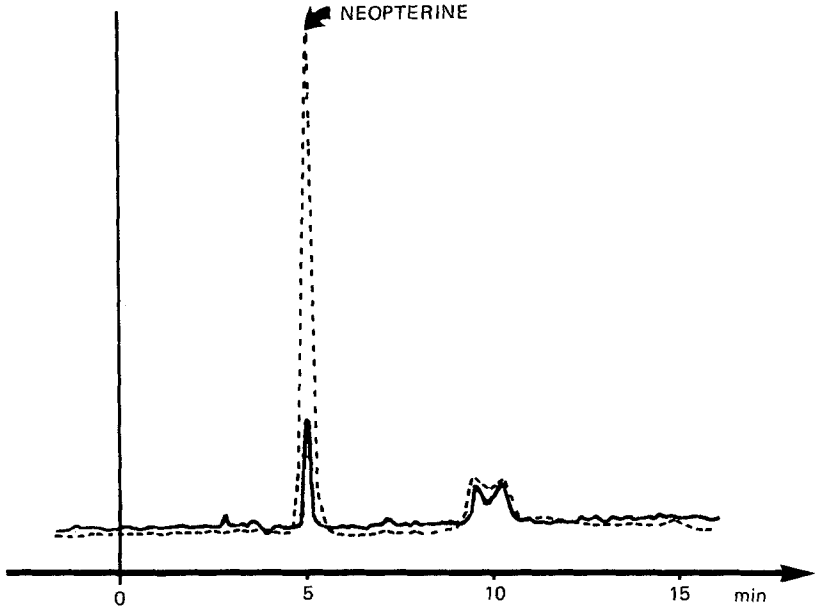

FIGURE 3. Chromatogram of a healthy adult's urine (————) containing 141 µmoles of Neopterine/mole of creatinine and of a patient's urine with ovarian cancer (------) containing 710 µmoles/mole.

excitation and 425 nm for emission. Wachter et al (7) used an excitation wavelength of 438 nm. The comparison of both methods shows that the second is twice as sensitive.

Under the conditions described, no interference from endogenous compounds in human urine was encountered : pretreatment clean-up sample procedure was therefore unnecessary. By avoiding this procedure, a better sensitivity was obtained and a detection limit of 0.02 µg/ml was observed.

Our experiments over a period of more than 5 months failed to reveal any significant changes in the separation efficiency of the µ-Bondapak C_{18} column. Due to the high buffering capacity of the mobile phase, the injection of this highly basic sample (pH 12) did not significantly shorten the column life.

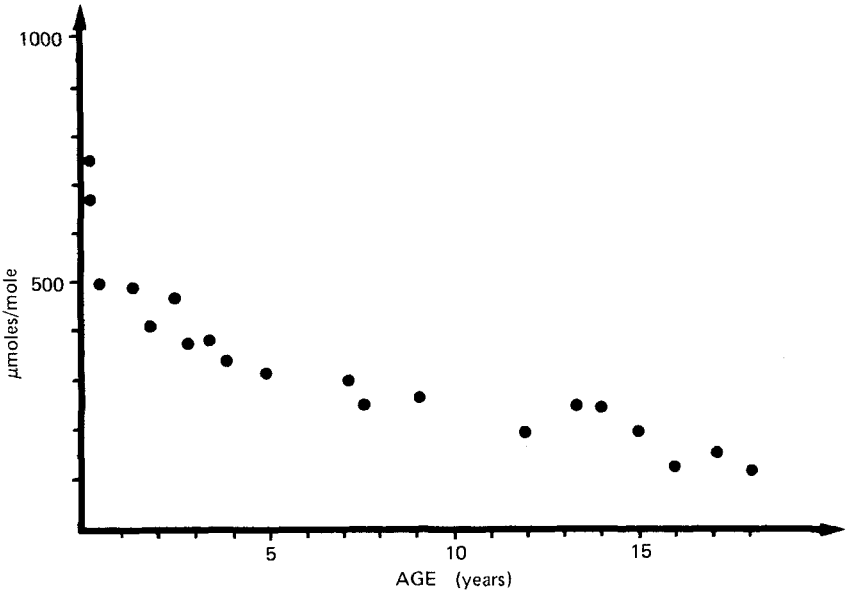

FIGURE 4. Physiological variations of Neopterine according to age.

An isocratic regime in combination with the appropriate phosphate buffer and flow-rate offers an improved analytical method for the assay of urinary Neopterine, which is well-suited for automation and clinical laboratory investigation.

Neopterine Values in Healthy Women and Men

Urinary Neopterine determinations were performed on 20 women and 20 men, all apparently healthy subjects from 30-40 years old. The values found in men were 118 ± 22 µmoles of Neopterine/mole of creatinine and 145 ± 18 µmoles/mole in women. The student t-test gives a t of 4.19, demonstrating that the mean values are significantly different. These results agree with previously published data (11), showing higher values in women than in men.

Neopterine Values in Connection with Age

Urinary Neopterine determination was performed on children of various ages. Fig. 4 shows very high values during the first months

of life and a decrease until the age of eighteen, when they correspond with established adult values.

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