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Short Communication

Simple column liquid chromatographic assay for serum neopterin

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ABSTRACT

A simple high-performance liquid chromatographic assay for serum neopterin with highly sensitive fluorimetric detection (limit 1 n*M*) is proposed. Comparison with results obtained by radioimmunoassay revealed a good correlation between the two techniques. The potential use of this method for the follow-up of liver transplant patients is discussed.

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INTRODUCTION

During the past fifteen years, increased concentrations of neopterin have been reported in the biological fluids of patients with infectious diseases. A pteridine synthesized from guanosine triphosphate [1], neopterin, occurs in various states of oxidation (Fig. 1). Fully oxidized dihydroneopterin is the main intracellular form, representing 70% of total neopterin in cerebrospinal fluid (CSF) [2].

Neopterin is synthesized by monocytes and macrophages following induction by the gamma interferon produced by activated T lymphocytes [3]. Serum and urinary neopterin levels rise in various pathologies implicating the immune system: viral and bacterial diseases, acquired immunodeficiency syndrome (AIDS), certain cancers, autoimmune diseases (rheumatoid polyarthritis) and transplant rejections [4–6]. Determination of the neopterin/creatinine ratio has been advocated for kidney transplant patients [6]. Neopterin also increases in some cases of phenylketonuria, the only instance in which cellular immunity is not stimulated [7].

Urinary neopterin assays have been proposed for the follow-up of patients who are seropositive for the human immunodeficiency virus (HIV) [8]. Similarly, serum neopterin reportedly has prognostic value, in association with immunoglobulin assays, for determining the incidence of AIDS in HIV seropositive subjects [9].

The present study was designed to develop a chromatographic method for serum neopterin assay after simple ultrafiltration using a sensitive HPLC method with fluorimetric detection. The specificity of the method was evaluated by comparing these results with data obtained by radioimmunoassay (RIA).

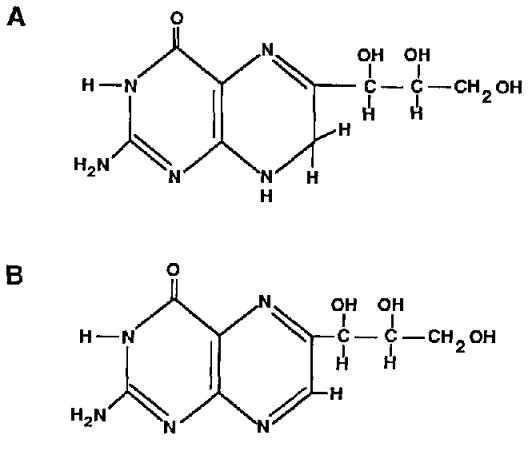

Fig. 1. Structures of (A) D-7,8-dihydroneopterin and (B) D-neopterin.

EXPERIMENTAL

Reagents and chemicals

Neopterin standard for HPLC was obtained from Sigma (Coger, Paris, France), methanol from Carlo Erba (Massy, France), and the other reagents from Prolabo (Paris, France).

Chromatography

Chromatographic separation was performed with an Ultrasphere ODS 5-μm column (250 mm × 4.6 mm I.D.) (Beckman, Gagny, France).

The mobile phase, identical with that for urinary neopterin assays [10], consisted of a 98% phosphate buffer (0.015 *M*; pH 6.4) and 2% methanol; it was filtered through a 0.2-μm filter (Elvetec, Nice, France) and deaerated before use. To check the identity of the neopterin peak, ten serum samples were analysed with another mobile phase: 50 mmol/l sodium acetate–5 mmol/l citric acid, (pH 5.22). Neopterin was analysed with a highly sensitive HPLC system and detected by natural fluorescence (DFL 450, Société Grégoire Service, Cap d'Ail, France and Société Cortiula, Saint-Raphaël, France). The detector was equipped with a high-pressure mercury vapour lamp; the excitation filter selected the bands between 340 and 370 nm, and a set of high-transmission filters centred between 430 and 470 nm allowed emission selection. The arc of the lamp was stabilized to ensure low background noise.

Subjects

Serum neopterin levels were assayed in eight liver transplant patients and analysed as a function of clinical status after transplantation and in *post mortem* samples. These six males and two

females, aged 20–40 years, had undergone transplantation for posthepatitic cirrhosis (n = 7) or hepatocarcinoma (n = 1). A total of 48 blood samples were obtained from day 1 until the day of death (from 4 to 25 days post-transplant). All patients died of bacterial septicemia.

Sample preparation

After ultrafiltration through membranes allowing the passage of only particles with a relative molecular mass below 10 000 (Minisart, Sartorius, Palaiseau, France), the serum was centrifuged for 10 min at 2000 g. The ultrafiltrate was injected directly.

Radioimmunoassay

RIA, performed using a Behring kit (Rueil-Malmaison, France), consists in competition between the neopterin to be assayed and the standard. The tracer neopterin is radiolabelled with ^{125}I for sheep antineopterin antibody [this antibody was first purified by precipitation with donkey anti-sheep antibody (IgG) and resolubilized]. The neopterin–antibody complex is separated from the excess antigen by precipitation, centrifugation, and rinsing. The radioacitivity of the precipitate is inversely proportional to the amount of neopterin in the test sample. The response is linear up to 780 nM and the coefficient of variation (C.V.) is 4%.

RESULTS AND DISCUSSION

Fig. 2 shows the chromatograms of neopterin in a reference solution (A) and an ultrafiltered plasma sample (B). The neopterin retention time (6 min) was increased by eliminating the methanol in the mobile phase; a single symmetrical peak was always obtained. The only minor difficulty concerned the preparation of the standard: neopterin is not soluble at a concentration of 0.5 mM in the mobile phase. However, in suspension, it can easily be removed with a pipette and remains stable for 2 months at −20°C. The 1

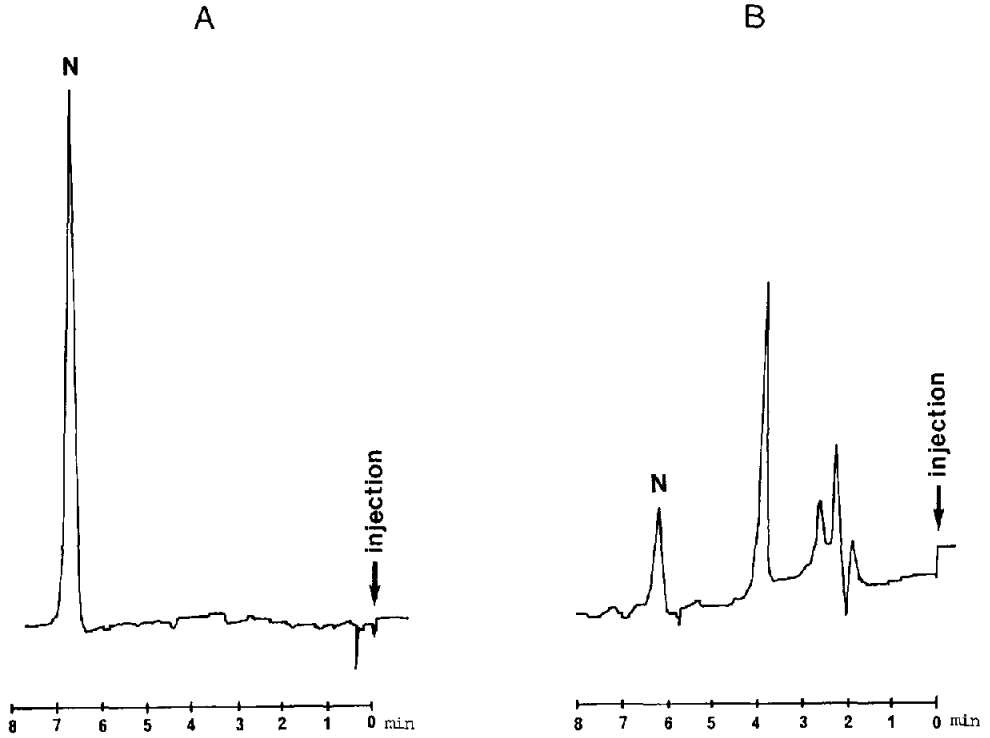

Fig. 2. Chromatograms of (A) neopterin (N) standard (25 nM) and (B) ultrafiltered plasma (3.3 nM). Injection volume, 50 μl; photomultiplier, 7.5; expansion 5.0.

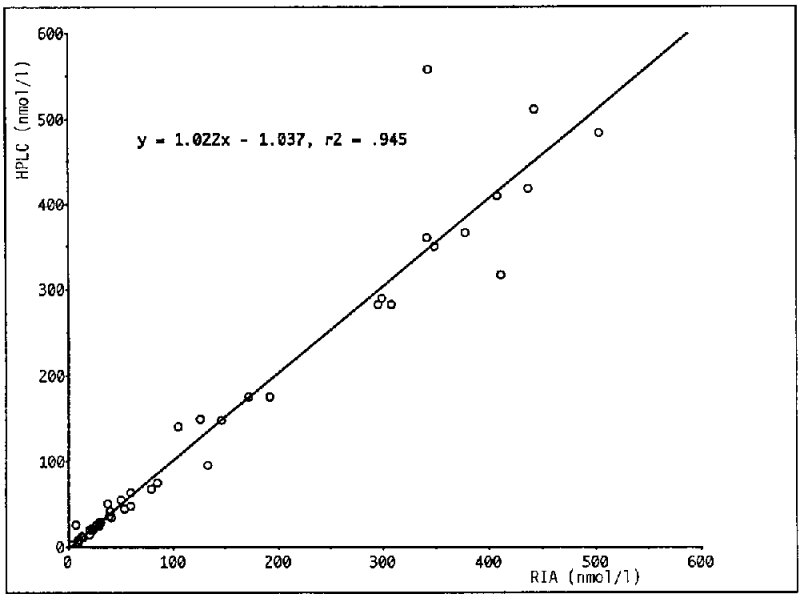

Fig. 3. Correlation between RIA and HPLC assays: abscissa, RIA results; ordinate, HPLC results. The equation is that of a straight line.

μM, 100 nM and 20 nM solutions were all clear. The 1 μM solution is stable for 1 week at 4°C, whereas the other solutions had to be freshly prepared. Although neopterin is reportedly soluble in 0.1 M NaOH solution [11], the retention times of subsequent dilutions (500 and 100 nM neopterin) in the mobile phase did not have the same retention time as the standard diluted in the mobile phase initially. However, this last retention time was valid for neopterin in the ultrafiltered plasma samples and in the diluted urine samples in the mobile phase [12].

The chromatographic method is highly sensitive: 1 nM detected per 100 μl injected, *i.e.* 0.1 pmol. The specificity and accuracy of the method were evaluated by comparing chromatographic results with RIA findings. The two methods correlated well, as shown in Fig. 3. The C.V. for a serum containing 10 nM neopterin was 4%, indicating good reproducibility. The accuracy of the method was determined by three dilutions of five different sera at 106, 52, 28, 17 and 12 nM. The results of the assay of their dilutions to 1:2 and 1:4 differed by a maximum of 4.5%. Under our analytical conditions, the upper limit of quantification was 1600 nM.

Previous HPLC methods proposed for neopterin assay require sample preparation by oxidation, which means that total neopterin is assayed (dihydroneopterin plus neopterin) [13,14], purification on a 4-propylbenzenesulphonic acid-modified silica sorbent cartridge, resulting in measurement of native neopterin alone [15], or use of specific chromatographic conditions for both columns [16]. In the literature [15], the upper normal limit of serum neopterin is 8.7 nM for subjects aged 19–74 years.

Early markers of organ rejection in the blood or urine, which could obviate the need for puncture biopsy, are of special interest for transplant surgeons. Elevation of serum and urinary neopterin levels is not specific for organ rejection because this molecule increases during infections, as demonstrated by our results. However, an increase in circulating neopterin can alert the clinician. It thus advantageously complements other non-invasive analyses. This simple and rapid assay technique of serum neopterin is also compatible with use in emergencies.

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