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Short communication

Dissociation of neopterin and 7,8-dihydroneopterin from plasma components before HPLC analysis

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Abstract

Measurement of plasma neopterin by HPLC with fluorescence detection is used clinically as a marker of immune cell activation in the management of a number of disease pathologies. HPLC analysis of neopterin requires the acidic removal of plasma proteins but we have found that 7,8-dihydroneopterin is oxidised to neopterin with varying yield. Using acetonitrile as the precipitant, we have measured substantially higher quantities of both total neopterin (7,8-dihydroneopterin and neopterin) and neopterin from plasma of healthy and septicemia patient's. Total neopterin concentrations were on average 50% and 200% greater in healthy and septicemia subjects, respectively, when measured after acetonitrile precipitation compared to trichloroacetic acid. Our data suggests that some pterin co-precipitates with proteins during acid treatment.

Keywords: Neopterin; 7,8-Dihydroneopterin; Protein precipitation; HPLC; Acetonitrile; Trichloroacetic acid

1. Introduction

Neopterin and its reduced form 7,8-dihydroneopterin, are synthesised and released primarily by human macrophages when stimulated with γ-interferon [1]. Elevated plasma and urinary neopterin levels are used as a marker of inflammation and the immune response. Measurement of plasma and urine neopterin are reportedly used in the clinical management of HIV infection [2], autoimmune diseases [3], bacterial infections [4] and post-operative transplant patients [5] to name a few of many studies. 7,8-Dihydroneopterin has been shown by us and other laboratories to be a potent antioxidant in vitro capable of inhibiting oxidative damage to both cells, proteins and lipoproteins [6–12].

The level of neopterin in biological fluids is measured either by ELISA [13] or by HPLC with fluorescence detection of the highly fluorescent neopterin. Though the initial purchase price of a HPLC is relatively high compared to ELISA equipment, the cost in consumables for each individual sample is relatively inexpensive making it the method of choice in many research laboratories.

HPLC analysis of plasma or tissue requires the removal of proteins. Original methods used ion exchange solid phase extraction to collect and concentrate the neopterin [14,15], but with changes to solid phase manufacturing these methods appear to have become unreliable and technically demanding. The more common methods now employed remove sample proteins by acid precipitation prior to HPLC analysis [16]. However, under acidic conditions, 7,8-dihydroneopterin is oxidised to neopterin with varying yield [14]. This oxidation can be partially prevented by the addition of ascorbate prior to acid precipitation but is not completely effective. Total neopterin levels (7,8-dihydroneopterin + neopterin) are usually determined by oxidising the 7,8-dihydroneopterin to highly fluorescent neopterin with an acidic iodide solution. We here report that the acid precipitation step also removes or co-precipitates varying levels of pterin with the plasma proteins which results in an under-estimation of neopterin and total neopterin levels in plasma. The use of the solvent acetonitrile to precipitate plasma proteins appears to eliminate these problems.

Abbreviation: TCA, trichloroacetic acid.

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2. Experimental

2.1. Chemical and reagents

Chemicals and reagents were AR grade or better and, unless otherwise stated, were obtained from either Sigma Chemical Company (USA) or BDH Chemicals New Zealand Ltd. Neopterin and 7,8-dihydroneopterin was supplied by Schirck's Laboratories (Switzerland). All solutions were prepared with high purity water from a NANOpure ultrapure water system, supplied by Barnstead/Thermolyne (IA, USA).

2.2. Sample collection

Blood samples were obtained by venipuncture from 10 randomly selected septicemia patients and 5 healthy control subjects. Septicemia patients were undergoing aminoglycoside therapy and blood samples were collected as part of their routine monitoring. All blood samples were surplus to clinical requirements and were anonymised before release for neopterin analysis as part of the diagnostic laboratory ethics approval for assay validation or establishing a reference range. This study has been approved by the Upper South A Regional Ethics Committee, New Zealand. Plasma was shielded from light and processed under red light illumination to prevent oxidative loss by UV light. The plasma was prepared by centrifugation and stored at −80 °C until analysis.

2.3. Precolumn plasma preparation

Where possible all treatments were carried out under red light illumination to prevent oxidative loss by UV light. Two differing sample preparation methods were employed for neopterin measurement. The first involved acid precipitation of protein by adding 100 μL of plasma, 10 μL of 0.6 M ascorbate and 10 μL of 50% trichloroacetic acid (TCA) followed by vortexing then centrifugation (4 °C and 10,300 × *g* for 15 min); 100 μL of the acid supernatant was then placed in an autosampler vial for HPLC analysis. The second method employed acetonitrile to precipitate the protein. 100 μL of plasma was combined with 100 μL of 100% acetonitrile, vortexed and centrifuged (4 °C and 10,300 × *g* for 10 min). 100 μL of the supernatant was then transferred to an autosampler vial for HPLC analysis.

For total neopterin analysis an oxidation step was included to convert 7,8-dihydroneopterin to neopterin for detection following the protein precipitation and centrifugation steps. 10 μL of acidic iodide solution (5.4% I_2/10.8% KI in 1 M HCl) was added to the supernatant and incubated for 20 min at room temperature in the dark. 10 μL of 0.6 mM ascorbate was added to oxidise the iodine before centrifugation of the samples (4 °C and 10,300 × *g* for 5 min). With the TCA treatment method, the ascorbate was not added before protein precipitation during total neopterin analysis. Except during the 7,8-dihydroneopterin oxidation to neopterin, all sample solutions were kept between 0 °C and 4 °C.

2.4. HPLC analysis

HPLC measurement of neopterin was performed using a Shimadzu Sil-10A HPLC with autosampler and RF-10Axls fluorescence detector [17]. 10 μL of sample was injected onto a Phenomenex Develosil reverse phase ODS-MG-5 4.2 mm × 250 mm column with a mobile phase of 5% methanol in 20 mM ammonium phosphate pH 6.0 pumped at 1 mL/min. Neopterin was detected by its native fluorescence at 438 nm, excitation 353 nm. The concentration and identity of the eluted neopterin was confirmed by comparison to a commercial standard and quantified by peak area using Shimadzu Class VP software. All analysis was conducted in triplicate and data is displayed as the mean ± the standard error of the mean of triplicate treatments.

3. Results and discussion

The use of acetonitrile to remove plasma proteins markedly improved the resolution and signal to noise ratio seen during HPLC compared to TCA treatment (Fig. 1). TCA-treated samples also showed a number of additional contaminant peaks during chromatography. With the acetonitrile-treated samples, neopterin was consistently observed to elute 1 min sooner than the neopterin from TCA-treated samples due to the presence of 50% acetonitrile in the injected sample. The neopterin peak identity was confirmed by spiking plasma samples with authentic neopterin. The recovery of this added neopterin was 100% showing that the changed elution time was not effecting the peak height.

In the acetonitrile-treated sample's chromatogram (Fig. 1B), the neopterin peak area is smaller than that seen in the chromatogram of the TCA-treated sample (Fig. 1A). This is due to the dilution of sample which occurs with the acetonitrile treatment. When dilution factors are taken into account the apparent concentration of neopterin in the plasma from the TCA-treated sample is 31.4 nM and in the acetonitrile-treated sample 55.3 nM. This apparent increase in neopterin levels with acetonitrile treatment was generally observed with all plasma samples examined. This increase in neopterin with acetonitrile treatment was not observed using protein free buffers suggesting the effect was due to the actual precipitation of the protein.

The linearity of the acetonitrile treatment and analysis was confirmed by measuring different concentrations of neopterin between 5 nM and 2 μM. The calibration curve ($y = 1.019x - 0.015$) was linear over this range with a correlation coefficient of $r^2 = 1.00$. Within-run precision, evaluated by 10 consecutive injects from the same plasma sample, and between-run precision, evaluated by injects on 6 separate days from the same plasma pool, showed repeatability of the assay is good (within-run CV% < 2.9; between-run CV% < 3.8).

Acidic conditions have been previously reported to cause significant oxidation of 7,8-dihydroneopterin to neopterin [14]. With TCA we observed up to 60% of 7,8-dihydroneopterin being oxidised to neopterin (data not shown). Similar problems were also encountered using perchloric acid. The effect of this oxidation would not be apparent when dealing with

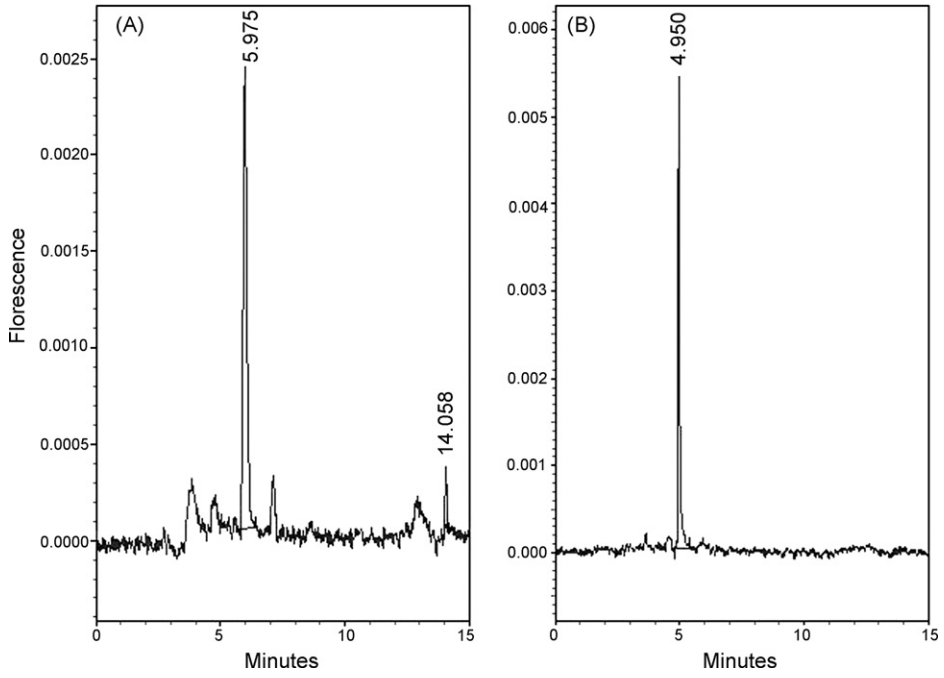

Fig. 1. HPLC chromatograms of plasma neopterin (without iodine oxidation) from (A) TCA- and (B) acetonitrile-treated plasma sample from a selected septicemia patient.

neopterin samples alone, but would cause a substantial underestimate of 7,8-dihydroneopterin concentrations and a slight additional overestimate of neopterin when working with biological samples. Ascorbate was found to reduce this oxidation but was not completely effective. This effect of ascorbate was previously described by Werner et al. [14]. Therefore acetonitrile was examined as an alternative to acid precipitation of proteins. We found that 7,8-dihydroneopterin was not oxidised by treatment with acetonitrile making it a more suitable reagent for protein precipitation (data not shown).

Analysis of 10 septicemia patients and 5 apparently healthy controls showed the acetonitrile treatment consistently returned higher levels of neopterin and total neopterin than the acid based treatment (Figs. 2 and 3). The acetonitrile precipitation treatment gave on average a 20 nM increase in neopterin levels compared to that obtained using TCA for both the healthy controls and septicemia patients (Figs. 2A and 3A). However, with total neopterin analysis, where the 7,8-dihydroneopterin is oxidised to neopterin, the increase in neopterin due to the acetonitrile treatment, compared to TCA, was less consistent (Fig. 3B). With healthy controls acetonitrile treatment gave on average a 50% increase in total neopterin compared to TCA, but this increase ranged from 4.3 nM to 12.9 nM.

With septicemia patients the measured total neopterin level also increased with acetonitrile treatment (Fig. 2B) but by approximately 100% compared to the TCA-treated samples. The size of this increase ranged from 15 nM to 120 nM.

TCA treatment shows a picture of healthy subjects having the majority of the pterin as 7,8-dihydroneopterin (75%) (Fig. 4) which is close to the published 2:1 ratio [18]. In the septicemia patients, this ratio is reversed where the majority of the pterin is neopterin (83%). This does agree with the hypothesis that

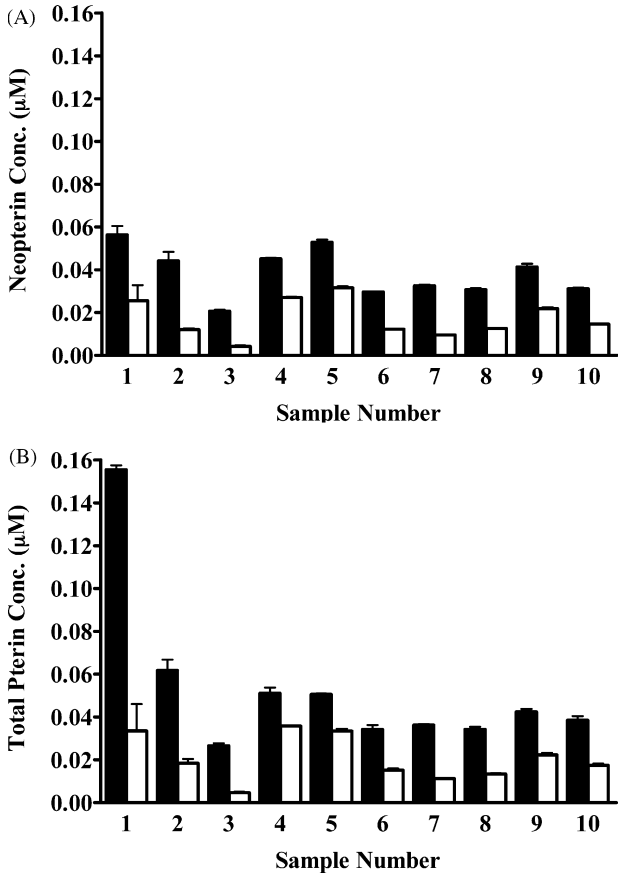

Fig. 2. Plasma neopterin (A) and total neopterin (B) concentrations for 10 septicemia patients. Samples were prepared for analysis using either acetonitrile (■) or TCA (□) for protein precipitation before HPLC analysis as described in Section 2. Values graphed are the mean + S.E. of three replicates for each sample.

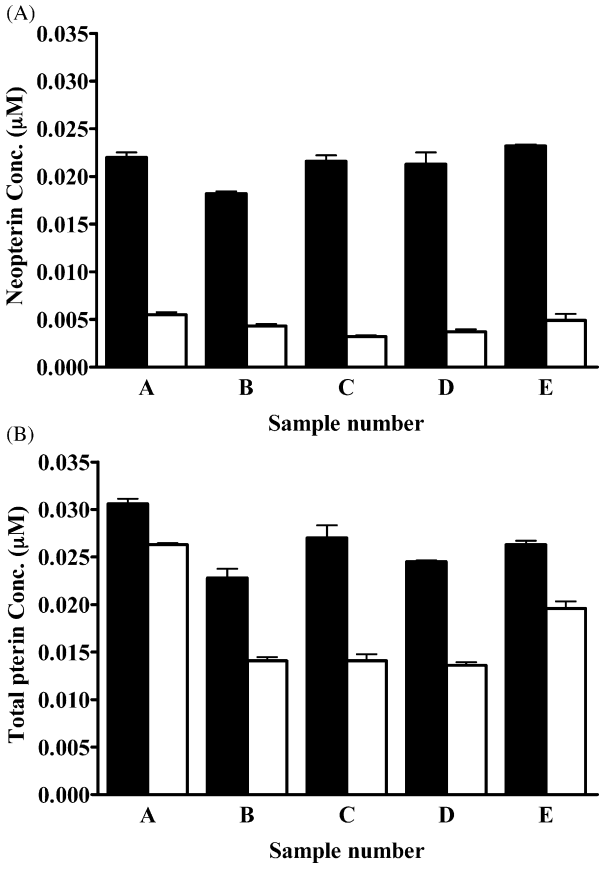

Fig. 3. Plasma neopterin (A) and total neopterin (B) concentrations for five apparently healthy control subjects. Samples were prepared for analysis using either acetonitrile (■) or TCA (□) for protein precipitation before HPLC analysis. Values graphed are the mean + S.E. of three replicates for each sample.

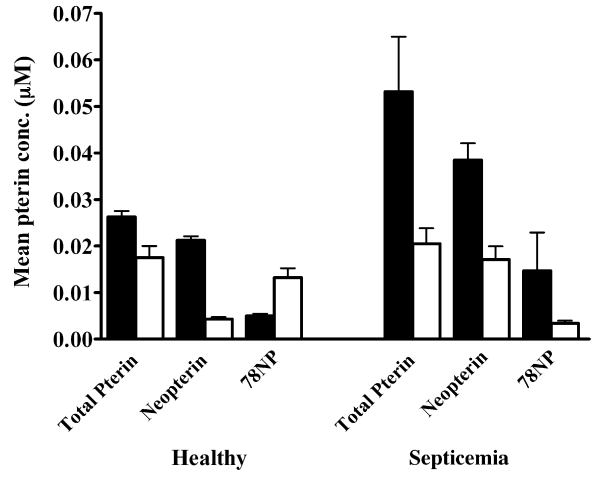

Fig. 4. Mean plasma total neopterin, neopterin and 7,8-dihydroneopterin concentrations for the two experimental groups (septicemia and healthy controls). The 7,8-dihydroneopterin concentration for each sample was calculated by subtracting the total neopterin concentration from the plasma neopterin concentration. The figure shows the mean of the acetonitrile-treated (■) and TCA-treated (□) samples from Figs. 2 and 3.

7,8-dihydroneopterin can be oxidised to neopterin by oxidants produced during inflammation. However, the TCA data does not show a significant overall increase in total neopterin when comparing the healthy controls and the septicemia patients (Fig. 4). This is not consistent with what is known about pterin release from macrophages during the immune response [1,19]. The acetonitrile data shows the overall level of pterins in the plasma increasing 203% with the level of 7,8-dihydroneopterin increasing from 19% to 28% which would be consistent with increased production by macrophage cells, especially if one of the roles of 7,8-dihydroneopterin is to provide protection to cells and biomolecules [9,20,21]. The overall increase in neopterin/7,8-dihydroneopterin is also consistent with the hypothesis that neopterin increases the potency of various cytotoxic agents [22–24].

The variation in levels of neopterin and total neopterin between the acetonitrile-treated and TCA-treated plasma samples cannot be fully explained by the acid-induced oxidation of 7,8-dihydroneopterin to neopterin, as the levels of both increased dramatically with the acetonitrile treatment. We suggest that in addition to the acid oxidation, a varying level of pterin is co-precipitating with the serum proteins and is lost from the sample.

Our data suggests that the current threshold of 10 nM, above which inflammation is suspected, should be raised when using acetonitrile for protein removal. The level at which this threshold is set will require further analysis of a larger pool of healthy subjects than presented here.

4. Conclusion

The use of acetonitrile rather than acidic conditions to precipitate and remove plasma proteins provides a significant increase in the amount of total neopterin and neopterin measured in clinical samples. The procedure also allows the accurate calculation of plasma 7,8-dihydroneopterin levels as this compound is not oxidised to neopterin during acetonitrile treatment. The chromatography of the acetonitrile-treated samples is also significantly improved without contamination from TCA.

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