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Assay of neopterin in serum by means of two-dimensional high-performance liquid chromatography with automated column switching using three retention mechanisms

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Abstract

An automated two-dimensional HPLC method for the determination of neopterin in serum is described. Neopterin is separated from proteins on a short octadecylsilica column by size exclusion and from the majority of the other serum components by adsorption. The fraction containing neopterin is transferred by column switching to a solvent-generated cation-exchange column using dodecylsulfonic acid as surface activator. Parameters influencing the separation performance and sensitivity of the fluorescence detection are discussed. The efficiency of the cleaning of the first column was optimized. The method was validated. It achieves a precision of 1% (R.S.D.) and a detection limit of about 0.3 nmol/l. The accuracy is nearly 100%. The method allows a high sample throughput, requiring 15 min per sample.

1. Introduction

During the last 15 years, neopterin has proved to be a marker for the cellular immune response and is used for diagnosis and therapy control in clinical practice [1]. It is released primarily by human macrophages after stimulation by interferon-gamma [2] and indicates the state of activation of the cellular immune system. The measurement of neopterin levels was developed for urine [3,4], serum [5–10] cerebrospinal fluid [11–14], synovial fluid [15,16] and saliva [17,18]. The assays were performed by HPLC [5–10,19–21] or immunological methods such as radioimmunoassay (RIA) [22–25] and enzyme-linked immunosorbent assay (ELISA) [26,27]. Because

of their easy handling and high sample-throughput capacity, immunoassays are suitable for daily clinical routine analysis. On the other hand, they suffer from inherent cross-reactivity. HPLC is therefore mainly used as a reference method.

In the analysis of serum samples by HPLC, plasma proteins are a common problem. Attempts have therefore been made to remove these proteins by ultrafiltration [20], micropartitioning [21] or on-line column deproteination using cation-exchange cartridges [10]. The final isolation of neopterin is usually carried out on an octadecylsilica column using an aqueous buffer system [5,10,20] or by ion-pair chromatography with cetyltrimethylammonium bromide [28], dodecylsulfonic acid [9] or octansulfonic acid [29], which requires extensive sample clean-up. A powerful approach is a two-stage HPLC, in

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which two octadecyl columns are connected by a switching valve. Neopterin is separated from macromolecules and most of the other compounds on the first column. The separation from other interfering compounds is completed on the second column [5].

Based on previous work [30], this paper deals with the automation and optimization of the two-dimensional automated HPLC column-switching method for the direct measurement of neopterin in serum without the need for sample clean-up.

2. Experimental

2.1. Chemicals

For the preparation of standard solutions, analytical-reagent grade neopterin (Fluka, Buchs, Switzerland), sodium dihydrogenphosphate, sodium azide and sodium hydroxide (Merck, Darmstadt, Germany) were used. Water was purified using a Model UHQ high-performance water-purification system (Millipore, Bed-

ford, MA, USA). Neopterin standard samples in aqueous buffer solution and serum were used for calibration (Henning, Berlin, Germany).

For HPLC analysis, acetonitrile (LiChrosolv; Merck) and analytical-reagent grade sodium acetate, acetic acid, sodium dodecyl sulfonate (SDS), 1-propanol, 2-propanol, hydrochloric acid and pure ethanediol (Merck) were used.

2.2. Instrumentation

The determination of neopterin was carried out by two-dimensional chromatography with column switching using two separation columns with different selectivity (Fig. 1).

In the first dimension, a high-pressure pump with a low-pressure gradient former (Model L-6200A; Merck) was used. Samples were injected into the separation system by an intelligent autosampler (Model AS 4000; Merck). Separation was performed on a 35×4 mm I.D. stainless steel column packed with octadecylsilica (ODS) of $5\text{-}\mu\text{m}$ particle size (LiChrospher 100 RP-18; Merck). For some experiments a 50×4

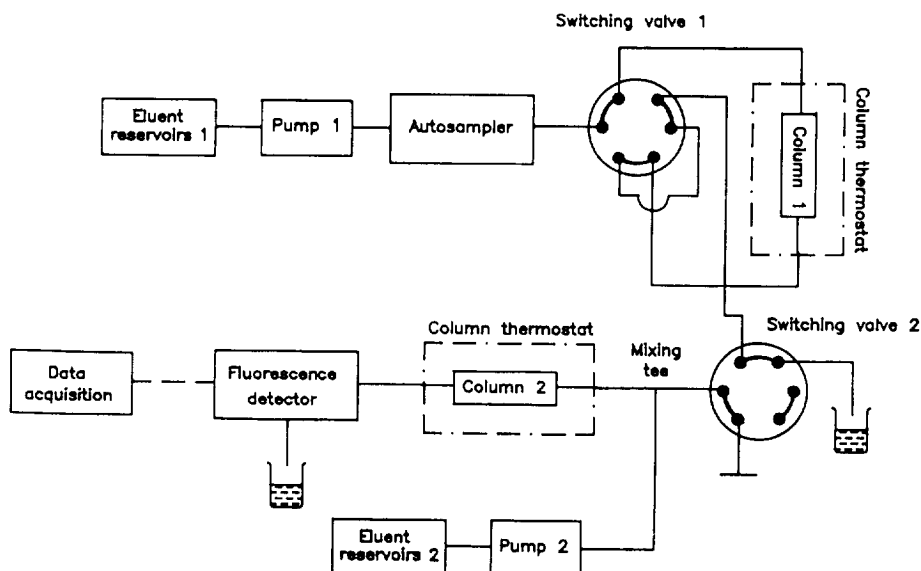


Fig. 1. Schematic diagram of the HPLC apparatus used.

mm I.D. column, packed with the same material, was used. The column was thermostated at 15°C by a column thermostat (W.O. Industrial Electronics, Langenzersdorf, Austria).

Back-flushing and column switching were performed by two electrically driven switching valves (Model ELV 7000; Merck Vienna, Vienna, Austria). For method development, a diode-array detector (Model L-6500; Merck) was coupled to the outlet of the first column.

Eluent for the second dimension was delivered by a high-pressure pump (Model L-6200; Merck). Separation of the transferred fraction was achieved on a 125 × 4 mm I.D. stainless-steel column (LiChroCART; Merck) packed with ODS (LiChrospher 100 RP-18) of 5-μm average diameter and thermostated by a column thermostat (Model T-6300; Merck). Detection was performed with a fluorescence detector (Model F-1000; Merck). The detector signal was digitized by a chromatographic data interface (Model 763; Nelson Analytical, Cupertino, CA, USA). Data were stored and processed on a Vectra personal computer (Hewlett-Packard, Waldbronn, Germany) by use of chromatographic software (Nelson Analytical).

2.3. Operation

Sample preparation

Blood was allowed to clot at 37°C for 30 min, centrifuged at 2000 g for 15 min and stored at -20°C. Before analysis, the serum samples were centrifuged for 3 min at 10 000 g. Exposure of standards and samples to sunlight was strictly avoided and samples were handled in dim light.

Preparation of neopterin standards in aqueous buffer solution

A 400–700 μg amount of neopterin was dissolved in 10 ml of 2 mM sodium hydroxide solution and a solution of 0.1 M phosphate buffer, containing 0.1% (w/v) sodium azide and 4 mM sodium chloride, was added to give a final volume of 50 ml. Further dilutions were prepared, portioned and stored at -20°C.

Separation and detection

After sample injection into the first column by the autosampler, data acquisition and the programme of pump 1 (Table 1) were started. Column switching was initiated 0.2 min before peak start and ended 0.4 min after peak end. To

Table 1
Linear gradient programme for column 1

Time (min)	Action
0.0	Injection of 20 μl of sample; start of programme; eluent 100% A, flow-rate 0.8 ml/min
1.0	Column 1 connected to column 2
2.2	Column 2 disconnected, reversal of flow in column 1 for back-flushing
2.5	Start of linear cleaning programme, 100% A, flow-rate 1.0 ml/min
3.0	Eluent: 70% A–15% B–15% C
3.9	Eluent: 45% A–25% B–30% C
4.9	Eluent: 20% A–45% B–35% C
5.3	Eluent: 80% B–20% C
5.5	Eluent: 80% B–20% C
6.0	Eluent: 100% A
8.0	Re-equilibration of column 1
9.5	Flow-rate 1.5 ml/min
14.2	Reversal of flow in column 1 for separation
15.0	Flow-rate 0.8 ml/min, end of cycle

Eluent: (A) aqueous solution of 9 mM sodium acetate buffer adjusted to pH 5.1 with acetic acid; (B) ethanediol–2-propanol (75:25, v/v); (C) acetonitrile–1-propanol (65:35, v/v).

Table 2
Influence of the flow-rate of pump 2 during the switching interval on peak broadening

Flow-rate of pump 2 during column switching (ml/min)	Peak variance, $\sigma_{\text{column 1+2}}^2$ (10^{-2} min^2)
0.0	2.92
0.2	3.06
0.4	3.27
0.6	3.38
0.8	3.42

First dimension: column bed 50×4 mm I.D., eluent aqueous solution of 0.6 g/l NaH_2PO_4 (pH 6.0); flow-rate, 0.8 ml/min; column temperature, 15.0°C, injection volume, 20 μl of neopterin standard solution; switching interval, 1.4–2.4 min. Second dimension: conditions as in Experimental except temperature, 32°C.

Variance of the neopterin peak at the outlet of column 1: $1.76 \times 10^{-3} \text{ min}^2$; $\sigma_{\text{column 1+2}}^2 = \sigma_{\text{column 1}}^2 + \sigma_{\text{column 2}}^2$. Retention time of neopterin on column 2: 9.92 ± 0.14 min.

minimize peak broadening, pump 2 was stopped during column switching (Table 2). An aqueous buffer of sodium acetate, containing organic solvents for cleaning of the column, was used as the eluent. Cleaning started immediately after column switching by back-flushing the column and applying a cleaning gradient. Re-equilibration of the column was performed at an increased flow-rate. The column was thermostated at 17°C. Because of the high degree of automation, unattended overnight analysis was possible.

The eluent for column 2 was 0.1 g/l SDS solution adjusted to pH 4.0 with acetic acid. It converts the original ODS packing into an SDS cation exchanger because of the adsorption of SDS [31]. Changes of the retention time were made by altering the temperature and changes of selectivity by pH adjustment. Minimal peak broadening was achieved when pump 2 was stopped during the switching interval (Table 2). The flow-rate was set to 1.2 ml/min and the temperature of the column thermostat to 30°C. Fluorescence detection was performed at an excitation wavelength of 353 nm by measuring the emission at 438 nm.

3. Results and discussion

3.1. Retention on column 1

In the first dimension, neopterin was separated from most of the serum components, such as serum proteins and less polar serum constituents. To achieve a short analysis time, the column length and operating parameters influencing the resolution, cleaning and re-equilibration of the column were optimized.

Aqueous acetate buffer was used as the eluent. Other buffer salts such as citrate, phosphate or phosphate-EDTA were found to have a similar influence on the retention of neopterin in the pH range 5–7. Spherical octadecylsilica with a 5- μm particle size was used as an adsorbent. A column of 3.5 cm length proved to give sufficient resolution for the separation of neopterin from most of the accompanying components and permitted a high sample throughput. A chromatogram of the separation of serum on column 1 is shown in Fig. 2. The retention time of neopterin was determined by injection of spiked serum and standard solutions.

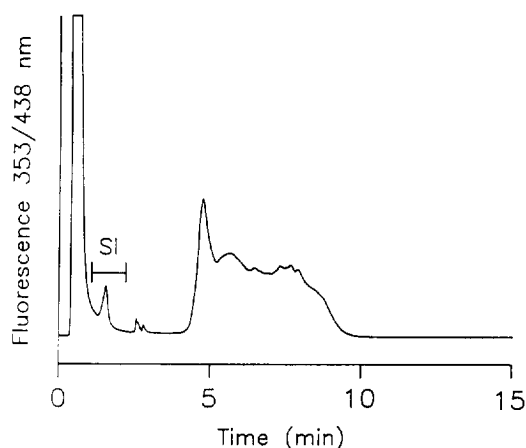


Fig. 2. Separation of serum on column 1. Conditions: eluent gradient programme as in Table 1; column, 35×4 mm I.D. packed with 5- μm LiChrospher 100 RP-18; injection volume, 20 μl of serum; temperature, 25°C; fluorescence detection at 353/438 nm. SI = switching interval.

Effect of sample solvent

The retention differences for neopterin dissolved in 0.01 and 0.1 M phosphate buffer and serum on an equilibrated column with aqueous eluents containing 0.001–0.1 M sodium acetate were less than 5%. To keep the analysis time short, the column is not fully equilibrated by pumping through only a low eluent volume. Therefore, in practice retention of neopterin depends on the cycle time (Fig. 3). On an equilibrated column, and also on a non-equilibrated column, the retardation of neopterin in serum is higher than in buffer solution. To achieve reproducible retention it is necessary to keep the cycle time constant. The reproducibility of the retention time for neopterin in serum was found to be better than 0.8% (R.S.D.) ($n = 30$).

Influence of temperature

The retention of neopterin decreased with increasing temperature (Fig. 4). A significant and almost linear drop was observed above 20°C. Below 20°C the retention dropped only slightly with increasing temperature. Identical results were obtained by thermostating the column in an air or water bath. Repeated injections of serum reduced the retention of neopterin owing to deactivation of the surface of the adsorbent. Minimum changes were observed at 35°C.

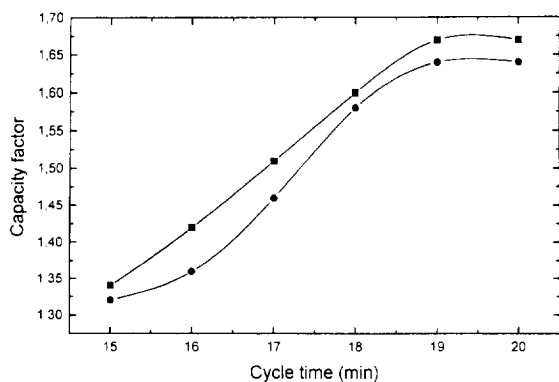


Fig. 3. Dependence of the retention of neopterin on column 1 on the reconditioning time. Conditions as in Fig. 2. Sample: ■ = neopterin in serum; ● = neopterin in 0.1 M NaH₂PO₄.

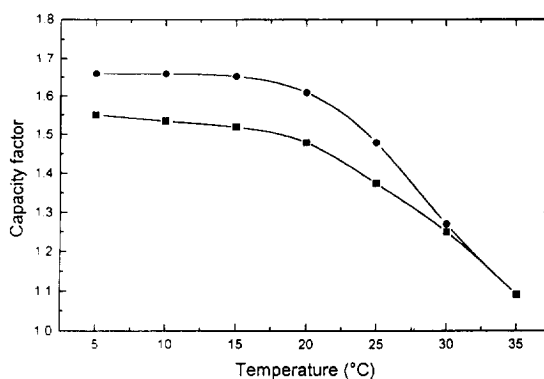


Fig. 4. Dependence of retention of neopterin on column 1 on temperature. ● = Before injection of serum; ■ = after 50 serum samples. Conditions as in Fig. 2, except column, 50 × 4 mm I.D.; flow-rate, 1.0 ml/min; cycle time, 18 min.

Cleaning of the column

To maintain the column performance it is necessary to keep the surface contamination low by cleaning the column after each injection of serum. For direct injection of untreated serum the main problem is the adsorption of plasma proteins, which are responsible for an increased back-pressure and decreased column performance. Using a strongly hydrophilic eluent and a non-polar adsorbent, many of the high-molecular-mass proteins may be partly denatured when they come into contact with the surface of the adsorbent. Non-polar parts, which are buried in the inner region of the folded molecule, cause partial unfolding in order to become accessible to the adsorption sites on the adsorbent [32].

For cleaning of the ODS column, organic solvents such as acetonitrile and methanol are frequently used. They have a high elution strength but are also strongly denaturing solvents for proteins. Especially acetonitrile acts as a strongly denaturing agent at concentrations above 50%. Denatured proteins elute as sharp peaks at high concentrations of organic modifier but cause a significant increase in the column back-pressure after each cycle. The pressure increase can be kept low when cleaning is carried out with a slow gradient [30]. The aim was

therefore to develop a short washing procedure for the rapid and efficient removing of adsorbed serum components under mild and non-denaturing conditions for proteins. This was achieved by back-flushing the column and applying a combination of non-denaturing solvents with good solubility properties and high elution strength.

In the first part of the cleaning gradient, stabilization of the protein conformation without further denaturation was sought. As polyalcohols are less denaturing than monoalcohols [33], ethanediol was chosen for this purpose. The main problem is the high back-pressure, due to the viscosity of the eluent. An overview of several cleaning gradients is given in Table 3. The efficiency of the cleaning procedure and its influence on system stability can be seen from the level of the baseline and the column back-pressure after cleaning, reflecting the removal of contaminants from the column. Peak asymmetry and theoretical plate height were not used as indicators for column contamination because irreproducible results were observed in this respect.

The increase in the column back-pressure is mainly caused by the inlet frit of the column, and in the case of a linear gradient of acetonitrile–methanol also by the outlet frit. Frits producing

an increased pressure drop can be reused after heating in 6 M hydrochloric acid. The addition of 1-propanol instead of methanol to acetonitrile reduces the pressure increase to an acceptable level after each injection. The addition of ethanediol in the cleaning gradient leads to a significant improvement of the cleaning efficient. The best results are obtained by reducing the concentration of acetonitrile in the gradient to 50% in favour of 1-propanol and by the addition of 2-propanol to ethanediol.

3.2. Retention on column 2

Solvent-generated ion exchange is used as the separation mechanism in the second column. Dodecylsulfonic acid is applied as an additive in the mobile phase. The parameters were optimized with regard to separation performance and detection sensitivity. Chromatograms of switched serum fractions on column 2 are shown in Fig. 5.

Influence of pH

Retention is achieved by adsorption of SDS on the ODS surface, dynamically creating a cation exchanger. For the amino group of neopterin, a pK_a of 5.7 was found by titration (Fig. 6A). At $pH < 5.7$ the protonation of neopterin gives a

Table 3
Efficiency of different cleaning procedures

Eluent	Column back-flush	Cycle time (min)	No. of samples injected	Pressure increase (bar per sample)	Baseline increase (μV per sample)
Linear gradient a ^a [B = ACN–MeOH (90:10, v/v)]	Yes	17	46	1.8	580
Linear gradient b ^b [B: ACN–1-propanol (80:20, v/v)]	Yes	20	45	0.6	700
Linear gradient c ^c [B = ethanediol; C = ACN–1-propanol (80:20, v/v)]	No	15	62	0.8	200
[B = ethanediol–2-propanol (75:25, v/v); C = ACN–1-propanol (65:35, v/v)]	Yes	20	73	0.5	80
	Yes	16	63	0.4	36

Conditions as in Experimental: eluent A, aqueous buffer of 9 mM sodium acetate (pH 5.1).

^a 0–2.5 min 100% A, 2.5–5.0 min to 100% B, 5.0–5.5 min 100% B.

^b 0–2.5 min 100% A, 2.5–4.5 min to 100% B, 4.5–4.7 min 100% B.

^c 0–2.5 min 100% A, 2.5–3.0 min to 70% A–15% B–15% C, 3.0–3.9 min to 45% A–25% B–30% C, 3.9–4.9 min to 20% A–45% B–35% C, 4.9–5.3 min to 80% B–20% C, 5.3–5.5 min 80% B–20% C, 5.5–6.0 min to 100% A.

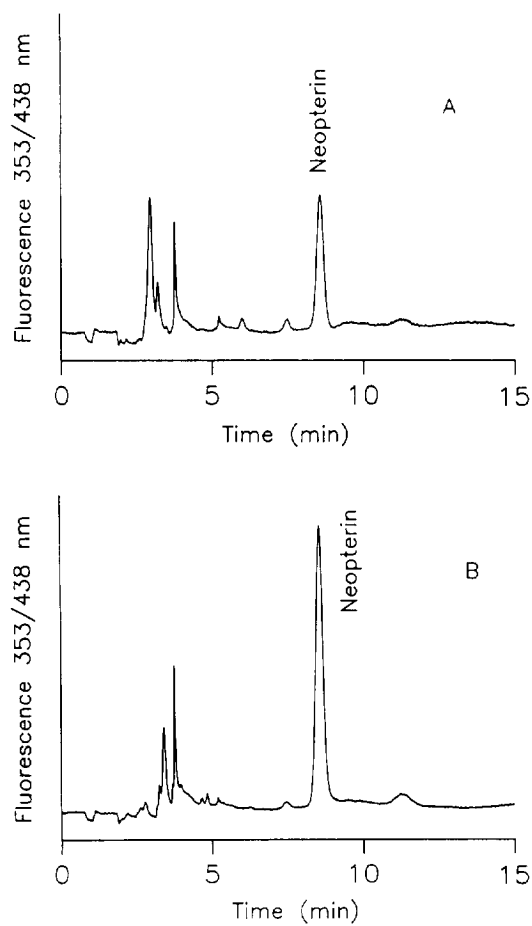


Fig. 5. Chromatograms of serum fractions containing neopterin switched from column 1 to column 2. Conditions: eluent, 0.1 g/l SDS adjusted to pH 4.0 with acetic acid; column, LiChroCART (125 × 4 mm I.D.) packed with 5- μ m LiChrospher 100 RP-18; flow rate, 1.2 ml/min; temperature, 30°C; detection, fluorescence at 353/438 nm. (A) Normal neopterin level (3.6 nmol/l); (B) elevated neopterin level (14.2 nmol/l).

cation which can replace the proton of the adsorbed SDS. With decreasing pH the retention increases (Fig. 7), but at the same time the quantum efficiency of fluorescence decreases (Fig. 6B). To attain a high signal response, it is best to perform the separation at pH \geq 4.0.

The pH of the eluent is adjusted by addition of acetic acid. Initially trifluoroacetic acid was used, but it induced the formation of a yellowish to brownish coating on the adsorbent, probably due

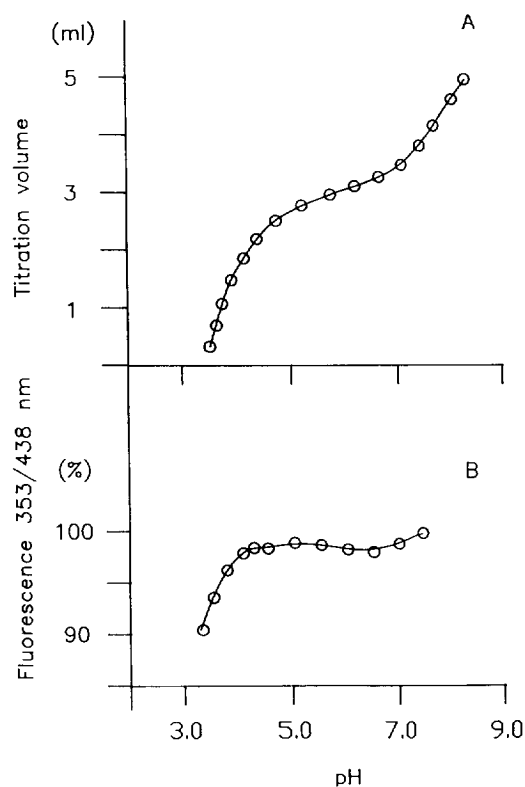


Fig. 6. Protonation and quantum efficiency of neopterin as a function of pH. (A) Titration curve of 7 mg of neopterin in aqueous solution with 0.03 M NaOH; (B) fluorescence response of neopterin in 9 mM sodium acetate.

to denatured proteins. Cleaning of the column could be accomplished with 1-propanol–acetic acid–water (60:30:10, v/v/v). Coating of the column was not observed with acetic acid.

Influence of temperature

The retention is further strongly influenced by temperature (Fig. 7). The capacity factor is halved on increasing the temperature from 25 to 45°C. Thermostating of the column is therefore required and temperature can be used to optimize the separation.

Effect of the SDS concentration

The retention of neopterin depends on the concentration of the surface-active additive SDS (Fig. 8). Maximum retention can be achieved at

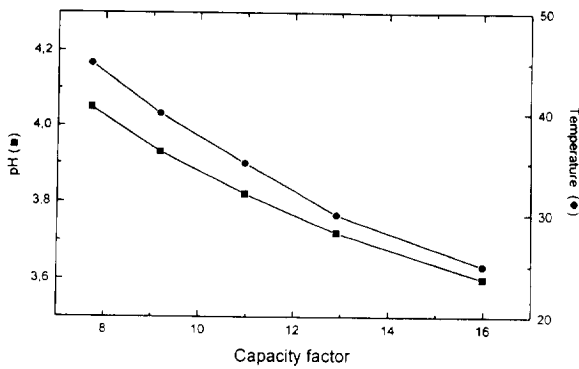


Fig. 7. Dependence of the capacity factor of neopterin on pH and temperature on column 2. Conditions as in Fig. 5, except for pH. (■) Eluent, 0.8 g/l SDS + 0.5% (v/v) MeOH; injection volume, 20 μ l of neopterin standard solution; temperature, 35°C. For temperature pH = 3.8; other conditions as for pH curve.

an SDS concentration of 0.075 g/l. The addition of methanol for better wetting of the adsorbent leads to a considerable decrease in the retention of neopterin owing to competitive adsorption of methanol. Changing the methanol content from 0 to 2% (v/v) decreases the retention of neopterin by a factor of 2.2.

Disturbances because of switching

The equilibrium in column 2 is disturbed by the transferred fraction depending on its volume. The ionic strength and pH influence the re-

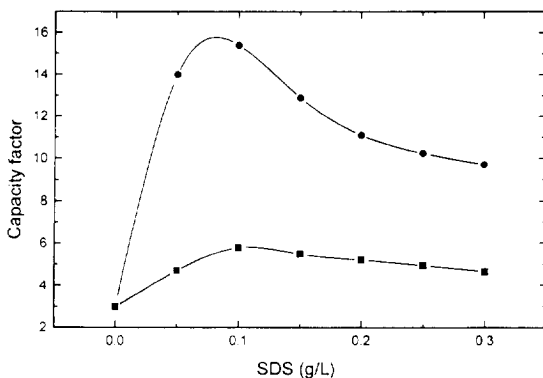


Fig. 8. Dependence of retention of neopterin on column 2 on SDS concentration. Conditions as in Fig. 5, except flow-rate, 0.8 ml/min; injection volume, 20 μ l of neopterin standard solution; temperature, 35°C. ● = pH 3.8; ■ = pH 4.0, 0.5% (v/v) MeOH.

tention of neopterin. The smallest influence is observed at a low ionic strength and low pH of the fraction. The local disturbance of the ion-exchange equilibrium and the short equilibration time make it necessary to keep all parameters constant in order to achieve a reproducible retention of neopterin. Under these conditions the reproducibility of the retention time is better than 0.6% (R.S.D.) ($n = 30$).

Blank reading

A blank reading of neopterin on column 2 is created by the first dimension of separation. It is caused by depositions of neopterin in the switching and injection valve. The contamination of the system can be reduced by cleaning procedures, but the best results are obtained by adjusting the pH of the eluent to 5.1. In comparison with an eluent of pH 6.1, a 90% decrease in the blank reading is found. During the analysis of a series of samples the neopterin background remained fairly constant under these conditions.

3.3. Column lifetime

Degradation of the column performance is primarily indicated by additional peak broadening, increased peak asymmetry and decreased retention. The end of the column lifetime is characterized by the occurrence of peak splitting.

First column

Applying the above-described conditions, at least 100 samples corresponding to 2 ml of serum can be injected before peak splitting is observed. When the column is back-flushed only during the cleaning cycle, the column lifetime is reduced to 80 samples. Overnight cleaning of a heavily used column with 1-propanol–formic acid–water (60:30:10, v/v/v) results in a slight improvement in peak performance, but the column lifetime could not be significantly improved.

Second column

Degradation of the second column is mainly caused by the components of the transferred fraction. Especially proteins interact with the

Table 4
Recovery of neopterin from spiked serum by column switching

Neopterin ^a concentration (nmol/l)	Amount spiked (nmol/l)	Concentration found (mean \pm S.D., $n = 5$) (nmol/l)	Precision (R.S.D.) (%)	Accuracy ^b (%)
9.01	4.92	8.86 \pm 0.13	1.4	98.2
13.54	9.45	13.38 \pm 0.13	1.0	98.8
21.49	17.40	21.45 \pm 0.17	0.8	99.8

Conditions as under Experimental.

^a Neopterin concentration in the unspiked sample: 4.09 nmol/l.

^b Accuracy = (found concentration/true concentration) · 100%.

adsorbed SDS and are strongly retained. Using a 35 \times 4 mm I.D. column in the first dimension, 80–90 serum samples can be analysed on the second column before cleaning of the column becomes necessary. The sample capacity can be increased by using a longer column in the first dimension, thereby increasing the resolution of neopterin from accompanying compounds and consequently reducing the amount of other transferred components. However, with this approach the separation time increases.

3.4. Method validation

Calibration factor

The chromatographic system was calibrated by injection of standard samples of neopterin in aqueous buffer solution in the range 1.5–517 nmol/l ($n = 8$). A linear calibration function was obtained with a slope of 9512 ± 18 counts/(nmol/l), an intercept of 6833 ± 1222 counts and a correlation coefficient of 1.0000. An overall standard deviation of 2518 counts can be estimated for the calibration function.

Equal slopes of the calibration function were found for switched standard samples and samples injected directly on to the second column. Neopterin is quantitatively transferred by column switching (Table 4) and no evidence of adsorption of neopterin on proteins could be found, as reported for other pterins [34]. The calibration and system performance were checked at regular intervals after 10–16 serum samples by injection of control samples of neopterin in buffer solution and serum.

Table 5
Reproducibility of the method ($n = 5$)

Concentration of neopterin in serum (nmol/l)	Precision (%)	
	Within-day	Day-to-day
4.5	1.1	0.8
8.7	1.4	1.5
14.4	1.7	2.2
20.8	0.7	0.7
102	1.7	1.2

Conditions as under Experimental.

Precision and detection limit

The precision of the HPLC method for the determination of neopterin in serum was about 1% for signal-to-noise ratios ≥ 100 and more

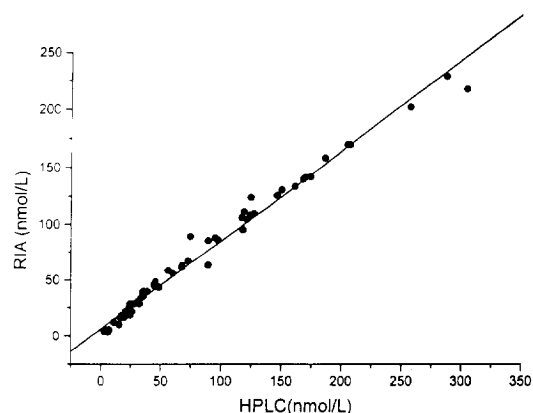


Fig. 9. Method comparison plot for the assay of neopterin in serum by RIA and HPLC ($n = 61$). Least-squares parameters: slope = 0.791, y -intercept = 6.40, correlation coefficient = 0.9926.

(Table 5). The detection limit for a signal-to-noise ratio of 3 was found to be 0.3 nmol/l.

Accuracy

A comparison of the results obtained by HPLC and by radioimmunoassay (RIA) is shown in Fig. 9. It can be seen that the concentrations found by RIA are smaller by a factor 0.791 than those obtained by HPLC. It may be assumed that HPLC is more accurate than RIA and can be used as a reference method.

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