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Determination of neopterin, kynurenine, tryptophan and creatinine in human serum by high throuput HPLC

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ABSTRACT

A new HPLC method for simultaneous determination of neopterin, creatinine, kynurenine and tryptophan in human serum was developed and validated. Monolithic stationary phase's technology (two monolithic columns RP-18e were connected with guard monolithic cartridge 4.6 mm × 50 mm + 3.0 mm × 100 mm and 4.6 × 10 mm) and special auto sampler for micro titration plates (samples are storage in dark cooled place protected against evaporation) were combined with easy sample preparation step. As mobile phase 15 mmol/L phosphate buffer at pH 4.50 was used. Neopterin and tryptophan were detected using fluorescent detection and kynurenine and creatinine were detected by diode-array detection. This method may be suitable for large sequences of samples in clinical research and routine practice.

1. Introduction

Neopterin is a pteridine compound synthesized, mostly by activated macrophages, from guanosine triphosphage (GTP) in a reaction catalyzed by the enzyme GTP cyclohydrolase I. Interferon-gamma (IFN-gamma) is the most potent stimulus for GTP cyclohydrolase I induction and neopterin production. Increased concentration of neopterin have been reported in a wide range of disorders associated with the activation of cellular immunity, including viral infections, cancer, autoimmune diseases or rejection following organ transplantation. Another enzyme induced by IFN-gamma is indoleamine 2,3 dioxygenase (IDO). IDO catalyzes the conversion of tryptophan into kynurenine. Tryptophan depletion induced by IDO activity is one of the mechanisms responsible for suppression of immune response in pregnancy as well as in cancer [1,2].

The measurement of neopterin, kynurenine and tryptophan may be used to assess the immune activation systemically or locally, in the microenvironment. Neopterin determination has been widely used in laboratory medicine. Neopterin measurement may be utilized diagnostically, e.g. for early detection of transplant rejection. Neopterin has also been used to detect viral infection in blood donors. In addition, neopterin determination may be used to predict prognosis in cancer patients or to monitor the effect of therapy [3].

In clinical studies, neopterin was measured both in the serum and in the urine. Methods for analysis of neopterin, tryptophan and kynurenine have mostly utilized high-performance liquid chromatography (HPLC).

A rapid and sensitive separation and quantitation method for the determination of urinary neopterin using high-performance liquid chromatography (HPLC) on reversed phase was developed in early 1980s [4]. Urinary neopterin concentrations are expressed as neopterin/creatinine ratio.

Determination of neopterin in serum by HPLC is more difficult than in urine because of the presence of protein and lower concentration of neopterin [5], and commonly used methods of HPLC determination of neopterin are based on acid deproteinization of biological samples. The use of neopterin/creatinine ratio has also been advocated to correct for renal function for serum samples in patients with kidney disease.

Immunoassays represent the other option for neopterin determination in biological fluids. Radioimmunoassay (RIA) and enzyme-linke immunosorbent assay (ELISA) kits for neopterin are commercially available.

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HPLC method for simultaneous determination of kynurenine and tryptophan in human serum has also been reported [6].

To the best of our knowledge no method for simultaneous determination of neopterin, kynurenine, tryptophan and creatinine in human serum was reported. The aim of this study was to develop and validate HPLC method for separation of neopterin, creatinine, kynurenine and tryptophan using simple sample preparation procedure suitable for large sequences of samples in clinical research and analysis in biochemical laboratories in routine practice.

2. Experimental

2.1. Chemicals

Ethanol (100%) for the deproteinization procedure was obtained from Lachema (Brno, Czech Republic) and redistilled water from system Goro (Prague, Czech Republic) was used. Neopterin, kynurenine, tryptophan and creatinine (99.5%) were supplied by Fluka Sigma–Aldrich, (Prague, Czech Republic). Mobile phase was phosphate buffer p.a. ($KH_2PO_4 + K_2HPO_4{\cdot}3H_2O$) purchased from Appplichem (Darmstadt, Germany).

2.2. Instrumentation

The analyses were performed using a HPLC set Prominence LC 20 Shimadzu (Kyoto, Japan) composed by degasser DGU 20A5, pump LC20-AB, special auto sampler SIL/20 AC for micro titration plates (rack changer), column oven CTO-20 AC, diode array detector SPD-M20A, fluorescence detector RF10 AXL, communication bus module CBM-20A.

Sample preparation technique was developed using micro titration plates with filters AcroPrep 96 filter Plate 0.2 μm/350 μl Pall (MI, USA), vacuum manifold Phenomenex (Aschaffenburg, Germany), vacuum pump VAC Space-50 Chromservis (Prague, Czech Republic), and centrifuge Minispin Eppendorf (Hamburg, Germany).

2.3. Chromatographic conditions

Analysis was performed at HPLC set Prominence LC 20 (Shimadzu, Kyoto, Japan) equipped by special auto sampler for micro titration plates. As stationary phase two monolithic columns RP-18e (4.6 mm × 50 mm, 3.0 mm × 100 mm) were connected together with monolithic security guard (4.6 mm × 10 mm). As the mobile phase 15 mmol/L phosphate buffer ($KH_2PO_4 + K_2HPO_4{\cdot}3H_2O$) was used at pH 4.51 and flow rate 1 mL/min (0–3.09 min) and 2.3 mL/min (3.10–8.20 min). Injection volume was 1 μL. Kynurenine and creatinine were detected using diode array detection (230 and 235 nm), neopterin and tryptophan were detected using fluorescent detection (excitation wavelength 353 nm, emission wavelength 458 nm for neopterin and excitation wavelength 254 nm, emission wavelength 404 nm for tryptophan). Separation was held at ambient temperature in 8.2 min.

2.4. Preparation of standard solutions

Stock standard solutions of neopterin, kynurenine, tryptophan and creatinine were prepared by the following methods. Compounds were dissolved in distilled water. Neopterin stock solution was prepared at concentration 100 μmol/L, kynurenine stock solution at concentration 0.6 mmol/L, tryptophan stock solution at concentration 3.0 mmol/L and creatinine stock solution at concentration 10 mmol/L. Stock solutions were stored at +4 °C and diluted to working solution at concentrations 50–0.5 nmol/L for neopterin, 150–5 μmol/L for tryptophan, 0.25–10 μmol/L for kynurenine and 100–6 μmol/L for creatinine.

2.5. Sample preparation

The protocol was approved by the institutional ethical committee, and all patients gave their consent. Blood samples were drawn from the peripheral vein and were obtained after 12 h of overnight fasting. The samples were then centrifuged (1600 × *g*, 10 min, +4 °C) and serum was separated. Then 200 μL of serum was diluted with 100 μl phosphate buffer (15 mmol/L, pH 6.5) deproteinised by 100 μL cooled ethanol (10 min, −25 °C). After centrifugation (14,000 × *g*, 10 min), the supernatant was filtered using 0.2 μm micro titration plate filters and vacuum manifold. Filtered solution was applied into the HPLC column.

3. Results

3.1. HPLC method development and optimization

HPLC method was based on modified separation conditions for neopterin and creatinine in human urine developed by Fuchs et al. [4].

For the separation of target analytes (neopterin, kynurenine, tryptophan and creatinine) three monolithic columns with different size and flow rates were tested:

Chromolith Performance RP-18e, 100 mm × 4.6 mm, MERCK (Darmstadt, Germany)
Chromolith Performance RP-18e, 100 mm × 3.0 mm, MERCK (Darmstadt, Germany)

Monolithic columns were tested separately using mixture of standards and in connection in these modes: 100 mm × 4.6 mm + 100 mm × 4.6 mm, 50 mm × 4.6 mm + 100 mm × 4.6 mm, and 50 mm × 4.6 mm + 100 mm × 3.0 mm, these connections of monolithic columns allow large separation capacity and better resolution. As the mobile phase phosphate buffer at concentration 15 mmol/L and pH 6.50 was used. As the best separately tested column with regard to tested parameters (HETP and peak symmetry – parameters were calculated using Europhean pharmacopoeia and FDA guidelines [7,8]. Chromolith Performance RP-18e, 100 mm × 4.6 mm was chosen (Table 1).

Due to very simple sample preparation technique (dilution, deproteinization, centrifugation and filtration) there were some problems with impurities especially in creatinine and tryptophan peaks. Improvement was achieved using 4.6 mm × 50 mm and 4.6 mm × 100 mm monolithic column connected together but time of analysis was so long. Better separation offers connection of 4.6 mm × 50 mm and 3.0 mm × 100 mm monolithic column and monolithic security guard (Fig. 1). After connection of security guard two main impurities were better separated form creatinine and tryptophan peak.

It was discover during robustness testing that pH of mobile phase can significantly influence elution of impurities in creatinine and tryptophan peak.

As the best pH for impurities separation and elimination, pH 4.51 was chosen. For speed separation flow rate switching mode was used (0–3.09 min: flow rate 1 mL/min and 3.10–8.20 min: 2.3 mL/min).

3.2. Method validation

Method validation was performed according to the European Pharmacopoeia [7] FDA guidelines for biological method validation,

Table 1
Testing of different monolithic columns, (HETP): height equivalent to a theoretical plate of different monolithic columns with various flow rates.

Column (size, flow rate)	Neopterin		Creatinine		Kynurenine		Tryptophan	
	Area	HETP	Area	HETP	Area	HETP	Area	HETP
Monolith 4.6 × 100 (1.5 ml/min)	20,705	54	668,210	31	1723	13	625,926	11
Monolith 3.0 × 100 (1.5 ml/min)	21,261	235	67,635	219	1343	74	627,613	23
Monolith 3.0 × 100 (1.0 ml/min)	29,149	165	109,923	134	2802	32	837,241	20
Monolith 3.0 × 100 (grad.)	54,520	93	2,010,612	56	4035	18	472,368	6

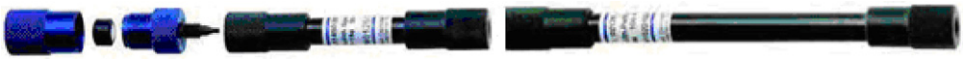


Fig. 1. Connection of two monolithic columns RP-18e (4.6 mm × 50 mm + 3.0 × 100 mm) and security guard (4.6 mm × 10 mm).

and the International Conference on Harmonization (ICH) guidelines Q2A and Q2B [8,9] consisting of two parts: system suitability test (SST) and validation parameters.

3.2.1. System suitability test (SST)

Within the system suitability test some chosen parameters describing the separation properties and precision of the chromatographic system were determined. Table 3 summarizes the calculated values of number of theoretical plates (N), height equivalent to theoretical plate (HETP), asymmetry (T), and peak resolution (R). Column performance was determined as number of theoretical plates by the equations $N = 5.545\,(t_R/W_{0.05})^2$ ($W_{0.05}$ is width at 5% of peak height, t_R is retention time) and HETP = L/N (N is column performance, L is length of the column). Asymmetry (tailing factor) was calculated by the equation = $W_{0.01}/2f$ ($W_{0.01}$ is width at 5% of peak height; f is distance between maximum and the leasing edge of the peak); see Table 2. Peak resolution was calculated by the equation $R_{ij} = 2(t_R - t_{Rj})/(W_i + W_j)$; t_R and t_{Rj} are retention times, W_i, W_j are peak widths). For the determination of injection repeatability, ten samples from one lyophilized human serum were analyzed. The repeatability of injection was expressed as the relative standard deviation (RSD) of peak area and retention time calculated from the obtained data.

3.2.2. Validation of parameters

In order to validate the developed method, precision, accuracy, linearity, detection, and LOD and LOQ, as well as ruggedness, were evaluated.

3.2.2.1. Precision. For the determination of the method precision, ten samples prepared individually from one human serum at three different concentration levels were analyzed. The method precision (the repeatability of injection, repeatability of extraction and inter-person repeatability) was expressed by the peak area and retention time and calculated as the relative standard deviation (RSD) of the obtained data.

3.2.2.2. Accuracy. The accuracy of the method was tested first as recovery (see Table 5), which was determined by performing three measurements of the serum pool spiked with neopterin (final concentration at level 1 was 50 nmol/L, at level 2 was 70 nmol/L and at level 3 was 100 nmol/L), tryptophan (final concentration at level 1 was 22.5 μmol/L at level 2 was 45 μmol/L and at level 3 was 75 μmol/L), kynurenine (final concentration at level 1 was 1.44 μmol/L at level 2 was 4.6 μmol/L and at level 3 was 9 μmol/L) and creatinine (final concentration at level 1 was 25 μmol/L at level 2 was 50 μmol/L and at level 3 was 150 μmol/L), and than as repeatability of injection, extraction and repeatability inter-person (see Table 4).

3.2.2.3. Linearity. Linearity of the calibration curves was determined using the LINREGRE program developed at the Department of Biophysics and Physical Chemistry at the Faculty of Pharmacy, Charles University, at six concentration levels in the range of 5–250 μmol/L for creatinine, 2.47–100 nmol/L for neopterin, 1–150 μmol/L for tryptophan, 0.25–10 μmol/L for kynurenine. Table 6 shows the calculated regression equations and correlation coefficients based on data obtained for all compounds.

3.2.2.4. Limit of detection and limit of quantification. The limit of detection (LOD) was defined as the compound concentration that produced a signal-to-noise ratio greater than five, and the limit of

Table 2
Retention time (t_R) and peak asymmetry (T) of each analyte.

Column	neopterin		creatinine		kynurenine		tryptophan	
	t_R	T	t_R	T	t_R	T	t_R	T
Monolith 4.6 × 100 (1.5 ml/min)	1.87	1.23	1.44	1.70	3.70	1.27	8.74	1.30
Monolith 3.0 × 100 (1.5 ml/min)	0.84	1.14	0.59	1.65	1.58	1.19	3.74	0.76
Monolith 3.0 × 100 (1.0 ml/min)	1.14	1.21	0.88	1.88	2.70	0.88	4.85	0.86
Monolith 3.0 × 100 (grad.)	2.24	1.30	1.70	1.51	4.12	1.01	6.22	0.81

Table 3
System suitability test parameters: t_R is retention time, W is peak width; $W_{0.05}$ is the width at 5% of peak height, N is the number of theoretical plates, HETP is the height equivalent to theoretical plate, $W_{0.01}$ is the width at 5% of peak height, f is distance between maximum and the leasing edge of the peak, T is asymmetry and R_{ij} is peak resolution.

	t_R (min)	W (min)	$W_{0.05}$ (min)	N	HETP (μm)	$W_{0.01}$ (min)	f (min)	T	R_{ij}
Neopterin	2.52	0.38	0.13	2083	72	0.25	0.11	1.14	8.52
Tryptophan	6.85	0.64	0.19	7207	21	0.41	0.19	1.08	
Kynurenine	3.91	0.32	0.09	10,465	14	0.21	0.09	1.17	8.49
Creatinine	1.74	0.19	0.07	3426	44	0.17	0.08	1.06	

Table 4
Repeatability of the method.

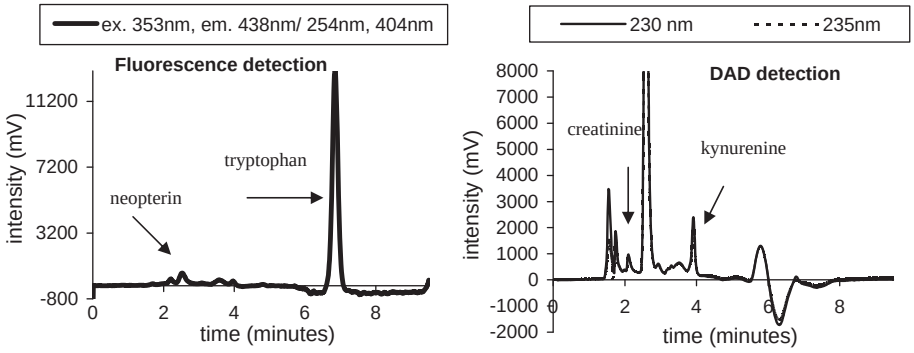
Repeatability RSD (%)	Neopterin		Tryptophan		Kynurenine		Creatinine	
	t_R	c	t_R	c	t_R	c	t_R	c
Injection (n = 15)	0.11	2.10	0.24	4.89	0.15	4.39	0.22	0.79
Extraction (n = 10)		3.66		4.17		1.12		1.14
Inter-person (1/2)		1.96/3.05		1.97/0.50		0.46/0.48		17.39/1.22

Table 5
Recovery of extraction (c – concentration: neopterin (nmol/L), creatinine (μmol/L), kynurenine (μmol/L), tryptophan (μmol/L); $c1$ – calculated, $c2$ – measured).

	Recovery								
	Level 1			Level 2			Level 3		
	$c1$	$c2$	R%	$c1$	$c2$	R%	$c1$	$c2$	R%
Neopterin	57.17	57.86	101.24	77.17	77.63	100.61	107.17	104.28	97.31
Creatinine	61.60	60.02	97.44	115.99	113.50	97.86	240.99	239.66	99.45
Kynurenine	2.48	2.52	101.72	5.64	5.83	103.53	9.94	10.19	102.52
Tryptophan	44.61	42.79	95.92	51.39	47.81	93.03	97.15	88.62	91.26

Table 6
Linearity, limit of detection (LOD) and limit of quantification (LOQ).

	Regression equations	Correlation coefficient	LOD (μmol/L)	LOQ (μmol/L)
Neopterin	$Y = 261.93x + 1284.53$	0.9999	0.00050	0.00167
Creatinine	$Y = 232.47x - 296.73$	0.9999	0.25948	0.98493
Kynurenine	$Y = 543.55x - 118.41$	0.9999	0.69505	0.23168
Tryptophan	$Y = 7521.98x - 5790.80$	0.9999	0.16116	0.53719



Figs. 2 and 3. Final separation conditions of target analytes in human serum: monolithic separation system RP-18e (4.6 × 50 mm + 3.0 × 100 mm) and security guard (4.6 × 10 mm), mobile phase pH 4.5 with flow rate switching (0–3.09 min: flow rate 1 mL/min and 3.10–8.20 min: 2.3 mL/min). Neopterin t_R = 2.52 min at the concentration c = 21.64 nmol/L and tryptophan t_R = 6.85 min at the concentration c = 27.86 μmol/L were detected using florescence detection. Kynurenine t_R = 3.91 min, the concentration c = 4.5 μmol/L and creatinine t_R = 1.74 min, the concentration c = 19.61 μmol/L were detected using diode-array detection.

quantification (LOQ) was evaluated as the concentration equal to ten times the value of the signal-to-noise ratio [9] (Table 6).

3.2.2.5. Selectivity. The good selectivity of this method for neopterin, kynurenine, tryptophan and creatinine is documented in Figs. 2 and 3 and in Table 3.

3.2.2.6. Ruggedness. For the determination of method ruggedness, the pH of mobile phase, mobile phase, concentration and temperature were varied. Retention time and peak area were determined. The retention time was clearly not affected by variation of mobile phase concentration. Temperature changes influence analytes concentration in standard solutions in the range of 94–107% (25 °C – 100%). Concentrations of analytes in serum were changing in the range of 76–111%. Most affected analyte was kynurenine.

4. Discussion

In the present study, a simple HPLC method for selective and sensitive simultaneous determination of neopterin, kynurenine, tryptophan and creatinine in human serum using a monolithic technology was developed and validated for the use in clinical setting. It is being increasingly recognized that the immune activation is present not only in patients with infections or autoimmune disease, but also across a spectrum of conditions or disorders ranging from atherosclerosis, malignant neoplasms or neurodegenerative disorder to pregnancy. Inflammatory phenomena are currently thought to play a major role in the pathogenesis or cancer, atherosclerosis or disorders associated with end-stage renal failure. Thus, combined analysis of neopterin, kynurenine, creatinine and tryptophan in the serum may be useful for the measurement of GTP cyclohydrolase and IDO activation and the assessment of systemic immune activation wide use in different medical and surgical specialties (e.g. medical oncology, gynecology and obstetrics, neurology, psychiatry or cardiology) [3,10–13].

The potential significance of simultaneous serum neopterin/creatinine and kynurenine/tryptophan determination could be illustrated on the example of cancer. Urinary and serum concentrations of neopterin are increased in patients with different malignant tumors, including lung cancer, gynaecological

malignancies, gastrointestinal and head and neck carcinomas [11,12,14]. In cancer patients, increased neopterin is a prognostic factor associated with inferior survival. Moreover, changes in neopterin concentrations have been observed in body fluids of cancer patients treated with cytokines or cytotoxic agents [15]. Much less is known about the significance of kynurenine, tryptophan or kynurenine/tryptophan ratio in cancer patients. Experimentally, IDO activation with kynurenine production and tryptophan depletion has been shown to result both in inhibition of antitumor immunity resulting in tumor progression [2] and inhibition of tumor growth [16,17]. Tryptophan depletion results in inhibition of T cell responses [1]. Similarly, high systemic neopterin production has been associated in ganges of lymfocyte phenotype and fiction in cancer patients [18,19]. It is possible that, similarly to neopterin, could kynurenine or kynurenine/tryptophan ratio determination represent an important tool for the assessment of patient prognosis or monitoring the disease course. In studies performed so far correlation between neopterin and kynurenine production has been demonstrated, but potential differences in the dynamics of these laboratory parameters should also be investigated in future studies. Gerontologic research represents another field for potential utilization of simultaneous neopterin, creatinine, kynurenine and tryptophan determination [10].

While early morning or random urinary samples are commonly used for neopterin determination, urinary kynurenine measurement has not been validated in wider laboratory practice. The kynurenine/tryptophan ratio that is more meaningful indicator of IDO activation than absolute kynurenine concentration is also practicable only in the serum. The concentration in first-morning or random urine samples reflects systemic events occurring over several hours, and compared to urine the use of serum also allows for a more precise timing and monitoring of acute changes. In addition, determination of serum neopterin/creatinine ratio could be of advantage in patients with end-stage renal disease and oliguria, where the interpretation of urinary neopterin/creatinine ratio could be problematic.

In 1982 Fuchs et al. developed a rapid and sensitive separation and quantitation method using high-performance liquid chromatography (HPLC) on reversed phase [4].

There were a lot of variation using this analysis as template usually separation on C18 or C16 particular stationary phases with phosphate buffer or combination of water and acetonitrile as a mobile phase [20–22].

Determination of neopterin levels in human urine is commonly used in clinical practice or in research, especially for its non-invasive character. However, the present newly developed HPLC method for simultaneous determination of neopterin, kynurenine, tryptophan and creatinine in human serum, handle with very small amount of sample (200 μL) and thus can be included as a part of other common blood examinations (e.g. standard biochemistry, vitamins and minerals serum concentrations).

Determination of neopterin in serum by HPLC is more difficult to perform than in urine due to the presence of protein and approximately 200-fold lower neopterin concentrations [5].

Methods of HPLC determination of neopterin are usually based on acid deproteinization of biological samples. This process can lead to destruction of neopterin and also results in the dilution of samples, decreasing the sensitivity of the method. A 100 mm × 4 mm column packed with Diasfer C16 (5 μm) particles has been used in combination with a 4 × 3 mm C18 precolumn (Phenomenex, USA). Eluent: 1:99 (vol.) acetonitrile–water. The flow rate was equal to 600 μL/min. The retention time of neopterin was found equal to ~3.6 min. The analysis was performed using a standard chromatographic instrumentation. Neopterin was isolated from blood serum and urine by solid-phase extraction on cartridges containing 30 mg of supercrosslinked polystyrene. The extraction rate was 96–113% [22].

Midttun et al. determined neopterin, kynurenine and tryptophan in human plasma by HPLC-MS. The mobile phase consisted of three components, solution A (650 mmol/L acetic acid), solution B (100 mmol/L heptafluorobutyric acid in A) and solution C (90% acetonitrile in water), time of analysis was 6.4 min. Plasma was deproteinized by mixing with an equal volume of trichloroacetic acid in water (60 g/L) containing the isotope labelled internal standards. The solution was mixed by a robot, incubated for 60 min on ice, and finally centrifuged at 5796 × *g* at 48 °C for 15 min. The supernatant was transferred to a new vial which was placed in a cooled auto sampler. Analytes were detected in positive-ion multiple-reaction monitoring (MRM) mode with unit resolution at Q1 and Q3 [23]. This method exhibits high performance and sensitivity, but is expensive, and therefore not suitable for routine analysis in biochemical laboratories for large sequences of samples. This method is not suitable for patients with kidney diseases and patient after kidney transplantation because of absence creatinine measurement [23].

Compared to HPLC the advantage of immunoassays is the ability to perform quickly large number of analyses. An RIA kit for neopterin is commercially available. The RIA is based on the competition of unlabelled neopterin of the serum samples or standards and radiolabelled neopterin for the binding sites of the neopterin specific antibody. The neopterin ELISA is based on the competition of unlabelled neopterin of the serum samples or standards and horseradish peroxidase labeled neopterin for the binding sites of the neopterin specific antibody. Correlation of RIA and ELISA is good, but for RIA very expensive equipment is needed and this method involved the use of radionuclides [5]. Immunoassay are determine only for neopterin, and creatinine or other analytes have be measured by other methods.

Ogiwara et al. developed an ELISA of neopterin and biopterin using polystyrene microtiter plate. A conjugate of neopterin or biopterin to bovine serum albumin was used to raise a specific antiserum against neopterin or biopterin in rabbits [24].

Werner et al. developed an HPLC method for simultaneous determination of neopterin and creatinine in serum. Acidified, but not deproteinized serum is applied to a 4-propylbenzene sulfonic acid modified silica sorbent cartridge, which quantitatively retains the analytes but not the serum proteins. The retained analytes are then eluted from the cartridge directly onto the liquid-chromatography column [25]. An HPLC method for simultaneous determination of kynurenine and tryptophan in human serum in parallel with use of 3-nitro-l-tyrosine as an internal standard was developed by Laich et al. For separation, a reverse-phase LiChroCART 55-4 cartridge (Merck), 55 mm in length, filled with Purosphere STAR RP18 (3 μm grain size; Merck) together with a reverse-phase C18 precolumn were used. The elution buffer was acetic acid–sodium acetate at concentration 15 mmol/L and pH 4.0, containing 27 mL/L acetonitrile with the flow rate 0.9 mL/min. One single chromatographic run was completed within 7 min. Serum samples were deproteinized by 2 mol/L trichloroacetic acid and centrifuged [6].

Emerging technologies, including capillary electrochromatography, ultra high pressure HPLC (UHPLC) and the use of monolithic silica columns, allows for very high separation efficiency and speed of analysis, surpassing the conventional particle-packed columns in high performance liquid chromatography (HPLC). Monolithic columns attracted attention because of their potential high performance under common operating conditions that rivals that of packed columns without high pressure requirements. The monolithic columns used in the present investigations (performance) provide rapid, high-quality separation of complex mixtures. The monolithic column possesses much larger through-pores than a

particle-packed column. High porosity leads to a high permeability or a low pressure drop, and a small skeleton size at a similar through-pore size can lead to higher column efficiency than what could be expected from the pressure drop. An additional advantage of a monolithic silica column is increased mechanical stability provided by the integrated network structure, which allows elution at high mobile phase linear velocities. Particle-packed columns often show problems in the permeability and/or in the stability of their packed bed at such linear velocities [26]. From our experience, monoliths are suitable for biological samples and one monolithic column is possible to use for more than 1000 biological samples (human serum, urine, etc.) [15,27–29].

Micro-titration plates with filters allow filtrating 96 samples at one time, reducing use of hazardous solvents, operator time, glassware and equipment and achieving high recoveries with low coefficients of variation. Time for one sample preparation (using multi channel pipette) was calculated as 25 s/sample. The combination of these modern technologies with modern HPLC equipment facilitates the processing of large sequences of samples, that in routine analyses as well as in clinical studies in research laboratories. A special auto sampler Rack Changer SIL-20AC is equipped with a sample cooler with a built-in dehumidifier to minimise condensation problems. Samples can be maintained at a fixed temperature from 4 °C to 40 °C. Temperature equilibration is rapid and uniform, so heat or cold sensitive sample constituents remain stable. Greater injection volume accuracy has been attained by incorporating a high-performance precision metering pump. Injection volumes of less than 1 μL are possible, and the direct injection method means valuable samples are never wasted. The 12-plate capacity ensures convenient processing of over 1000 samples.

The present method for simultaneous determination of neopterin, kynurenine, tryptophan and creatinine profits from main quality of monoliths which allows flow rate switching and no equilibration time is needed. This possibility is very useful for speed analysis in routine medicine practice. Connection of two monoliths allows large separation capacity and better separation efficiency with low back pressure which is in combination with simple and speed sample preparation procedure very advantageous. Connection of 4.6 mm × 50 mm offers preliminary separation and 3.0 mm × 100 mm monolithic column provides analytical separation and improves peak shape. Security guard protects HPLC columns, also improves separation of two main impurities of creatinine and tryptophan peaks and makes this system longlife for large sequences of biological samples. The other good results in method optimization were achieved by using pH 4.51 of mobile phase for separation.

Presented method may also be suitable for other biological fluids, such as exudates and amniotic fluid. Examination of malignant ascites or pleural effusions offers an opportunity to investigate immune activation in the tumor micro-environment [17,30].

5. Conclusions

In the present study, a new and simple HPLC method for selective and sensitive simultaneous determination of neopterin, kynurenine, tryptophan and creatinine in human serum using a monolithic technology for clinical monitoring was developed and validated.

The main advantage of this method is the combination of two different size monoliths, for preliminary and analytical separation with peak shape improvement and using of flow rate switching with low back pressure resulting in short time of analysis. Very simple sample preparation using micro titration plates that allow handling 96 samples during one extraction step. This method is suitable for routine analyses (low price, small volume of samples and solvents) of large sequences of samples which have to be measured in a short time.

Conflict of interest

The authors declared no conflict of interest.

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