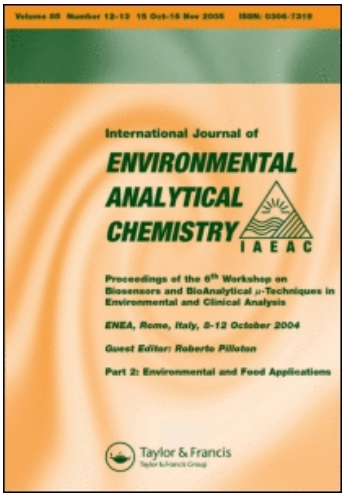

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Miniaturisation of solid phase extraction method for determination of retinol, alpha- and gamma-tocopherol in human serum using new technologies

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Miniaturisation of solid phase extraction method for determination of retinol, alpha- and gamma-tocopherol in human serum using new technologies[†]

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The aim of this study was to develop rapid and simple solid phase extraction (SPE) and HPLC methods for simultaneous determination of retinol, gamma- and alpha-tocopherol in human serum using a special auto sampler with micro titration plates.

Separation of vitamins was performed at ambient temperature using monolithic column on a HPLC containing rack changer for micro titration plates. As the mobile phase methanol with flow rate 2.5 mL min^{-1} was used. The injection volume was 20 μL. Retinol was detected at 325 nm, gamma- and alpha-tocopherol were carried out at 295 nm, respectively. The total time of analysis was 1.8 minutes. Extraction method was developed using Spe-ed 96 C18, 100 mg/2 mL micro titration plates and SPE vacuum manifold. The consumption of the sample was 50 μL. Time of the analysis for 96 samples on one micro titration plate was 1.5 hour. In order to validate the developed method, precision, accuracy, linearity, detection and quantitation limits were evaluated. This method is suitable for rapid automated large-batch analysis of retinol, alpha- and gamma-tocopherol in small sample volumes of human serum.

Keywords: solid phase extraction; HPLC; monolithic column; liposoluble vitamins; micro titration plates

1. Introduction

At the present time there are ever higher requirements governing biomedical analyses. The groups of the patients for statistical survey have to be larger, which means that clinical research laboratories have to deal with a higher amount of the samples. The timesaving, modern and effective methods are proving still more useful. Consumption of only small amounts of the sample and small volumes of the chemicals is necessary.

The miniaturisation of the sample preparation process has been increasingly focused because it must provide a solution to the requirements such as high performance, rapid analysis with a low running cost, and no environmental pollution [1].

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The heart of each HPLC method is the column, which enables the resolution of compounds based upon selectivity and column performance. In contrast to such HPLC columns packed with particulate silica materials, monolithic columns are made of a single piece of porous silica, which is also called a 'silica rod'. A monolithic column can be defined as a column consisting of one piece of solid that possesses interconnected skeletons and interconnected flow paths (through-pores). This special porous character of the monolithic column allows relatively high mobile phase flow rates (1–9 mL min^{-1}) while keeping the backpressure low [2].

Another unique feature of monoliths is the high column efficiency, even at high linear flow velocities [3].

Actually, monolithic columns were first reported for organic-polymer-based materials. Major chromatographic features of monolithic silica columns are high permeability based on large through-pores and a large number of theoretical plates per unit pressure drop based on small-sized skeletons [4].

The vitamins A (retinol) and E (alpha-tocopherol) serve as reducing agents being able to inactivate the toxic effects of free radicals and protect the organism against oxidative stress.

Oxidative stress may also be important for the host response to tumour. Increased vitamin A has been associated with increased natural-killer-cell function, which plays an important part in antitumour immunity. Decreased serum vitamin A levels may have a negative effect on the host-immune response and may affect, by unchecked tumour growth or increased susceptibility to infection, the prognosis of the individual patient. It was investigated that serum concentrations of vitamin A are significantly decreased in patients with colorectal cancer [5].

Tocopherols (both alpha- and gamma-tocopherol) are the major antioxidants found in serum and are commonly referred to as vitamin E. Alpha-tocopherol is responsible for most of the antioxidant activity in animal tissues [6].

Disorders of antioxidant balance involving tocopherols may be involved in the pathogenesis of some of the toxic effects of radiotherapy [7,8] or chemotherapy [9].

A decrease in serum alpha-tocopherol has been observed during systemic chemotherapy [10–13] but an increase in serum tocopherols has also been reported, probably resulting from tumour control [14,15].

Alpha-tocopherol may have chemo preventive activity [16]. The monitoring of retinol and alpha-tocopherol blood levels contributes to a closer knowledge of different antineoplastic drug effects and investigates the possible utilisation of these drugs in the prevention and therapy of cancer [17,18].

Epidemiological studies have confirmed that dietary supplementation with vitamin E significantly inhibits oxidation of low-density lipoproteins and reduces the risk of atherosclerosis and coronary heart disease. Retinol and beta-carotene are important micronutrient vitamins in reducing the risk of age-related macular degeneration and cancer [19].

Vitamins A and E are usually determined by the means of high performance liquid chromatography (HPLC), which is one of the most important separation techniques used in clinical laboratories [2,19–22].

Concerning sample preparation, it is recommended to use a short time and gentle extraction methods. Deproteinisation of the plasma is mainly achieved by acetonitrile, ethanol, methanol, and liquid–liquid extraction (LLE) for sample clean-up is performed by *n*-hexane or heptan [23]. Bee-Lan Lee developed an extraction method for determination

of retinol, tocopherol and carotenoids using liquid–liquid extraction and isocratic separation with two monomeric C18 columns maintained at 35 and 4°C coupled with ultraviolet–visible and fluorometric detection [24]. Urbanek *et al*. developed LLE for determination of retinol and alpha tocopherol. In this LLE 500 µL of human serum and 2500 µL of hexan as extraction solution were used [3]. Abahusain extracted vitamins using LLE 200 µL of serum into 400 microliters of ethyl acetate– butanol (1 : 1). Retinol, alpha-tocopherol, alpha- and beta-carotene were separated using Supelco stainless steel column (250 mm × 34.6 mm, i.d.) and precolumn (50 mm × 4.6 mm, i.d.) packed with ODS Supelcosil LC 18, 5 µm particles, in 20 minutes [25]. Chatzimichalakis *et al*. developed SPE method using Cyclohexyl (C_6H_{11}) cartridges (500 mg/3 mL). After sample application vitamin E was retained on the sorbent and subsequently eluted by passing methanol. For the separation of vitamins a Phenomenex (Torrance, USA) column, type Luna 3 C18 (150 mm × 4.60 mm, 3 µm) coupled with a Phenomenex security guard pre-column was used [23].

In our laboratory Urbanek *et al*. developed a HPLC method for determination of retinol and alpha-tocopherol using monolithic column. We transformed and revalidated this method for determination of retinol and alpha-tocopherol after solid phase extraction using microtitration plates and Prominence LC 20 (Shimadzu) HPLC set [3].

In this work the novel, simple and timesaving HPLC method for liposoluble vitamins A and E (retinol, alpha- and gamma-tocopherol) in human serum was developed and validated for routine and clinical research laboratories. Main advantages of this method are a combination of several up-to-date technologies – monolithic column technology, solid phase extraction on micro titration plates and Rack Changer for micro titration plates.

2. Experimental

2.1 Instrumentation and chemicals

The analyses were performed using a HPLC set Prominence LC 20 (Shimadzu, Kyoto, Japan) composed by rack changer/C, 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, communication bus module CBM-20A.

Solid phase extraction method as extraction technique was developed using Spe-ed column cartridges micro titration-plates C18 500 mg/3 mL Applier Separations (Allentown, USA), vacuum manifold Phenomenex (Aschaffenburg, Germany), vacuum pump VAC Space-50 Chromservis (Prague, Czech Republic), vacuum concentrator Eppendorf (Hamburg, Germany) and centrifuge Labofuge 400R Heraeus (Hanau, Germany).

All solvents were of HPLC grade. Both methanol and *n*-hexane used for the preparation of standard solutions and as elution solution were obtained from Scharlau (Sentmenat, Spain). Ethanol for the deproteinisation procedure was obtained from Lachema (Brno, Czech Republic) and distilled water Goro (Prague, Czech Republic) was used.

Retinol, alpha-tocopherol and gamma-tocopherol were supplied by Fluka Sigma Aldrich, (Prague, Czech Republic). A lyophilised human serum kit for HPLC analysis of vitamins A and E from Chromsystems (Prague, Czech Republic) was used for validation process.

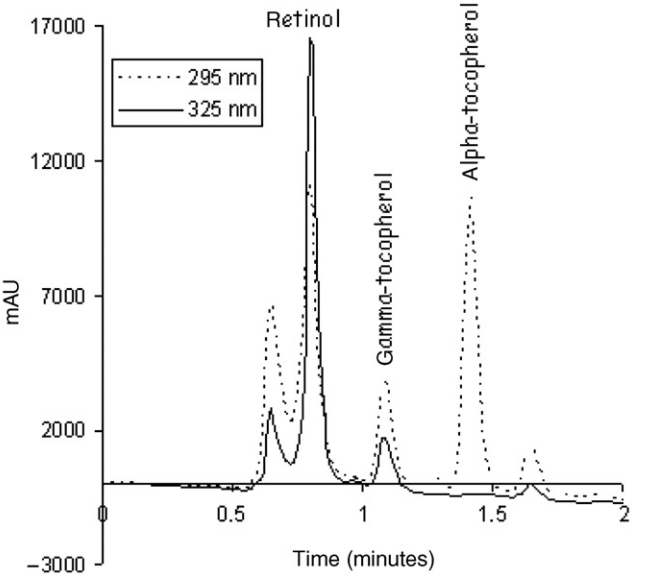

Figure 1. Chromatogram of human serum (retinol 1.95 μmol L^{-1}, gamma-tocopherol 0.55 μmol L^{-1}, alpha-tocopherol 28.51 μmol L^{-1}).

2.2 Chromatographic conditions

Separation of vitamins was performed using a Chromolith Performance RP-18e, 100 × 4.6 mm monolithic column, Merck (Darmstadt, Germany) at 25°C. The mobile phase methanol with flow rate 2.5 mL min^{-1} at the column pressure 2.7 MPa (400 PSI) was used. The injection volume was 20 μL. The DAD detection of retinol was carried out at 325 nm and 295 nm for gamma- and alpha-tocopherol, respectively. The total time of analysis was 1.8 minutes (Figure 1).

2.3 Preparation of standard solutions

Stock standard solutions of retinol, alpha- and gamma-tocopherol were prepared by the following methods. Retinol solution was prepared by dissolving it into methanol (1000 μmol L^{-1}, 572,92 mg L^{-1}). Alpha- and gamma-tocopherol standards were firstly dissolved in n-hexane (1000 μmol L^{-1}, 430,72 mg L^{-1} and 416,68 mg L^{-1}) and then diluted by methanol to get 500 μmol L^{-1} stock solutions. These standard solutions were stored at −25°C (retinol) and at 4°C (alpha- and gamma-tocopherol). For the calibration, working solutions of all standards were diluted by methanol in volumetric flasks in the concentration range: 0.25–10.00 μmol L^{-1} for retinol, 0.5–50.00 μmol L^{-1} for gamma-tocopherol and 0.5–50.00 μmol L^{-1} for alpha-tocopherol. The calibration was accomplished at six concentration levels. The stability of stock and working solutions was 6 months.

2.4 Sample preparation – solid phase extraction procedure

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 hours of overnight fasting. The samples were then centrifuged (1600× *g*, 10 min, 4°C) and serum was separated. Then 50 µL of serum was deproteinised by cooled ethanol (170 µL, 8 minutes, −25°C). After centrifugation (2000× *g*, 20 minutes, 4°C), the supernatant was separated and applied on the SPE column. Before application of the sample the SPE column was activated by 500 µL of methanol and then washed by 500 µL of water. Hexane 400 µL was used as elution solution. The hexane was evaporated at 45°C in vacuum evaporator. The residuum was diluted in 50 µL of methanol and applied into the analytical column.

3. Results and discussion

3.1 Method validation

The method validation was performed according to the European Pharmacopoeia [26] and the International Conference on Harmonization (ICH) guidelines Q2A and Q2B [27,28] consisting of two parts: System Suitability Test (SST) and Validation parameters.

3.1.1 *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 1 summarises the calculated values of number of theoretical plates (N), Height Equivalent of Theoretical Plate (HETP), asymmetry (T) and peak resolution (R).

Column performance was determined as number of theoretical plates by the equations $N = 5.545.\ (t_R/W_{0.5})^2$ ($W_{0.5}$ is width at half 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 $= W_{0.05}/2f$ ($W_{0.05}$ is width at 5% of peak height, 2f is twice the front part of the peak (distance between maximum and the leading edge of the peak). Peak resolution was calculated by the equation $R_{ij} = 2.\ (t_R - t_{RJ})/W_i + W_j)$ (t_R, t_{RJ} are retention times, W_i, W_j are peak widths). For the determination of injection repeatability, 10 samples with standard solutions of one concentration level and 10 samples from one lyophilised human serum were analysed. The repeatability of injection was expressed as the relative standard deviation (RSD) of peak area and retention time calculated from the obtained data.

3.1.2 *Validation of parameters*

In order to validate the developed method, precision, accuracy, linearity, detection and quantification limits were calculated.

Table 1. The System Suitability Test: number of theoretical plates (N), Height Equivalent of Theoretical Plate (HETP), asymmetry (T) and peak resolution (R).

Compound	$W_{0.5}$ (min)	t_R (min)	N	HETP (µm)	$W_{0.05}$ (min)	F (min)	T	W (min)	t_R (min)	R
Retinol	0.04	0.799	2212.5	45.1	0.127	0.053	1.198	0.210	0.799	1.58
Gamma-tocopherol	0.053	1.100	3279.6	30.4	0.0530	0.021	1.262	0.170	1.100	1.68
Alpha-tocopherol	0.053	1.412	3935.5	28.25	0.128	0.042	1.524	0.202	1.412	

3.1.2.1 *Precision*. For the determination of the method precision 10 samples were prepared individually from lyophilised human serum. The method precision expressed by the repeatability of the peak area and retention time was determined as the relative standard deviation (RSD), calculated from the obtained data. Table 2 comprises RSD values of retinol, gamma-tocopherol and alpha-tocopherol.

3.1.2.2 *Accuracy*. The accuracy of the method was tested first as recovery, which was determined by performing three measurements of the Chromsystems control serum for vitamin A and E pool spiked with gamma-tocopherol (final concentration was 10.00 μmol L^{-1}). A mean recovery of $n = 3$ was determined. The results from measurements on concentration level 1 are shown in Table 2.

Secondly the accuracy of the method was determined by using the Chromsystems control set for vitamin A and E. The values obtained were always in the acceptable range (Table 3).

3.1.2.3 *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, Czech Republic, in six

Table 2. Validation parameters of retinol, alpha-tocopherol and gamma-tocopherol.

	Retinol	Gamma-tocopherol	Alpha-tocopherol
Repeatability-concentration (RSD %)	5.65	1.66	5.51
Repeatability-retention time (RSD %)	0.41	0.38	0.83
Accuracy (% recovery)	93.9	85.6	73.4
Accuracy (RSD %)	5.65	1.66	5.51
Calibration range (μmol L^{-1})/(mg L^{-1})	0.25–10.00/ 0.071–2.87	0.5–50.00/ 0.21–20.83	0.5–50.0/ 0.22–21.83
Equation	$y = 24488.31x + 7277.149$	$Y = 1681x - 26.198$	$y = 1380.74x - 85.139$
Correlation coefficient	0.9998	0.9999	0.9999
LOD (μmol L^{-1})/(mg L^{-1})	0.130/0.037	0.006/0.0025	0.09/0.039
LOQ (μmol L^{-1})/(mg L^{-1})	0.27/0.077	0.01/0.0041	0.19/0.083

Table 3. Test of method accuracy using control set Chromsystems.

Chromsystems control set	Level 1 Measured value/acceptable range μmol L^{-1}	Level 2 Measured value/acceptable range μmol L^{-1}
Vitamin A (retinol)	1.67/1.42–2.13	3.22/2.75–4.12
Vitamin E (alpha-tocopherol)	14.1/14.1–24.2	27.3/26.7–46.1

concentration levels in the range of 0.25–10.00 μmol L^{-1} for retinol, 0.5–50.00 μmol L^{-1} for gamma-tocopherol and 0.5–50.00 μmol L^{-1} for alpha-tocopherol. Each solution was injected into the column three times. Table 2 shows the calculated regression equations and correlation coefficients based data obtained for all compounds.

3.1.2.4 *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 quantification (LOQ) was evaluated as the concentration equal to 10 times the value of the signal-to-noise ratio (Table 2).

3.1.2.5 *Selectivity*. Selectivity of the method was tested using DAD detection. Spectrums of each analytes in biologic samples were compared with spectrums of analytes in standard solutions. Injection of mobile phase, injection of standard solution and preparation blank sample (extraction procedure was done without serum) were tested.

3.1.2.6 *Stability*. The stability of stock and working standard solutions of retinol, alpha- and gamma-tocopherol was tested at room temperature, 4°C, −25°C and −80°C. The stability of stock and working solutions of retinol was 6 months at −25°C.

The stock and working solutions of alpha- and gamma-tocopherol were stable for 6 months at 4°C.

The novel, simple and rapid HPLC method with SPE micro-extraction using a small amount of biological sample and allowing us to process many samples in one extraction step was developed.

The used monolithic (Performance) columns provide rapid, high-quality separation of complex mixtures. The columns can offer a variable external porosity and through-pore size/skeleton size ratios that are impossible to achieve with particle-packed columns. These characteristics give monolithic columns very high permeability that allows their operation at pressures much lower than traditional HPLC. 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 [29]. Combination of advantages of monoliths with modern SPE stationary phases, which make solid phase extraction sample preparation and HPLC analysis easier, faster, and more economical. With micro titration plates it is possible to prepare 96 samples at one time, reduce use of hazardous solvents, operator time, glassware and equipment and achieve high recoveries with low coefficients of variation. The connection of these modern technologies and modern HPLC equipment allows working with large sequences of samples, which are typical for routine and large 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 optional rack changer automatically loads microplates into the SIL-20A/AC sample compartment. The 12-plate capacity ensures convenient processing of over 1000 samples.

The main advantage of this method is connection of monoliths (which allows short time of analysis) and SPE micro extraction in micro titration plates which enable us to handle 96 samples during one extraction step. In contrast to the special auto sampler for microplates (Rack Changer) where samples are stored during analysis in a dark, cooled place, protected against evaporation is this method is suitable for routine analyses of large sequences of samples which have to be measured in a short time.

4. Conclusion

In this work, a novel, fast and selective HPLC method for determination of retinol alpha- and gamma-tocopherol in human serum for clinical monitoring using a combination of new technologies – monolithic column technology, solid phase extraction on micro titration plates and Rack Changer auto samples for micro titration has been developed and validated.

This method could be used for analysis of retinol, alpha-tocopherol and gamma-tocopherol in human serum in monitoring of anticancer therapy.

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