1 PŘÍLOHY I – PUBLIKACE

1.1 Analytické publikace

1.1.1 Příloha 1

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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.

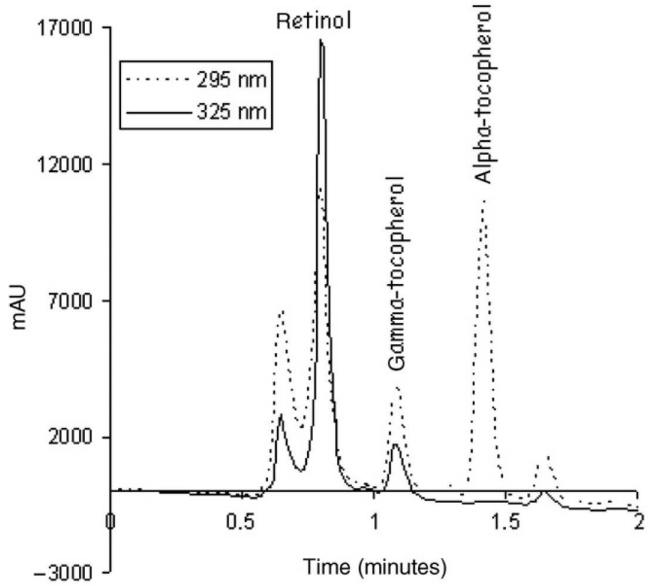

Figure 1. Chromatogram of human serum (retinol 1.95 μmol L^{-1}, gamma-tocopherol 0.55 μmol L^{-1}, alpha-tocopherol 28.51 μmol L^{-1}).

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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1.1.2 Příloha 2

Krcmova L., Stjernlof A., Mehlen S., Hauser P.C., Abele S., Paull B., Macka M.: Deep-UV-LEDs in photometric detection: A 255 nm LED on-capillary detector in capillary electrophoresis, ANALYST, Volume: 134 Issue: 12, 2394-239, 2009, počet citací bez autocitací: 0, IF = 3,272

Deep-UV-LEDs in photometric detection: A 255 nm LED on-capillary detector in capillary electrophoresis

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A 255 nm deep-UV-light-emitting diode (deep-UV-LED) is investigated as a novel light source for photometric detection in view of fundamental properties of UV-LEDs, in particular emission spectra and energy conversion. Its performance in on-capillary photometric detection in capillary electrophoresis (CE) is determined and the potential of deep-UV-LEDs in optical detection is discussed.

Utilising the benefits of solid state light technology,[1–4] LEDs are exceptionally stable light sources offering a number of potential advantages over traditional light sources including robustness, small size, generally lower heat production and price for well established visible (Vis) region LEDs. Since their introduction in the 1970s,[1,2] the wavelength range of LEDs has gradually extended from the original red, down the wavelength scale and into the UV region, while their emission intensities have increased and their prices have reduced, making LEDs attractive light sources in a broad variety of uses including science.[1,2,5,6] The spectrum of analytical utilisation of LEDs has been growing rapidly through the last decade with the subject reviewed by Dasgupta and co-workers,[5] and life-time fluorescence applications covered in a book chapter by Landgraf and co-workers.[6] From the optical methods it is mostly fluorescence that profited from the introduction of UV-LEDs and especially high-powered LEDs in the 350–370 nm range,[7,8] followed more recently by deep-UV-LEDs (emission 200–300 nm).[9,10]

For absorbance photometric detection in liquid chromatography and other separation techniques, Vis-LEDs have been shown to be excellent light sources.[5] This includes capillary electrophoresis (CE), where on-capillary photometric detection with Vis-LEDs is well established[11–31] since Tong and Yeung's[11] first report in 1995. Reviews by Gotz and Karst and by Xiao *et al.* focused on the use of LEDs in photometric and fluorimetric detection in CE and microfluidic chips.[32–34] In CE, deep-UV-LEDs have so far been reported only for fluorescence detection, and rather scarcely. For example by Yeung and co-workers at 280 nm,[35] or in an investigation on light delivery systems with optical fibres at 260–280 nm.[36] For on-capillary photometric detection, however, the only UV-LED used so far has been a 370 nm LED,[20,30] which is too high for most analytical applications. Recently, a deep-UV-LED was reported for photometric detection in a macro-analytical method, namely HPLC using a commercial *z*-cell.[37] Clearly, deep-UV-LEDs would considerably extend the use of LEDs as light sources for photometric detection in capillary or miniaturised analytical systems. When considering using CE as a portable instrument[38] capable of conducting on-site analysis in forensic, environmental and other areas of increasing interest,[30,38] LEDs offer the advantage of small size, eliminate the need for a monochromator and thus allow relatively simple, more compact designs, low current consumption, and increased robustness, which would be very useful in portable, battery powered instruments.

This work reports the first use of a deep-UV-LED for photometric detection in a capillary or miniaturised analytical system and presents an initial fundamental investigation into the role of the parameters of UV-LEDs for their utilisation in photometric detection. The specific aim of this work was to explore the possibility of a deep-UV-LED photometric detection in CE using a commercially available 255 nm deep-UV-LED (Table 1) in an experimental setup successfully used with a number of Vis-LEDs previously,[25] and to investigate the impact of the LED spectra and power conversion on detection performance. The nucleotides adenosine monophosphate (AMP), adenosine diphosphate (ADP) and guanosine diphosphate (GDP) were selected as model analytes as they are known to absorb well at 255 nm.[39]

Deep-UV LEDs have relatively low optical power output compared to LEDs in the Vis range.[41] Furthermore, photodiodes, which are commonly used as detectors in LED-based devices, have poor sensitivity in the UV-range. For this reason a miniature photomultiplier (PMT) module was used for measuring the transmitted UV-light intensity (Hamamatsu Photonics, Hertfordshire, UK,[40] Figure 1).

An important parameter of an LED is the optical power of its emission. This depends on the overall energy conversion of the LED (also called wall-plug efficiency),[43] which in turn determines the heat production. In this case noticeable heating manifested itself in a short stabilisation period (of approx. 10–15 min) which is needed after switching on the LED, as the emission intensity of LEDs generally decreases with rising temperature.[1,2] After this period the light output was stable (within ±1%) as documented later by the stable baselines in the obtained CE separations. As the manufacturer's information

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Table 1 Deep-UV-LED parameters (manufacturer specified/measured or used). Deep-UV-LED: Seoul Optodevice 255 nm from Sensor Electronic Technology, Columbia, SC, USA, cat.# UVTOP255-HL-TO39)[41a]

Em_{max} (nm)	Current (mA)	Diameter (mm)	$P_{optical}$ (μW)	$P_{electric}$ (μW)	Price (US $)[42]
255/257	20/30	9.2	25–40/14	265	*ca.* 300–400

[a] Legend: Em_{max} = emission maximum, P = power.

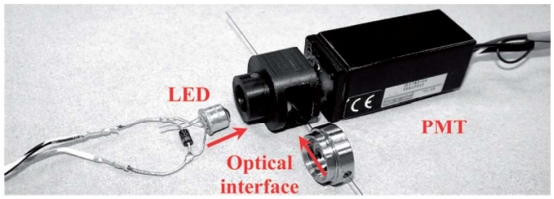

Fig. 1 Detection setup. The arrangement was similar to one previously described[25] except PMT was used here.

does not give any data on energy conversion or heating, the energy conversion was determined by means of the ratio between the optical power emitted (measured by a UV-photodiode, Hitachi Edmund Optics, York, UK, using its optical *vs.* electric power calibration graph), estimated to be 0.014 mW, and the electric power, 0.265 mW, delivered at the same time under the same conditions (see Table 1). Thus the energy conversion was about 5.3%, which is a value much lower than for most Vis-range LEDs, implying that current deep-UV-LEDs have an unfavourable ratio of supplied electrical power converted to optical power and heat respectively. This is in accordance with literature reporting heating to be associated with the current generation of deep-UV-LEDs.[44] In practical applications it can be solved by using a passive heat sink or active cooling[35] and appropriately adapted detector designs including these capabilities will be considered in future work.

The emission spectrum of the used LED is another key parameter and the emission spectrum for the 255 nm deep-UV-LED (Table 1) is shown in Figure 2.

The emission spectrum shows a somewhat surprising but important feature: significant parasitic emission bands in the Vis spectral region are present around 400–500 nm (consistent with a visual observation of off-white Vis light emitted), which is in contrast to relatively quasi-monochromatic emission spectra from Vis-LEDs free of any such parasitic emission bands. Although this

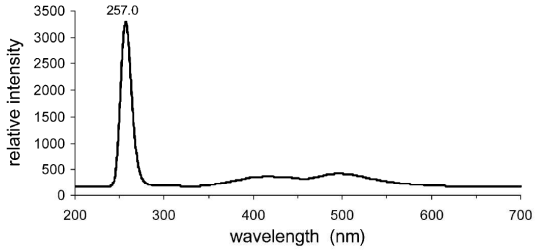

Fig. 2 Emission spectrum of the 255 nm UV-LED, emission band width (half-height) 25 nm. Conditions: OceanOptics 2000 fibre optics CCD spectrophotometer, $i = 20$ mA.

undesirable feature of deep-UV-LEDs, to the best knowledge of the authors, has not been discussed in the analytical literature, this phenomenon is well known in the physics and engineering literature concerning LEDs. The reasons for the parasitic Vis-spectrum emission are known to be from undesirable luminescence of some of the materials used in the LED, or (in the case of narrower parasitic emission bands) in additional unwanted electron transitions present.[45]

This parasitic emitted Vis light will not be absorbed by the UV-absorbing analytes and will therefore be effectively contributing to detector stray light, thus compromising the detection linearity. Therefore to investigate this effect, detection linearity was measured and plotted as absorbance *vs.* concentration, and also as detection sensitivity A/c *vs.* absorbance graphs (Figure 3). The latter, as demonstrated previously,[19,24] allows direct evaluation of the level of stray light and effective path-length. While the calculated effective path-length was 59 μm, which is a satisfactory result for a capillary of I.D. of 75 μm,[19,24] the level of stray light (calculated as 30.5%) is high as a result of the parasitic Vis-range emission of the deep-UV-LED. While this Vis-range portion of light could be filtered away by a suitable band-pass filter such as an interference filter, this example illustrates well the importance of a quasi-monochromatic emission spectrum free of higher-wavelength parasitic emissions. It is important to stress that the compromised linearity has a greater impact at higher absorbance values and therefore has a negligible effect on the typically low absorbance values of the peaks detected in CE.

Separation of nucleotides (AMP, ADP and GDP) using detection with the 255 nm deep-UV-LED based detector installed in a previously reported in-house designed portable CE instrument[38] is shown in Figure 4.

The detection shows a satisfactory performance with baseline noise determined under these real CE conditions as 0.1 mAU and limits of detection (LODs) of 40, 43 and 107 μmol.L^{-1} for AMP, ADP and GDP respectively, which are comparable with LOD data in the μM range from a comprehensive literature review.[39] The reproducibilities of peak heights and areas with about 3% relative standard deviation (RSD) confirm an acceptable detection performance.

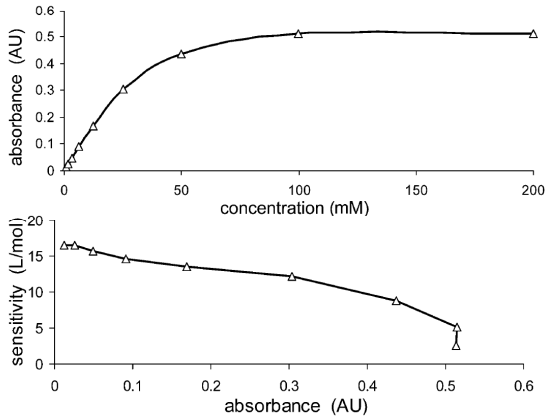

Fig. 3 Detection linearity as absorbance *vs.* concentration (top) and sensitivity *vs.* absorbance graph (bottom). Conditions: test analyte para-nitrophenol (pNP), effective path-length calculated for sensitivity extrapolated to $A = 0$ equal to $s = 17.7$, and pNP absorptivity = 3000 cm.L.mol^{-1} and the stray light % from the extrapolated high-end of the curve at $A = 0.51$.[19,24]

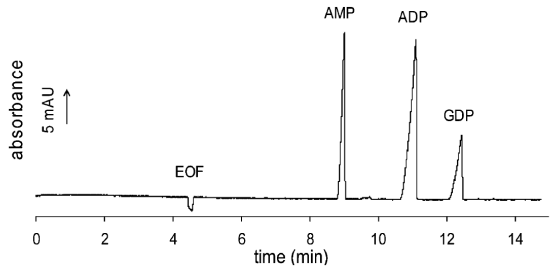

Fig. 4 CE separation of nucleotides using detection with 255 nm deep-UV-LED. Conditions: electrolyte: 25 mM ammonium acetate pH 10; fused silica capillary (75 μm I.D., length total/effective 39.0/31.0 cm); t = 20 °C; separation: 4 kV (50 μA); injection: hydrostatic, 2 cm, 30 s; sample: AMP and ADP 2.10^{-3} and GDP 1.10^{-3} mol.L^{-1}; detection: 255 nm deep-UV-LED; signal as PMT intensity converted to absorbance using the logarithmic function of the eDAQ data acquisition station (eDAQ, Sydney, Australia).

The demonstrated example showing a 255 nm deep-UV-LED utilised as a light source for on-capillary photometric detection in CE illustrates the potential that deep-UV-LEDs obviously have as light sources for photometric detection in miniaturised analytical devices.

An additional contribution this investigation makes is pointing out the problems in terms of parasitic Vis-spectra emission and heating that have to be overcome for the current generation of UV-LEDs. In terms of an outlook on the future of deep-UV-LEDs as lights sources for optical detection, the advance of newer generations of deep-UV-LEDs is expected with higher energy conversion and correspondingly higher optical power and lower heating, as well as providing quasi-monochromatic emission spectra free of Vis-parasitic emissions.[4,45]

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1.1.3 Příloha 3

Krcmova L., Urbanek L., Solichova D., Kasparova M., Vlckova H., Melichar B., Sobotka L., Solich P., HPLC method for simultaneous determination of retinoids and tocopherols in human serum for monitoring of anticancer therapy, JOURNAL OF SEPARATION SCIENCE, Volume: 32 Issue: 15-16 Special Issue: Sp. Iss. S, 2804-2811, AUG 2009, počet citací bez autocitací: 0, IF = 2,551

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Original Paper

HPLC method for simultaneous determination of retinoids and tocopherols in human serum for monitoring of anticancer therapy

A simple and rapid HPLC method requiring small volumes (250 μL) of human serum after C18 SPE sample preparation was developed using monolithic technology for simultaneous determination of all-*trans*-retinoic acid, 13-*cis*-retinoic acid, retinol, gamma- and alpha-tocopherol. The monolithic column, Chromolith Performance RP-18e (100 × 4.6 mm), was operated at ambient temperature. The mobile phase consisted of a mixture of acetonitrile (ACN) and 1% ammonium acetate in water (AMC) at pH 7.0. The mobile phase started at 98:2 (v/v) ACN/AMC (column pre-treatment) at a flow rate of 2 mL/min, then changed to 95:5 (v/v) ACN/AMC for 4 min at a flow rate of 1.5 mL/min and a further 3 min at a flow rate of 3.2 mL/min. Detection and identification were performed using a photodiode array detector. Retinol, 13-*cis*- and all-*trans*-retinoic acid were monitored at 325 nm. Both alpha- and gamma-tocopherol were detected at 295 nm. The total analysis time was 7.2 min. Tocol (synthesized tocopherol, not occurring in humans) was used as internal standard. The method was linear in the range of 0.125–10.00 μmol/L for all-*trans*-retinoic acid, 0.125–5.00 μmol/L for 13-*cis*-retinoic acid, 0.25–10.00 μmol/L for retinol, 0.5–50.00 μmol/L for gamma-tocopherol, and 0.5–50.00 μmol/L for alpha-tocopherol. The present method may be useful for monitoring of retinoids and tocopherols in clinical studies.

Keywords: Anticancer therapy / Monolithic column / Retinoic acid / Retinol / Tocopherol

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1 Introduction

All-*trans*-retinoic acid (ATRA) and 13-*cis*-retinoic acid (Fig. 1) are naturally occurring retinoids produced from retinol. Retinoic acids (RAs) have multiple roles in reproduction, embryogenesis, cellular differentiation, vision, and immune response. ATRA is used therapeutically in acute promyelocytic leukemia (APL) [1], and encouraging data have been reported on the activity ATRA in other tumors [2, 3]. After oral administration, RAs have variable pharmacokinetic profiles. Administration of ATRA induces accelerated metabolism, resulting in the lowering of the serum ATRA concentration and development of resistance [4, 5]. Despite these facts, all-*trans*-retinoic acid is not routinely determined in APL patients treated with this drug. 13-*cis*-Retinoic acid has also been used in clinical studies of chemoprevention, or in combination with other anticancer agents, mostly in patients with malignant or premalignant tumors of the head and neck region [6–8].

Tocopherols (both alpha-tocopherol and gamma-tocopherol) (Fig. 1) 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 [9]. Disorders of antioxidant balance involving tocopherols may be involved in the pathogenesis of some of the toxic effects of radiotherapy [10, 11] or chemotherapy [12]. Alpha-tocopherol may have chemopreventive activity and may also alleviate toxicity of retinoic acid administration.

Retinoic acid and tocopherols have been determined so far in different clinical samples using a variety of techniques [13]. In most studies, the method of determination of these compounds was based on HPLC (both normal and reversed phase) [14, 15]. HPLC technology provides rapid, sensitive, and accurate methods for vitamin determination and has the advantages of solvent economy and easy coupling with other techniques. Coupling

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Abbreviations: AMC, ammonium acetate in water

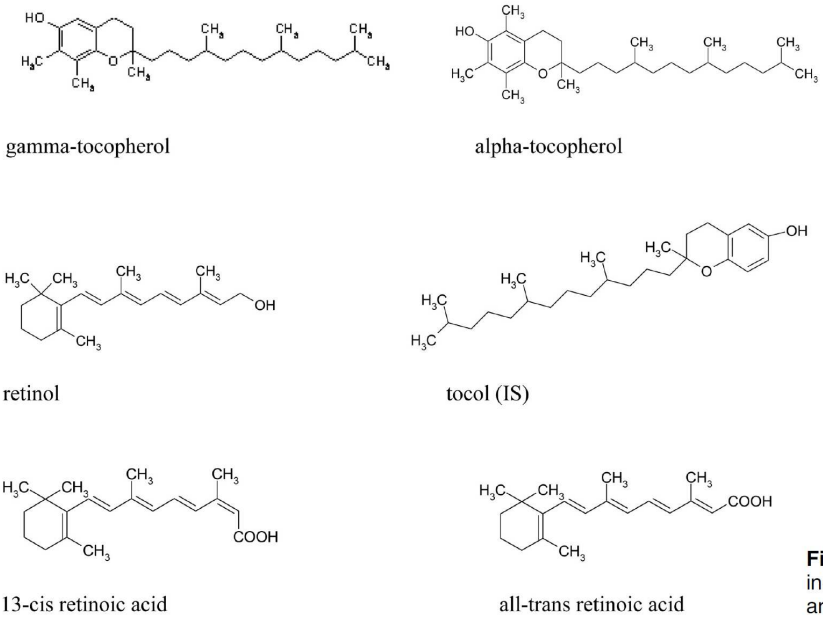

Figure 1. Chemical structures of examined fat-soluble vitamins, internal standard, all-*trans*- and 13-*cis*-retinoic acid.

the HPLC method with MS detection allows for low detection limits and small sample consumption [16, 17]. Determination of 13-*cis*- and all-*trans*-retinoic acid and tocopherols in one analysis may be beneficial especially in patients treated with retinoic acid derivatives because of the need to monitor retinoid concentrations as well as the concentrations of tocopherols that represent a major antioxidant system in the serum. Correlations between retinoic acids and tocopherols in human blood have been amply documented [18–21]. Moreover, combinations of retinoic acids with alpha-tocopherol have been studied in clinical trials [6, 8], but there is only one method for determination of retinoic acids and tocopherols in human serum in a single step. In 30 min 14 compounds are separated [22]. The long analysis time is due to the different polarities of the compounds. For analysis of samples from cancer patients it is not necessary to determine all these compounds and 30 min analysis is unsuitable for routine clinical practice. Use of simple gradient mobile phase and monoliths allows determination of different polarity samples in a short time.

The recently introduced monolithic technology is based on a unique sorbent material allowing good quality of separations in a minimal time. This special porous character allows relatively high mobile phase flow rates (1–9 mL/min) while keeping the back pressure low. Due to the favorable properties of monolithic materials, the risk of destruction and damage of the column by movement of the sorbent inside the column is eliminated and reliability as well as reproducibility of the analysis is improved [23].

Many examples can be found of increased separation speed at a high flow rate [23]. However, an increase in flow rate means consumption of a larger amount of solvents, unless smaller sized columns are used [24]. The main advantages of monoliths, apart from short analysis times, are long lifetime and immense robustness: All monolithic columns display very high mechanical stability and long operative lifetimes, in most cases far exceeding those of particulate columns. The next advantage is an improvement in HPLC system security because using monolithic columns also reduces maintenance work on HPLC pump and injector seals as well as the need for sample preparation because monolithic columns are very resistant to blocking even with biological samples [23, 24].

The aim of this study was to develop a simple and practical HPLC method using monoliths for the quantification of retinoic acid in pharmacokinetic studies that would also allow simultaneous determination of tocopherols. No method has yet been published for determination of retinoic acids and tocopherols in one step using monolithic technology.

2 Experimental

2.1 Instrumentation and chemicals

The analyses were performed using a Perkin Elmer HPLC set-up (Norwalk, USA) comprising an LC 200 pump, an LC 200 autosampler, an LC Column Oven 101 thermostat, and an LC 235C diode-array detector attached to the Per-

kin Elmer Turbochrom Chromatography Workstation version 4.1. All solvents were of HPLC grade. Both the methanol and the *n*-hexane used for the preparation of standard solutions were obtained from Scharlau (Sentmenat, Spain) and the ethanol for the deproteinization procedure from Lachema (Brno, Czech Republic). Acetonitrile was supplied by Scharlau (Sentmenat, Spain), ammonium acetate by Fluka–Sigma Aldrich (Prague, Czech Republic), distilled water by Goro (Prague, Czech Republic). Spe-ed SPE cartridges C18 500 mg/3 mL for sample extraction were obtained from Applied Separations (Allentown, USA). Retinol, alpha-tocopherol, and gamma-tocopherol were supplied by Fluka–Sigma Aldrich (Prague, Czech Republic), tocol by Matreya (Pleasant Gap, USA). ATRA and 13-*cis*-retinoic acid were purchased from Sigma –Aldrich (Prague, Czech Republic). A lyophilized human serum kit for HPLC analysis of vitamins A and E from Chromsystems (Munich, Germany) was used for the validation process.

2.2 Chromatographic conditions

Separation of vitamins and retinoic acids was performed using a Chromolith Performance RP-18e, 100 × 4.6 mm monolithic column, Merck (Darmstadt, Germany). Acetonitrile (ACN): 1% ammonium acetate in water at the pH 7.0 (AMC) in the ratio 95:5 (v/v) was used as the mobile phase. The column was pre-treated with ACN/AMC 98:2 (v/v) for 1 min at a flow rate of 2.0 mL/min. During separation ACN/AMC 95:5 (v/v) was used as the mobile phase for 4 min at a flow rate of 1.5 mL/min, then for 3 min at a flow rate of 3.2 mL/min. The column pressure was 3.32 MPa (1300 psi). The block heater LC Oven 101 Perkin Elmer (Norwalk, USA) was utilized to keep the analytical column temperature at 25°C. The injection volume of sample was 20 µL. Diode array detection of retinol and all-*trans*- and 13-*cis*-retinoic acid was at 325 nm and for alpha- and gamma-tocopherol and tocol it was at 295 nm. The total analysis time was 7.2 min.

2.3 Preparation of standard solutions

Stock solutions of all-*trans*- and 13-*cis*-retinoic acid, retinol, tocol, alpha- and gamma-tocopherol standards were prepared by the following methods. All-*trans*-retinoic acid and 13-*cis*-retinoic acid solutions were prepared by dissolution in 1000 µmol/L methanol. Tocol, alpha- and gamma-tocopherol standards were first dissolved in *n*-hexane (1000 µmol/L) and then diluted with methanol to give 500 µmol/L stock solutions. These standard solutions were stored at −25°C (retinol, all-*trans*- and 13-*cis*-retinoic acid) and at 4°C (tocol, alpha- and gamma-tocopherol). For calibration, working solutions of all standards were diluted with methanol in volumetric flasks in the concentration range: 0.25–10.00 µmol/L for retinol, 0.125–5.00 µmol/L for 13-*cis*-retinoic acid, 0.125–10.00 µmol/L for all-*trans*-retinoic acid, 0.5–50.00 µmol/L for gamma-tocopherol, 0.5–50.00 µmol/L for alpha-tocopherol. Calibration was accomplished at six concentration levels. The stability of stock and working solutions was 6 months for retinol, tocol, alpha- and gamma-tocopherol, 22 days for all-*trans*-retinoic acid and 16 days for 13-*cis*-retinoic acid.

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 after twelve hours of overnight fasting. The samples were then centrifuged (1600 × *g*, 10 min, 4°C) and serum was separated. Before solid phase extraction (SPE), an internal standard (20 µmol/L of tocol) was added and evaporated in the tube. Then 250 µL of serum was added and deproteinized by cooled ethanol (650 µL, 8 min, 4°C). After centrifugation (2000 × *g*, 15 min, 4°C), the supernatant was separated and applied to the SPE column. Before application of the sample the SPE column was activated by 1000 µL of methanol and then washed with 1000 µL of water. Hexane (2000 µL) was used as elution solvent. After evaporation of the hexane at 35°C, the residue was diluted in 250 µL of methanol and applied to the analytical column.

3 Results

3.1 Method validation

Method validation was performed according to the European Pharmacopoeia [25] and the International Conference on Harmonization (ICH) guidelines Q2A and Q2B [26, 27] 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 summarizes 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 $N = 5.545\,(t_R/W_{0.05})^2$ ($W_{0.05}$ 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 leading edge of the peak). Peak resolution was calculated by the

Table 1. System Suitability Test: number of theoretical plates (*N*), height equivalent of theoretical plate (HETP), asymmetry (*T*), and peak resolution (*R*).

Compound	$W_{0.05}$ (min)	t_R (min)	*N*	HETP (µm)	$W_{0.01}$ (min)	*f* (min)	*T*	*W* (min)	t_R (min)	*R*
Retinol	0.0658	1.77	3744.9	26.7	0.1667	0.0688	1.21	0.2000	1.77	1.68
13-*cis*-Retinoic acid	0.0606	2.13	4312.5	23.2	0.1563	0.0625	1.25	0.2286	2.13	2.99
all-*trans*-Retinoic acid	0.0517	2.90	8289.8	12.1	0.2500	0.109	1.15	0.2857	2.90	3.71
Tocol	0.0781	3.96	8821.0	11.3	0.4688	0.2344	1.00	0.2857	3.96	5.64
gamma-Tocopherol	0.1156	5.49	8235.5	12.1	0.2500	0.1155	1.08	0.2571	5.49	2.83
alpha-Tocopherol	0.1184	6.34	9668.4	10.3	0.3433	0.125	1.38	0.3429	6.34	

Table 2. Repeatability of injection of the method (serum).

n = 10	Mean of area (unit) ± SD	RSD (%)	Mean of retention time (min) ± SD	RSD (%)
Retinol	17155 ± 401	2.34	1.74 ± 0.01	0.52
13-*cis*-Retinoic acid	15933 ± 270	1.69	2.00 ± 0.01	0.50
all-*trans*-Retinoic acid	36071 ± 515	1.43	2.76 ± 0.02	0.74
Tocol	25001 ± 607	2.43	3.88 ± 0.09	2.22
gamma-Tocopherol	1563 ± 340	2.26	5.37 ± 0.06	1.14
alpha-Tocopherol	34489 ± 666	1.93	6.15 ± 0.08	1.23

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, 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. Table 2 compiles the RSD values of all-*trans*- and 13-*cis*-retinoic acid, retinol, tocol, alpha- and gamma-tocopherol in the serum sample.

3.1.2 Validation of parameters

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

3.1.2.1 Precision

For the determination of the method precision, ten samples prepared individually from one lyophilized human serum at two different concentration levels were analyzed. 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 3 compiles RSD values of all-*trans*- and 13-*cis*-retinoic acid, retinol, tocol, 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 serum pool spiked with 13-*cis*-retinoic acid (final concentration at Level 1 was 1.00 µmol/L and at Level 2 was 2.20 µmol/L), all-*trans*-retinoic acid (final concentration at Level 1 was 1.00 µmol/L and at Level 2 was 2.20 µmol/L), gamma-tocopherol (final concentration at Level 1 was 10.00 µmol/L and at Level 2 was 20.00 µmol/L). All the data are presented in Table 3.

Secondly, the accuracy of the method was determined by using the Chromsystems control set for vitamins A and E. The values obtained were always in the acceptable range (Level 1 retinol: 1.15–1.73 µmol/L, alpha-tocopherol: 15.00 – 21.3 µmol/L, Level 2 retinol: 2.75–4.12 µmol/L, alpha tocopherol: 28.0–40.1 µmol/L).

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, at six concentration levels in the range of 0.125–10.00 µmol/L for all-*trans*-retinoic acid, 0.125–5.00 µmol/L for 13-*cis*-retinoic acid, 0.25–10.00 µmol/L for retinol, 0.5–50.00 µmol/L for gamma-tocopherol, and 0.5–50.00 µmol/L for alpha-tocopherol. Tocol was used as an internal standard at a concentration of 20.00 µmol/L. Each solution was injected onto the column three times. Table 4 shows the calculated regression equations and correlation coefficients based on 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 ten times the value of the signal-to-noise ratio (Table 4).

Table 3. Precision, accuracy, and recovery in lyophilized human serum at two different concentration levels: For precision, ten samples were prepared individually from one lyophilized human serum at two different concentration levels, the accuracy of the method was tested first as recovery, which was determined by performing three measurements of the pool spiked serum and secondly the accuracy of the method was determined by using the Chromsystems control set for vitamin A and E.

	Level			Precision (Intra-day) Repeatability of the method		
	Added (µmol/L)	Found (µmol/L)	Recovery (%)	Area SD (units)/retention time SD (min)	*n*	RSD area/RSD retention time
Retinol	1.64	1.35	82.30	36464 ± 872/1.70 ± 0.03	10	2.39/1.95
	3.58	3.05	85.19	71697 ± 2075/1.75 ± 0.01	10	2.89/0.64
13-*cis*-Retinoic acid	1.00	1.00	100.00	20992 ± 274/2.10 ± 0.03	10	1.31/1.22
	2.20	2.06	93.64	43378 ± 1260/2.05 ± 0.02	10	2.90/0.95
all-*trans*-Retinoic acid	1.00	0.74	74.00	23833 ± 637/2.84 ± 0.06	10	2.67/2.13
	2.20	1.55	70.50	48388 ± 1059/2.90 ± 0.06	10	2.19/2.10
gamma-Tocopherol	10.00	9.81	98.10	10724 ± 231/5.25 ± 0.07	10	2.16/1.38
	20.00	17.39	87.00	17834 ± 551/5.14 ± 0.08	10	3.09/1.59
alpha-Tocopherol	17.6	15.17	85.71	16153 ± 313/6.09 ± 0.09	10	1.94/1.49
	36.70	28.03	77.10	29517 ± 95/6.02 ± 0.09	10	2.69/1.44

Table 4. Linearity and limit of detection and quantification.

Compound	Equation	Coeff. of correlation	Reliability (%)	LOD (µmol/L) signal-to-noise = 5	LOQ (µmol/L) signal-to-noise = 10
Retinol	$y = 0.8247x + 0.0177$	0.9998	99.5	0.03	0.06
13-*cis*-Retinoic acid	$y = 0.779x - 0.0104$	0.9997	99.5	0.04	0.09
all-*trans*-Retinoic acid	$y = 0.7038x + 0.0434$	0.9995	99.5	0.06	0.12
gamma-Tocopherol	$y = 0.0341x + 5E\text{-}05$	0.9999	99.5	0.60	1.20
alpha-Tocopherol	$y = 0.0278x - 0.0133$	0.9998	99.5	1.00	2.00

3.1.2.5 Selectivity

For the quantification of the method, tocol was used as an internal standard. The good selectivity of this method for retinol, 13-*cis*-retinoic acid, ATRA, and other compounds is documented in Fig. 2 and in Table 1.

3.1.2.6 Ruggedness

For the determination of method ruggedness, the pH of mobile phase and mobile phase composition were varied. Retention time and peak area were determined. The retention time was clearly not affected by variation of pH. But the area of the retinoic acids was clearly affected thereby (variations over 14%). Small changes of mobile phase composition (± 2.2%) influence the retention times of tocopherols (variations over 9%) and the areas of all compounds (variations over 5%). But there is still adequate resolution for all compounds.

3.1.2.7 Stability

The stability of stock and working standard solutions of all-*trans*- and 13-*cis*-retinoic acid, retinol, tocol, and alpha- and gamma-tocopherol was tested at room temperature, 4°C, −25°C, and −80°C. Concentrations of compounds were calculated with the aid of an internal standard using calibration curve. The best stability for all-*trans*-retinoic acid was at −25°C for 22 days and for 13-*cis*-retinoic acid at −80°C for 16 days (Figs. 3 and 4). The stock and working solutions of alpha- and gamma-tocopherol were stable for 6 months at 4°C. The best stability for retinol was at −25°C for 6 months.

4 Discussion

The newly developed method presented here combines the advantages of HPLC, monolithic technology, and solid phase extraction.

In this study, using a PDA detector, reverse phase, and flow rate gradient HPLC procedure, very polar and non-polar analytes were separated in a single run within 7 min. The PDA detector enables analysis of a variety of analytes of interest while monoliths allow high flow rates and then short time of analyses. It is easier to regenerate the column because compounds of all types of polarity are eluted during each run. In our method there is a re-equilibration time of only 1 min which is suitable for routine analyses of many samples. Ammonium acetate, which is used to reduce the tailing of compounds with carboxyl functions such as retinoic acids, had several advantages over other buffering systems, such as phosphates and acetic acid, in being chemically stable in

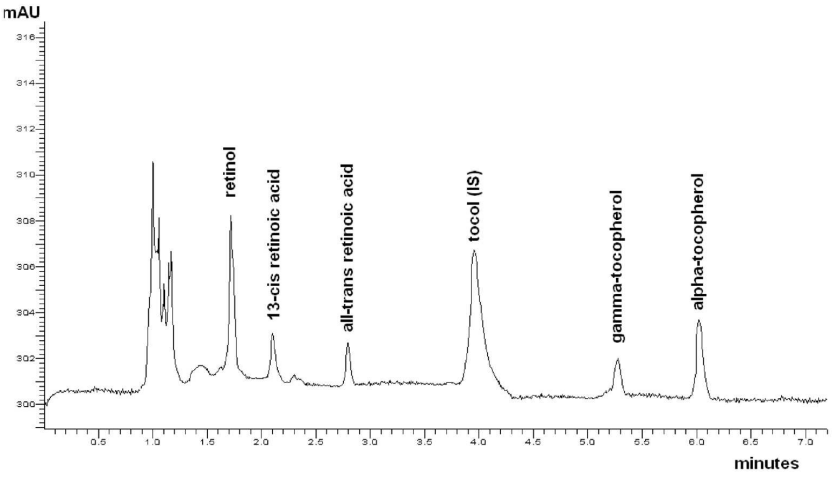

Figure 2. Separation of retinol, 13-*cis*-retinoic acid, all-*trans*-retinoic acid, gamma- and alpha-tocopherol in human serum (1.74 min retinol 0.99 μmol/L, 2.09 min 13-*cis*-retinoic acid 0.50 μmol/L, 2.81 min all-*trans*-retinoic acid 0.33 μmol/L, 3.09 min tocol (IS), 5.38 min gamma-tocopherol 7.44 μmol/L, 6.05 min alpha-tocopherol 22.56 μmol/L).

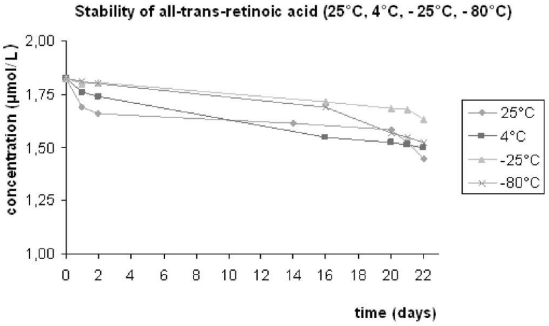

Figure 3. Stability of all-*trans*-retinoic acid in methanolic solution at concentration 1.82 μmol/L at different temperatures (calculated by internal standard).

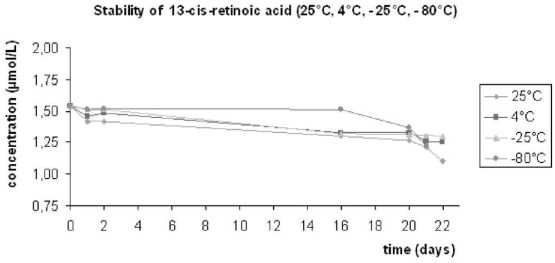

Figure 4. Stability of 13-*cis*-retinoic acid in methanolic solution at concentration 1.54 μmol/L at different temperatures (calculated by internal standard).

methanol and water, and suitable for masking the residual silanol functions [22]. Thus symmetrical peaks and better separations of analytes were possible.

Extraction of retinoids by use of the SPE techniques has been known to give low recoveries which are most probably caused by their highly protein-binding nature and their poor solubility in aqueous media [16].

Several procedures for sample pretreatment have been developed. Sample preparation is usually restricted to a liquid extraction procedure to liberate retinoids from the matrix and to solubilize them for subsequent injection onto a HPLC system. Some investigators used an on-line solid-phase extraction in combination with column switching [28]. Retinoids are sensitive to light, oxygen, and heat, all of which have to be considered in relation to sample collection and storage [13, 29]. Only a few examples exist where biological samples were applied to an analytical column without prior extraction [13, 30]. Gundersen and Sakhi used on-line SPE [16, 30]. Various methods for liquid–liquid extraction of retinoids were performed [31–33]. To extract all retinoids efficiently from the biological matrix, a mono-phase liquid extraction utilizing isopropanol was employed [34]. Tocopherols were extracted into hexane using ethanol or ethanol with 1% of methanol as deproteinization solution [23, 35, 36].

Solid phase extraction of retinoids is very complicated. The reason may be the lack of good recovery when untreated plasma samples are applied to reversed phase stationary phases [13]. McPhilips *et al.* precipitated plasma proteins with acetonitrile and diluted the supernatant with acetic acid before SPE on C18 cartridges [37]. Plasma was extracted with *tert*-butyl methyl ether, using Extrelut columns, followed by a second clean-up on amino columns [13]. Vitamin E was extracted with other vitamins using a robotic station for liquid–liquid extraction, connected on-line with an automatic system for solid-phase extraction [38].

4.1 Application of the method

A simple, reproducible, and rapid method using monoliths that allows high throughput determination of phys-

iological and pharmacological concentrations of retinoids and tocopherols in serum samples is presented. Potential utilization of the method includes monitoring of drug concentrations in patients treated with retinoic acid for acute promyelocytic leukemia or for other malignancies in clinical trials. The monitoring of serum all-*trans*-retinoic acid concentrations is important in patients treated with this substance because of variations in pharmacokinetics and autoinduction of catabolism [4, 5]. Moreover, retinoic acid metabolism could be altered because of comorbidity, which is frequent in cancer patients. All-*trans*-retinoic acid is currently the standard therapy for acute promyelocytic leukemia, but 13-*cis*-retinoic acid may also be active in other tumors, *e.g.* squamous cell carcinoma of head and neck or uterine cervix [2, 6]. Retinoids are also under active investigation for cancer prevention, and monitoring of serum retinoic acids concentrations may also be useful in chemopreventive trials. Retinoic acids have also been studied in non-neoplastic disorders, such as emphysema [39].

The available data on serum retinoic acid concentrations in normal subjects or patients are limited, especially when compared with the data on other retinoids, *e.g.* retinol. Serum retinoic acid concentrations in normal subjects are in the nanomolar range, usually between 3 and 8 nmol/L [28, 39, 40], but slightly lower concentrations have been reported elsewhere [41]. Serum retinoic acid concentrations two orders of magnitude higher have been observed after oral administration [4, 5, 42]. A less dramatic increase of serum retinoic acid has been described as a result of a diet rich in retinoids or vitamin A supplements [43]. Lower retinoic acid concentrations have been reported in newborns [44] or during fasting [45]. Lower levels of serum retinoic acid have been observed in patients with lung cancer [46]. In animals, an increase of serum retinoic acid has been associated with inflammation [47]. However, there is little data on serum retinoic acid in patients with other tumors and in cancer patients during different phase of the disease, or before and after surgery, chemotherapy, or radiation.

So far, in most publications, alpha-tocopherol has been investigated in epidemiological studies in relation to cancer risk. For example, lower vitamin E concentrations have been reported in patients with breast cancer [48]. An association of low vitamin E intake and toxicity of chemotherapy has been reported in children with acute lymphoblastic leukemia [39]. We reported a similar observation in patients with breast and ovarian carcinoma treated with paclitaxel/platinum combination [49]. In addition, the administration of vitamin E has been shown to alleviate toxic effects of radiotherapy [10] or chemotherapy [13]. Although serial monitoring of vitamin E levels is a necessary prerequisite for any therapeutic use of this antioxidant vitamin, serum vitamin E is still not being routinely measured in cancer patients.

5 Concluding remarks

In this work, a new fast and simple HPLC method for selective and sensitive determination of all-*trans*-retinoic acid, 13-*cis*-retinoic acid, retinol, gamma- and alpha-tocopherol in human serum using a monolithic column for clinical monitoring was developed and validated.

This method may be useful for pharmacokinetic monitoring of patients treated with retinoic acid for acute promyelocytic leukemia or patients with other tumors treated in clinical trials. This method will allow analysis of a large number of samples in clinical studies at relatively low cost, resulting in a better understanding of the metabolism of these compounds.

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1.1.4 Příloha 4

Urbanek L., Krcmova L., Solichova D., Melichar B., Opletalova V., Solich P., Development and validation of a liquid chromatography method for the simultaneous determination of alpha-tocopherol, retinol and retinyl esters in human serum using a monolithic column for the monitoring of anticancer therapy side effects, JOURNAL OF SEPARATION SCIENCE, Volume: 29 Issue: 16, 2485-2493, NOV 2006, počet citací bez autocitací: 4, IF = 2,535

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Original Paper

Development and validation of a liquid chromatography method for the simultaneous determination of α-tocopherol, retinol and retinyl esters in human serum using a monolithic column for the monitoring of anticancer therapy side effects

Among other side effects, administration of anticancer agents is accompanied by manifestations of gastrointestinal toxicity and disturbances of antioxidant balance. The monitoring of these toxic effects in clinical practice is impeded by a dearth of reliable laboratory methods. Therefore, a simple and rapid reversed-phase high-performance liquid chromatography procedure for selective and sensitive determination of retinol, α-tocopherol, and retinyl esters (retinyl-palmitate and retinyl-stearate) in blood serum has been developed and presented in this study. A Series 200 LC HPLC instrument from Perkin Elmer (Norwalk, USA) with diode-array detector (DAD) was used for the analysis. Separations of retinol, α-tocopherol, retinyl-palmitate, and retinyl-stearate were performed using a Chromolith Performance RP-18e, 100 × 4.6 mm monolithic column from Merck (Darmstadt, Germany). Gradient elution was used at a flow rate of 3 mL/min; the mobile phase was methanol-water (95:5, v/v) for 0–2.1 min and methanol-2-propanol (60:40, v/v) for 2.1–4.9 min. The total time of analysis was 6 min. The injection volume was 20 μL and the analysis was performed at ambient temperature. Detection of retinol, α-tocopherol, and retinyl esters was carried out at 325, 295, and 330 nm, respectively. For practical assessment of the method, the vitamin A absorption test was performed on seven healthy controls as well as on six patients with non-small cell lung carcinoma or head and neck carcinoma previously treated by chemotherapy and/or radiotherapy, six patients with rectal carcinoma before chemoradiotherapy, four patients with gastrointestinal stromal tumor (GIST) before treatment with imatinib, and a breast cancer patient with chemotherapy-induced diarrhea. Present data demonstrate the feasibility of large scale HPLC determination of vitamin E, vitamin A, and retinyl esters in human serum using a silica monolithic column, and this method may represent a valuable aid in the laboratory monitoring of the toxicity of anticancer therapy.

Keywords: HPLC / Monolithic column / Retinol / Retinyl esters / α-Tocopherol

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1 Introduction

The remarkable progress achieved during the past decades in medical therapy of malignant disorders has been at the price of significant toxicity that accompanies cancer treatment. The myriad different side effects of pharmacological agents used for cancer therapy include manifestations of gastrointestinal toxicity and disorders of antioxidant balance.

Although gastrointestinal toxicity represents one of the most common side effects of administration of cytotoxic agents, the assessment of gastrointestinal toxicity still relies mostly on anamnestic data. There is an obvious need for laboratory methods for objective assessment of

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Abbreviations: HETP, height equivalent of a theoretical plate; **LLE,** liquid-liquid extraction; **SST,** system suitability test

such gastrointestinal toxicity. Previous studies have demonstrated that the measurement of intestinal permeability may be used for detection and monitoring of the gastrointestinal toxicity of cytotoxic agents [1]. However, there are several factors limiting the use of intestinal permeability measurements in the assessment of gastrointestinal toxicity, and laboratory tests based on different principles are needed.

Vitamin A is absorbed in the small intestine [2]. The resulting marked rise in serum retinyl esters may be used for assessment of the function of the small bowel [3, 4].

Vitamin E represents a major antioxidant in serum [5, 6]. Administration of cytotoxic agents and, to a lesser extent, biological agents, may be associated with antioxidant stress. Disorders of antioxidant balance involving vitamin E may also be involved in the toxicity associated with radiotherapy [7] or chemotherapy [8].

In the present report we describe the development and validation of an HPLC method for determination of vitamin E, vitamin A, and retinyl esters in human serum using a silica monolithic column for the monitoring of side effects of anticancer therapy.

2 Experimental

2.1 Chemicals and reagents

All-*trans*-retinol and DL-α-tocopherol were obtained from Fluka (Prague, Czech Republic). Retinyl-acetate, retinyl-propionate, and retinyl-palmitate were purchased from Sigma-Aldrich (Prague, Czech Republic). Retinyl-stearate was synthesized at the Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy in Hradec Králové, using the modified method described by Huang and Goodman [9]. Slovakofarma vitamin A capsules (30 000 IU) from Zentiva (Hlohovec, Slovakia) were administrated to patients during vitamin A absorption tests.

Vitamin A/E by HPLC Control Set from Bio-Rad (Bio-Rad Laboratories, Munich, Germany) and the ClinChek-Control lyophilized human serum kit for HPLC analysis of vitamins A and E from Recipe (Recipe Chemicals + Instruments Labortechnik, Munich, Germany) were used for the validation process. HPLC-grade methanol and *n*-hexane for the preparation of standard solutions were obtained from Merck (Darmstadt, Germany). Ethanol denatured with 5% methanol for the deproteinisation procedure was obtained from Lachema (Brno, Czech Republic). Methanol and toluene of p.a. purity for the HPLC mobile phase were supplied by Penta (Prague, Czech Republic). HPLC gradient 2-propanol was obtained from Scharlau Chemie (Sentmenat, Spain). Helium 4.6 was purchased from Linde (Prague, Czech Republic).

2.2 Chromatography

The analyses were performed using a Perkin Elmer HPLC set-up (Norwalk, USA) comprising an LC 200 pump, an LC 200 autosampler, an LC Column Oven 101 thermostat, and an LC 235C Diode Array Detector attached to the Perkin Elmer Turbochrom Chromatography Workstation version 4.1. Separation of retinol, α-tocopherol, retinyl-palmitate, and retinyl-stearate was performed using a Chromolith Performance RP-18e, 100 × 4.6 mm monolithic column Merck (Darmstadt, Germany). Gradient elution was used at a flow rate of 3 mL/min; mobile phase methanol-water (95 : 5, v/v) for 0–2.1 min and methanol-2-propanol (60 : 40, v/v) for 2.1–4.9 min. The total time of analysis was 6.0 min.

A block heater LC Oven 101 (Perkin Elmer, Norwalk, USA) was utilized to keep the analytical column temperature at 25°C. The injection volume was 20 µL. The DAD detection of retinol, α-tocopherol, retinyl-palmitate, and retinyl-stearate was carried out at 325, 295, and 330 nm, respectively.

2.3 Preparation of standards and samples

2.3.1 Standard solutions

Stock solutions of retinol and α-tocopherol standards were prepared as follows: a 500 µmol/L retinol solution was prepared by dissolution in methanol; α-tocopherol standard was dissolved in *n*-hexane to prepare a solution of concentration 2000 µmol/L. The stock solutions of retinyl esters (2000 µmol/L) were prepared by dissolution in *n*-hexane. The stock solutions of retinol and retinyl esters were stored at −25°C. The stock solution of α-tocopherol standard was stored at 4°C.

For the calibration, working solutions of stock standards were diluted with methanol in volumetric flasks in the concentration ranges: 0.10–10.00 µmol/L for retinol; 0.5–50.0 µmol/L for α-tocopherol; 0.5–40.0 µmol/L for retinyl-palmitate; and 0.5–20.0 µmol/L for retinyl-stearate. The calibration was accomplished at six concentration levels. The stability of stock and working solutions of retinol and α-tocopherol was six months. The stability of stock and working solutions of retinyl esters was one month.

2.3.2 Vitamin A absorption test

For practical assessment of the method, the vitamin A absorption test was performed. Blood samples were drawn from the peripheral vein after a twelve-hour overnight fast and they were marked as sample one. These samples were centrifuged (1600 × *g*, 10 min, 16°C) and the serum was separated and stored at −85°C. Then, a single oral dose of vitamin A (360 000 IU) was administered to patients. The second blood sample was collected five

Table 1. System suitability test.

	Theoretical plates (*N*)	Height equivalent of theoretical plate (HETP) (mm)	Tailing factor	Resolution
Retinol	3 771	26.52	1.16	7.456
α-Tocopherol	2 372	42.15	1.16	8.639
Retinyl-palmitate	60 814	1.64	1.06	3.380
Retinyl-stearate	73 202	1.37	1.25	–

Table 2. Intra-day precision of the method.

n = 10	Mean of area (unit) ± SD	RSD (%)	Mean of retention time (min)	RSD (%)
Retinol	15 821.25 ± 836.7	5.29	0.68 ± 0.010	0.73
α-Tocopherol	13 899.25 ± 929.1	6.68	2.18 ± 0.041	1.92
Retinyl-palmitate	56 057.99 ± 4539.4	8.10	4.48 ± 0.014	0.33
Retinyl-stearate	19 783.51 ± 1117.5	5.65	4.76 ± 0.014	0.32

hours after administration of vitamin A and processed by the same procedure.

The institutional ethical committee approved the protocol of this study and all patients signed an informed consent to participate in the study.

2.3.3 Sample preparation

In the liquid-liquid extraction (LLE) procedure, 2500 µL of *n*-hexane-toluene (8:2, v/v) mixture was added to 500 µL of serum and extraction performed for 5 min with shaking. The mixture was deproteinized by cool ethanol denatured with 5% methanol (500 µL, 5 min, 4°C) and extracted for 5 min by the same apparatus. After centrifugation (1600 × *g*, 10 min, 0°C), an aliquot (2000 µL) of the clear extract was taken. Another 2000 µL of *n*-hexane-toluene (8:2, v/v) mixture was added to the rest of the serum sample to repeat the extraction process. The collected clear extract was evaporated down in a vacuum concentrator. Then the residue was dissolved in 100 µL of *n*-hexane and 300 µL of methanol and finally 20 µL of the sample was injected onto the chromatographic column (The residues obtained after evaporation can be stored at −25°C and these samples are stable for three weeks under these conditions.)

3 Results

3.1 Method validation

Method validation was performed according to the European Pharmacopoeia [10] and the International Conference on Harmonization (ICH) guidelines Q2A and Q2B [11, 12] consisting of two parts: System Suitability Test (SST) and Validation parameters.

3.1.1 System suitability test

Within the system suitability test (SST), some chosen parameters describing the separation properties of the chromatographic system were determined. Table 1 summarizes the calculated values of the number of theoretical plates, height equivalent of a theoretical plate (HETP), tailing factor, and resolution.

3.1.2 Validation parameters

3.1.2.1 Precision

For the determination of method precision, ten samples prepared individually from one lyophilized human serum at a single concentration level were analyzed. The method precision expressed by repeatability of peak area and retention time was determined as the relative standard deviation (RSD) calculated from the obtained data. Table 2 lists the RSD values of retinol, α-tocopherol, retinyl-palmitate, and retinyl-stearate.

3.1.2.2 Accuracy

The accuracy of the method was tested as recovery, which was determined by performing three measurements at three concentration levels. The serum pool was spiked with 0.5 µmol/L and 1.0 µmol/L of retinol, 5.0 µmol/L and 10.0 µmol/L of α-tocopherol, 10.0 µmol/L, 5.0 µmol/L, and 10.0 µmol/L of retinyl-palmitate, and 5.0 µmol/L, 2.5 µmol/L, and 5.0 µmol/L of retinyl-stearate. The mean recovery (*n* = 3) was determined. All data are presented in Tables 3, 4, and 5.

3.1.2.3 Linearity

The 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 0.1 – 10.0 µmol/L for retinol, 0.5 – 50.0 µmol/L for α-tocopherol, 0.5 – 40.0 µmol/L for retinyl-palmitate, and 0.5 – 20.0 µmol/L for retinyl-stearate. Each solution was injected into the column three times. The regression equations and correlation coefficients calcu-

Table 3. Recovery – Level 1.

$n = 3$	c_0 (μmol/L)	c_i (μmol/L)	R_i (%)
Retinol	0.98	0.82	83.7
α-Tocopherol	14.40	13.87	96.3
Retinyl-palmitate	10.00	9.70	97.0
Retinyl-stearate	5.00	4.50	90.0

c_0 – expected (calculated) concentration, c_i – real concentration (measured value). $R_i = 100 \cdot c_i/c_0$

Table 4. Recovery – Level 2.

$n = 3$	c_0 (μmol/L)	c_i (μmol/L)	R_i (%)
Retinol	0.50	0.48	96.0
α-Tocopherol	5.00	4.50	90.0
Retinyl-palmitate	15.00	15.60	104.0
Retinyl-stearate	7.50	11.06	88.5

c_0 – expected (calculated) concentration, c_i – real concentration (measured value). $R_i = 100 \cdot c_i/c_0$

Table 5. Recovery – Level 3.

$n = 3$	c_0 (μmol/L)	c_i (μmol/L)	R_i (%)
Retinol	1.00	0.96	96.0
α-Tocopherol	5.00	4.39	87.8
Retinyl-palmitate	20.00	19.26	96.3
Retinyl-stearate	10.00	10.06	100.6

c_0 – expected (calculated) concentration, c_i – real concentration (measured value).

lated on the basis of data obtained for retinol, α-tocopherol, and retinyl esters are shown in Table 6.

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 ten times the value of the signal-to-noise ratio (Table 7).

3.1.2.5 Selectivity

For quantitation, the external standard method was applied instead of using retinyl-acetate or retinyl-propionate as internal standard, as is commonly done, because of possible contamination of patients' serum by these compounds. Vitamin A was administered to patients in the form of its acetate during the vitamin A absorption test. The good selectivity of this method for retinyl-acetate and retinyl-propionate and other compounds is documented in Fig. 1 and Table 1 together with the resolution values.

Table 7. LOD and LOQ.

	LOD (μmol/L)	LOQ (μmol/L)
Retinol	0.04	0.08
α-Tocopherol	0.50	1.10
Retinyl-palmitate	9.40[a)]	0.02
Retinyl-stearate	0.05	0.10

[a)] nmol/L.

3.1.2.6 Stability

The stability of stock and working standard solutions of retinol, α-tocopherol, retinyl-palmitate, and retinyl-stearate was tested at room temperature, 4°C, and −25°C. The best stabilities were recorded at −25°C, *i.e.*, six months for retinol and one month for retinyl esters. The stock and working solutions of α-tocopherol were stable for six months at 4°C.

3.1.2.7 Application of method

For practical assessment of the method, the vitamin A absorption test was performed on seven healthy controls as well as on six patients with non-small cell lung carcinoma or head and neck carcinoma previously treated by chemotherapy and/or radiotherapy, six patients with rectal carcinoma before chemoradiotherapy, four patients with gastrointestinal stromal tumor (GIST) before treatment with imatinib, and a breast cancer patient with chemotherapy-induced diarrhea.

Serum concentrations of retinol, of retinyl esters before and after the vitamin A absorption test, and of α-tocopherol are shown in Table 8. Only a small increase in serum retinol was observed after oral administration of vitamin A. In contrast, the concentrations of retinyl esters rose markedly from pre-test concentrations that were often around the limits of detection. The increase

Table 6. Linearity.

	Equation	Correlation coefficient R	Residual deviation S_{res}	Reliability (%)
Retinol	$y = 18341x + 738.52$	0.9997	174	99.9
α-Tocopherol	$y = 1280x + 429.36$	0.9998	339	99.9
Retinyl-palmitate	$y = 6340x + 468.9$	0.9995	2210	99.9
Retinyl-stearate	$y = 5565x - 716$	0.9998	482	99.9

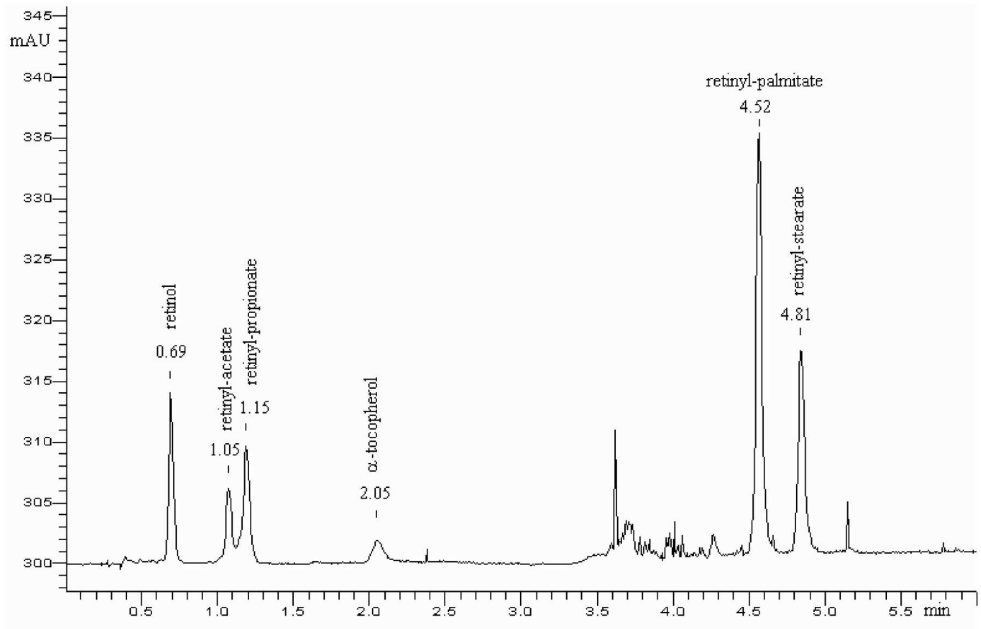

Figure 1. Selectivity of retinol, retinyl-acetate, retinyl-propionate, α-tocopherol, retinyl-palmitate and retinyl-stearate standards

Table 8. Clinical study.

	Healthy controls (n = 7)	Head and neck cancer patients and non-small cell lung cancer patients before gefitinib therapy (n = 6)	Metastatic GIST (n = 4)	Rectal carcinoma patients before therapy (n = 7)
Retinol pre-test (μmol/L)	2.962 ± 0.708 (1.935 – 3.864)	2.107 ± 0.739 (1.054 – 3.254)	2.681 ± 1.027 (1.679 – 4.109)	3.229 ± 1.248 (2.032 – 5.779)
Retinyl-palmitate pre-test (μmol/L)	0.142 ± 0.176 (0 – 0.496)	0.483 ± 0.567 (0 – 1.326)	0.185 ± 0.290 (0 – 0.610)	0.223 ± 0.253 (0 – 0.670)
Retinyl-stearate pre-test (μmol/L)	0.277 ± 0.211 (0 – 0.497)	0.198 ± 0.174 (0 – 0.406)	0.172 ± 0.200 (0 – 0.364)	0.173 ± 0.168 (0 – 0.390)
α-Tocopherol post-test (μmol/L)	18.300 ± 6.824 (9.839 – 29.804)	13.294 ± 8.225 (1.485 – 26.362)	13.495 ± 3.295 (11.085 – 18.366)	18.933 ± 7.335 (5.311 – 28.446)
Retinol post-test (μmol/L)	3.485 ± 1.169 (2.167 – 5.381)	2.164 ± 0.888[c] (0.564 – 3.150)	3.317 ± 0.806 (2.261 – 4.188)	3.625 ± 1.545 (2.312 – 6.747)
Retinyl-palmitate post-test (μmol/L)	23.205 ± 21.068 (2.808 – 63.231)	4.491 ± 3.968[b] (0 – 10.905)	31.491 ± 29.767 (6.312 – 67.964)	37.473 ± 36.706 (4.211 – 107.593)
Retinyl-stearate post-test (μmol/L)	10.361 ± 8.816 (1.503 – 25.773)	2.004 ± 1.938[a] (0.233 – 5.602)	14.264 ± 13.241 (2.698 – 31.013)	15.046 ± 13.490 (1.900 – 38.897)
Retinol increase (μmol/L)	0.523 ± 0.638 (–0.619 – 1.517)	0.057 ± 0.342 (–0.490 – 0.390)	0.636 ± 0.476 (0.079 – 1.242)	0.396 ± 0.673 (–0.523 – 1.472)
Retinol increase (%)	16.5 ± 19.0 (–21.8 – 39.3)	–0.33 ± 25.0 (–46.5 – 23.8)	28.6 ± 21.3 (1.9 – 53.1)	12.8 ± 21.0 (–14.8 – 51.0)
Retinyl-palmitate increase (μmol/L)	23.057 ± 21.073 (2.725 – 63.009)	4.008 ± 3.869[a] (0 – 10.809)	31.297 ± 29.717 (6.293 – 67.945)	37.241 ± 36.707 (4.192 – 107.574)
Retinyl-palmitate increase (fold)	500.8 ± 695.2 (16.0 – 1993.5)	24.0 ± 44.0[a] (0 – 113.5)	1010.9 ± 1715.3 (62.9 – 3577.1)	969.6 ± 2074.6 (24.6 – 5662.8)
Retinyl-stearate increase (μmol/L)	10.055 ± 8.751 (1.011 – 25.390)	1.772 ± 1.886[a] (0.133 – 5.233)	14.042 ± 13.185 (2.600 – 30.649)	14.830 ± 13.450 (1.602 – 38.507)
Retinyl-stearate increase (fold)	39.4 ± 24.7 (3.1 – 68.4)	9.0 ± 6.3[a] (1.541 – 15.324)	78.3 ± 78.6 (14.3 – 186.8)	86.9 ± 83.8 (6.5 – 249.9)

[a] p <0.05 compared to control group and both other cancer patient groups.
[b] p <0.05 compared to control group and patients with rectal carcinoma.
[c] p = 0.05 compared to control group.

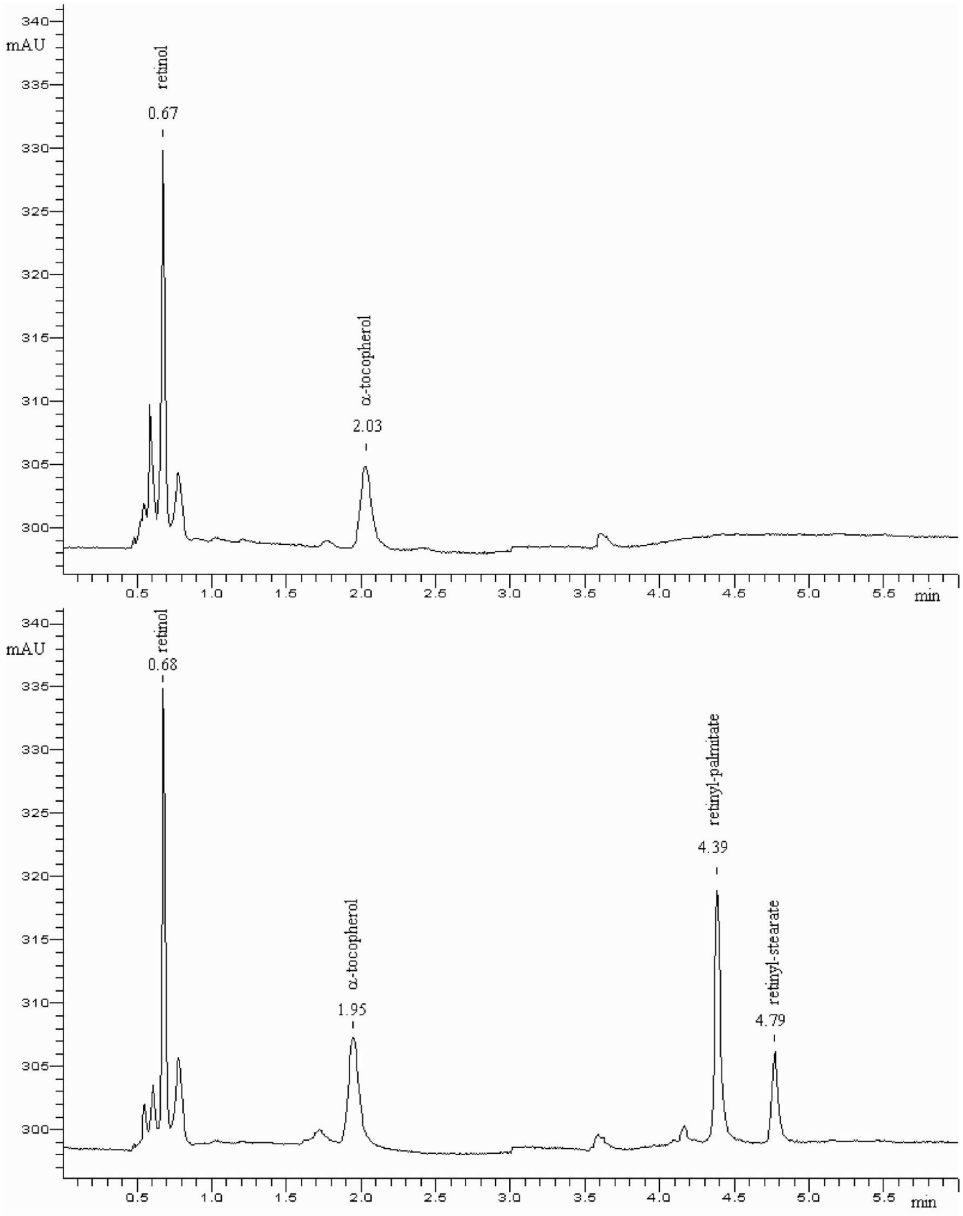

Figure 2. Retinol, retinyl-palmitate and retinyl-stearate before and after administration of vitamin A in a normal subject

was usually several hundred-fold for retinyl-palmitate and several dozen-fold for retinyl-stearate (the limit of quantitation was used to calculate the increase in the case of pre-test concentrations below the limit of quantitation). No differences were observed in post-absorption retinol and retinyl ester concentrations between the chemotherapy-naive patients with rectal carcinoma and GIST and controls. In contrast, compared to controls, patients with lung, head, and neck carcinomas examined before gefitinib therapy (all of them had been previously treated by chemotherapy and/or radiotherapy) had significantly lower concentrations of post-absorption retinyl esters while lower concentrations of post-absorption retinol were only of borderline significance. The retinyl esters, whether studied as post-absorption concentrations, absolute increase in concentration, or an increase expressed as a multiple of the original value, even in the present limited cohort allowed a clear dis-

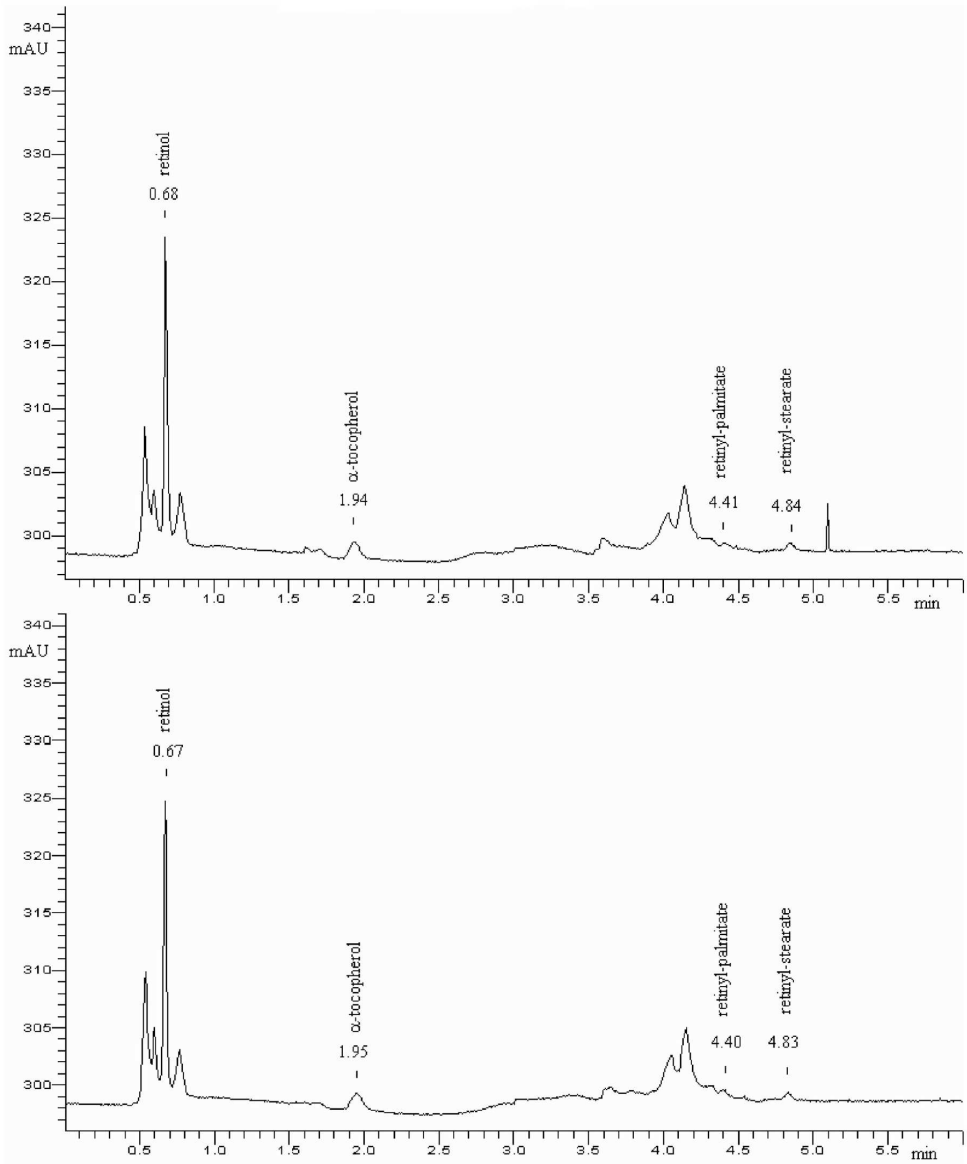

Figure 3. Retinol, retinyl-palmitate and retinyl-stearate before and after administration of vitamin A in a breast cancer patient with chemotherapy-induced diarrhea

tinction between pretreated patients with lung or head and neck carcinomas, and other groups. The typical chromatograms of retinol, retinyl-palmitate, and retinyl-stearate before and after administration of vitamin A in a normal subject and a breast cancer patient with chemotherapy-induced diarrhea are shown in Figs. 2 and 3.

4 Discussion

Currently, many different methods are available for the determination of retinol, α-tocopherol, and retinyl esters in biological samples [13–20]. Most of these procedures are expensive in terms of time and resources and the utilization of monolithic columns might thus be advantageous [21]. Although a number of studies have been published on the preparation, testing, and physical and chromatographic properties of monoliths [22–27], the number of articles dealing specifically with applications of monolithic columns in clinical practice is still relatively low despite a marked increase in the last three years [28]. These recent publications mostly cover analysis of drugs and metabolites [29–33], environmentally relevant sub-

stances [34], food additives [35 – 37], chiral separations [38], as well as bioanalytical separations [39 – 41]. In order to determine the above-mentioned micronutrients in biological material, a new, fast, and simple HPLC method using a monolithic column for clinical monitoring was developed and validated.

The determination of vitamin E, vitamin A, and retinyl esters may represent a valuable method for monitoring the toxicity of anticancer therapy. Present data indicate that the determination of retinyl esters (retinyl-palmitate and retinyl-stearate) may represent a sensitive tool to detect small bowel dysfunction associated with anticancer therapy. Moreover, the administration of vitamin E has been shown to alleviate some side effects of radiotherapy [7] or chemotherapy [8]. Serum vitamin E is not being routinely measured in cancer patients, but serial monitoring of vitamin E levels may be a prerequisite for any therapeutic use of this antioxidant vitamin. In routine monitoring, large numbers of samples need to be processed and a major advantage of the present method is the simultaneous determination of α-tocopherol, retinol, retinyl-palmitate, and retinyl-stearate in a relatively short time. The present method may therefore find use in large prospective studies evaluating the toxicity of currently used anticancer drugs or in monitoring the toxicity of a new agent.

Laboratory tests evaluating the intestinal toxicity of cytotoxic agents and the effect of interventions aimed at alleviating these side effects are urgently needed in medical practice. Intestinal permeability measurements may sometimes be associated with logistical problems, and may be impossible to perform or to interpret in some patients. Vitamin A absorption may represent an alternative to intestinal permeability measurement. Although vitamin A absorption has been used to monitor gastrointestinal disorders associated with malabsorption [3], this method has so far not been used to investigate gastrointestinal side effects of anticancer therapy. Present data indicate that the determination of retinyl-palmitate and/or retinyl-stearate represents a potentially useful test to detect gastrointestinal toxicity of chemotherapy. Prospective longitudinal studies should evaluate the utility of this method in monitoring the gastrointestinal toxicity of anticancer agents.

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