Rapid HPLC method for the determination of vitamin A and E and cotinine concentration in human serum in women with CIN and cervical cancer

Szybka metoda oceny stężenia witaminy A, witaminy E oraz kotyniny w surowicy krwi kobiet z śródnabłonkową neoplazją (CIN)i rakiem szyjki macicy

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Abstract

Objective: The aim of this study was to elaborate on the analytical method for quantitative determination of retinol and α-tocopherol in serum of women diagnosed with CIN and cervical cancer. The basic problem in the analysis of the vitamins content in biological material is their low physiological concentration level and instability. Liquid chromatography with diode array detector (DAD) was applied.

Material and methods: The material consisted of serum and urine collected from 12 women diagnosed with cervical intraepithelial neoplasia (CIN) and 16 diagnosed with cervical cancer.

The method was evaluated for the following parameters: linearity, recovery, sensitivity, precision, accuracy, selectivity, stability, limit of quantification (LOQ) and limit of detection (LOD).

Results: Results showed good linearity ($r^2 \geq 0.99$) in the range 0,1μg/ml-10mg/ml for retinol and 0,25μg/ml-15μg/ml for α-tocopherol. The Lower Limit of Detection was 0,15μg/ml for vitamin E and 0,05μg/ml for vitamin A. The within-run R.S.Ds were below 5,2% at all concentration levels and the between-run R.S.Ds were below 10,0% at all concentration levels.

Conclusions: The advantage of this method is that it measures both compounds in a more rapid, reproducible and accurate manner when compared to the previous HPLC studies. The compounds (vitamin A and E and internal standards) are measured in the same sample at the same time.

Quantitative determination of cotinine may reveal active smokers and subjects exposed to environmental tobacco smoke, which is independent measurable carcinogenetic co-factor.

The following study is a part of a project determining non-viral causative agents in cervical carcinogenesis.

Key words: vitamin A / vitamin E / retinol / alpha-Tocopherol / high pressure liquid chromatography – HPLC / cotinine /

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Streszczenie

Cel pracy: Celem niniejszej pracy jest ocena przydatności analitycznej metody oznaczania ilościowego retinolu i α-tokoferolu w surowicy kobiet z rozpoznaną śródplonową neoplaszą (CIN) i rakiem szyjki macicy. Głównym problemem w analizowaniu zawartości witaminy w materiale biologicznym jest ich niskie fizjologiczne stężenie i niestabilność. W niniejszym badaniu zastosowano metodę cieczowej chromatografii z detektorem diodowym (DAD).

Materiał i metodyka: Materiał badawczy stanowiło osoczce i mocz, 12 kobiet z rozpoznaną śródplonową neoplaszą szyjki macicy (CIN) i 16 z rakiem szyjki macicy.

Metodę oceniono pod kątem następujących parametrów: liniowość, powtarzalność, czułość, dokładność, selektywność, stabilność, granica kwantyfikacji (limit of quantification - LOQ) i detekcji (limit of detection - LOD).

Wyniki: Uzyskano wysoką liniowość (r ≥0,99) w zasięgu 0,1 μg/ml-10 mg/ml dla retinolu i 0,25 μg/ml-15 μg/ml dla α-tokoferolu. Dolna granica wykrywalności wynosiła 0,15 μg/ml dla witaminy E i 0,05 μg/ml dla witaminy A. Zasięg metody R.S.Ds wyniósł 5,2% we wszystkich poziomach stężenia a interwałowy zasięg R.S.Ds poniżej 10,0% we wszystkich poziomach stężenia.

Wnioski: Zaletą badanej metody jest fakt, iż można przy jej pomocy uzyskać wyniki w szybko, bardziej powtarzalny i dokładniejszy sposób, niż w opisywanych w literaturze badaniach z użyciem HPLC. Dodatkową zaletą stosowanej metody jest możliwość pomiaru z tej samej próbki witaminy A i E.

Ilościowa ocena stężenia kotyniny może wskazać pacjentki, będące aktywnymi palaczami lub na razem na paleście i bierne. W aspekcie karcinogenezy w obrębie szyjki macicy może być to cenna wiadomość dotycząca istotnego a często zatajającego kofaktora nowotworzenia.

Niniejsze badanie jest częścią projektu oceniającego pojawianie się czynników onkogenicznych w procesie karcinogenezy w obrębie szyjki macicy.

Słowa kluczowe: witamina A / witamina E / retinol / α-tokoferol / wysokosprawna chromatografia cieczowa – HPLC / kotynina /
The most important function in visual process involves predominantly one form of vitamin A, namely retinal. Retinal enters into the composition of rhodopsin, the most light-sensitive pigment and thus highly important in perception of reduced light. Rhodopsin, under the influence of light, undergoes a sequence of changes to ultimately disintegrate to trans-retinal and opsin. In the process, a nerve impulse is generated which assures good vision. Vitamin A deficiency leads to deprivation of rhodopsin, night blindness and xerophthalmia. Vitamin A prevents the keratinization of mucous-secreting epithelium of gastrointestinal and respiratory tract. It accelerates cell division and growth of fibroblasts, thus acting as the growth factor, predominantly in young organisms. Vitamin A plays an important role in spermatogenesis and embryogenesis. There is evidence that vitamin A may inhibit the growth of experimental breast cancer.

Recommended daily intake of vitamin A is 500 IU. The demand for vitamin A increases significantly during pregnancy and breast feeding.

**Vitamin E**

Vitamin E is widely distributed in nature, particularly large amounts may be found in vegetable oils (soy), vegetables, dairy products and nuts. A group of eight closely related compounds, known as tocopherols, exhibit vitamin E activity, but a tocopherol is the most active one. It constitutes (up to) 90% of all body tocopherols.

As it is currently known, tocopherols are exclusively produced by plants. The absorption of vitamin E, as of all fat-soluble vitamins, takes place in ileum, requires normal biliary tract and pancreatic function, and depends on diet. If the intestinal absorption is impaired, the intramuscular oil solutions of vitamin E may be administered. Vitamin E is excreted with bile, as glucuronide, and by the kidneys, as tocopheronic acid.

This essential nutrient belongs to the group of antioxidants whose function is scavenging free radicals formed in redox reactions throughout the body. It plays a role in termination of oxidative-generated lipid peroxidation chain reactions, particularly in cellular membranes that are rich in polyunsaturated lipids. Vitamin E is a component of LDLs and protects them from oxidative damage.

Hypovitaminosis E is associated with inadequate bile release and small intestinal disease. Vitamin E deficiency contributes to neurologic manifestations, peripheral and central neuropathies. In case of adults, unlike in children, neuropathies are not a feature of the deficiency state and may be present after many years of inadequate intake. Vitamin E deficiency may affect mature red cells by shortening their life span. Fertility disorders may be seen in women (impaired implantation), as well as men (reduced spermatogenesis). It has been suggested that vitamin E may have protective effects against atherosclerosis and cancer, the two most common causes of death in developed countries. It is prescribed in treatment of anemia, blood vessel spasm disorders, collagenoses and atherosclerosis. Vitamin E can be used in recurrent abortions, oligospermia and muscular atrophies. However, therapeutic effect of vitamin E has not yet been conclusively stated and defined.

Daily requirement of vitamin E is 10-3 mg.

**Cotinine**

Nicotine is a pyridine alkaloid that constitutes 85 to 90% of all alkaloids in tobacco leaves. Its main metabolite (80%) is cotinine; the metabolism is shown in Figure 1.

The sources of nicotine and cotinine in biological fluids are tobacco smoking and medicines containing nicotine (chewing-gum, plasters, inhalers) [11, 12].

Cotinine – the main nicotine metabolite – is eliminated from the body for a longer period of time than nicotine.
Its biological half-life is 17 hours, clearance 0.04 l/min, distribution volume 55 l (0.9 l/kg) [13]. The main way of cotinine and its metabolites elimination is via urine. Cotinine is one of the best and most useful biomarkers of tobacco smoke exposition [14, 15, 16].

The urine cotinine concentration reflects exposition to nicotine and tobacco smoke in the last 2-3 days. Research showed that urine cotinine concentrations may be useful in distinguishing active smokers from the ones exposed to environmental tobacco smoke [15]. The cotinine concentration in active smoker urine is higher than 100ng/ml and can be higher than 3000ng/ml. The cotinine concentration in the urine of a no-smoker exposed to environmental tobacco smoke one ranges from 25 to 100ng/ml [15, 16, 17].

Material and methods

28 patients, aged 19 to 67 years, diagnosed with pre-invasive or invasive cervical lesion were included in the study in accordance with ethical approval regulation by Local Research Ethic Committee of Jagiellonian University in Krakow. The basis for the diagnosis were: punch or cone biopsy specimens obtained from consecutive pre-invasive CIN1 (n=3), CIN2 (n=3), and CIN3 (n=3) lesions at the Department of Gynecology of Jagiellonian University Medical College, Krakow between 2004 and 2006. Additionally, specimen of primary squamous cell carcinoma of the cervix CaCx (n=16) were included into the study from patients who had undergone the radical hysterectomy with pelvic lymph node dissection. The material for this pilot study included 20ml of blood and 100ml of morning urine.

Chemicals and reagents

Retinol, α-tocopherol, retinol acetate, α-tocopherol acetate and ascorbinic acid in substance were provided from Sigma-Aldrich. Methanol, acetonitril, hexane, water, and dichloromethane used in this determination were of analytical grade (Merck). Ethanol was from PoCH (Gliwice). Chemicals used for cotinine determinations were: methanol HPLC Grade (Merck), octanesulfonic acid sodium salt monohydrate (Fluka), dichloromethane HPLC Grade, acetonitril HPLC Grade, phosphoric acid, K2HPO4, 2-propanol, cotinine standard (Sigma), NaOH, HCl (POCh), deionized water.

Chromatographic conditions

The chromatographic separations and quantitative determination were performed on a high performance liquid chromatograph Agilent 1100 series equipped with a pump, degasser, autosampler and DAD detector. A Supelco analytical column (Discovery C18 column, 5mm particle size, 250x4.6mm) as the stationary phase was used.

Methanol was used as the mobile phase. The flow rate was 1.0 ml/min. Detection was monitored at two different wavelengths: λ=292nm for tocopherols and λ=325nm for retinols determination.

Cotinine was determined by high performance liquid chromatography (HPLC) with UV detector (254nm).

The equipment included: HPLC Merck Hitachi Pump L6200A, Detector Spectra 100 Thermo Separation Products, 4880 integration system, centrifuge MPW 220, multi-block heater Lab-Line, ultrasonic cleaner Sonic-2, vacuum apparatus Alltech, vortex TH-3S TechnoCartel, pH meter MAT1202-SM.

The resolution was performed on Supelcosil LC-8 column (25cm, 4.6mm ID). The mobile phase was deionized water 88%, acetonitril 12 %, sodium octasulphoniane 1.1g/l, dicalium phosphate 5.95 g/l; pH was adjusted to 4.7 with the use of phosphoric acid. The HPLC analysis was isocratic with constant flow rate 1ml/min. The Rheodyne injector loop was 100μl. The detector wavelength was 254nm.

Standards and samples preparation

The blood was extracted and left to spontaneous coagulation. Then the samples were centrifuged at 300 r.p.m. for 15 minutes, becoming excluded haemolysed samples. The clean serum was frozen and stored at the temperature of about -40°C. “Stock solutions” of retinol, retinol acetate, α-tocopherol and α-tocopherol acetate were prepared first in the mixture of hexane/dichloromethane (50/50 v/v) containing ascorbic acid (0,025%) to protect vitamins against oxidation.

After this, standard solution was diluted in a mixture of dichloromethane/methanol (50/50 v/v) and, finally, in methanol (100%) and stored at -22°C, without the access of light. The QC samples were prepared in human plasma and stored in the same conditions as the analytical samples. The calibration samples were prepared immediately before the analysis.

The preparation of samples was done following the procedure described by M.A. Rodriguez-Delgado at al. [13]. 100ml retinol acetate (internal standard I) and 100ml α-tocopherol acetate (internal standard II) and 200ml of ethanol were added at 200ml volume of serum.

The mixture was shaken for 5 minutes. Subsequently, 200ml water and 800ml of hexane was added to the mixture and after blending for 15 minutes it was centrifuged for 5 minutes at 4000 r.p.m., and next 600ml of the upper organic layer was extracted. The organic phase was evaporated to dryness and the residue was dissolved in 100ml of methanol/ethanol/hexane (88:10:2, v/v/v). All samples were run in duplicate, one with and the other without internal standards.

At 20ml volume of this solution was injected directly into the butcher. The chromatographic separation took 18 minutes.

Cotinine extraction procedure

The liquid-liquid extraction was used for the preparation of the samples. 0.5ml of 1M NaOH and 50μl of norephedrine (internal standard) was added to 5 ml of urine and mixed. The samples were extracted with 5 ml mixture of dichloromethane: 2-propanol (9:1) (v/v) for 15 minutes. The organic phase was collected to cone tubes and evaporated in the air stream in 40°C. The residues after evaporation were dissolved in 400ml of mobile phase and injected to HPLC.

Calibration curves

For calibration curves the stock solutions were diluted with methanol to obtain a mixture of working standards. Water containing measured amounts of retinol and α-tocopherol was used for the analysis.
The samples of water were spiked with \( \alpha \)-tocopherol to the following concentration: blank, 0.15; 0.25; 0.75; 1.5; 5.0; 10.0; 15.0; 20.0 mg/ml and with retinol to the following concentration: blank, 0.05; 0.10; 0.25; 0.5; 1.0; 2.5; 5.0; 10.0 mg/ml. Retinol acetate and \( \alpha \)-tocopherol acetate were used as the internal standards. The concentration of retinol was 10 \( \mu \)g/ml and \( \alpha \)-tocopherol acetate was 100 \( \mu \)g/ml. The blank samples were water and serum. Calibration for cotinine was made using blank urine spiked with cotinine to obtain standards: 10, 50, 100, 200, 500, 1000 ng/ml.

These calibration samples were analysed following the procedure described for sample preparation (2.3 section). The number of QC's per batch was six (three concentrations level in duplicate). They were assessed according to 4-6-20 rule.

Results
Specificity / selectivity
The specificity/selectivity of the method can be illustrated by comparing the chromatograms obtained after analysis of a test solution of the pure compound with the chromatograms of the independent blank serum. No interfering peaks can be seen if blank serum is used. The peaks of the retinol, retinol acetate (Figure 2), \( \alpha \)-tocopherol and \( \alpha \)-tocopherol acetate (Figure 3) were well-resolved and showed no interferences with endogenous or exogenous materials.

The retention time was: 4.54 minutes for retinol; 5.46 minutes for retinol acetate, 9.4 minutes for \( \alpha \)-tocopherol and 11.35 minutes for \( \alpha \)-tocopherol acetate.

Sensitivity
The lower detection limit depends on several parameters such as the mobile phase and the DAD monitor used. Under the experimental conditions described, the LOD (defined as three times the baseline noise) was approximately 0.15 \( \mu \)g/ml for vitamin E and 0.05 \( \mu \)g/ml for vitamin A. The lowest concentration of the calibration graph for vitamin E was 100 ng/ml and for the vitamin A was 250 ng/ml and it was the Lower Limit of Quantification.

For cotinine the limit of detection and the limit of quantification were 5 ng/ml and 10 ng/ml respectively.

Linearity
Detector response for vitamin A, E and internal standards was linear to at least 100 mg/ml. The resulting data was plotted as peak height and peak area versus concentration and studied by the linear regression analysis. Tables 1 and 2 present the results for the assessment of the goodness of fit/lack of fit for both compounds (vitamin A and vitamin E) in serum. The goodness of fit was highly significant. A test for lack of fit indicated that the linear model is appropriate for establishing a relationship between the concentration and the response. In general, correlation coefficients above 0.98 were observed during the validation experiments.

The linearity for cotinine was 10-1000 ng/ml. The urine samples with cotinine levels higher than 1000 ng/ml were dissolved.

Precision and accuracy
A summary of the results on precision and accuracy as derived from the measured concentration for the validation samples is presented in tables I and II. The within-run R.S.D.s. were below 9.44% for tocopherol and below 9.52% for retinol at all concentration levels in serum. Criteria of acceptance are ±15% at all level concentrations only at the LLOQ are ±20% [13, 21, 22]. The coefficient of variance (CV) for series of cotinine 200 ng/ml controls was 5.13%.
Recovery
For determination of recovery of the analysis, the mean peak heights obtained for triplicate measurements were compared with the mean peak heights obtained from triplicate direct injections performed in the same run. The five recoveries thus obtained were used to calculate the mean recovery.

Discussion
Squamous cervical cancer arises from the metaplastic epithelium of the transformation zone (TZ) (squamocolumnar junction) and develop slowly through progressive dysplastic changes to carcinoma in situ and invasive cancer.

Cervical intraepithelial neoplasia (CIN) is divided into three stages according to the degree of epithelial dysplasia and differentiation.

Over the past few decades significant advances have been made in understanding the molecular genetics underlying the development of human cancers. However, we are still far from constructing complete sequences of events leading to the development of invasive cervical cancer. There are many associated risk factors including number of sexual partners, parity, oral contraceptives use, smoking, immunological system alterations and lack of antioxidants [23] but persistent human papillomaviruses (HPV) 16 and 18 infection is the most significant factor in aetiology of this disease [24].

The applied method to determine retinol and α-tocopherol in serum uses an isocratic separation with only methanol as mobile phase at 1.0ml/min as flow rate which allows a fast elution of both compounds from the column. This method does not require complex mobile phase. Applied DAD detector allows measuring both vitamins in the same chromatographic separation. As can be observed the matrix effect obtained at 292nm can be eliminated measuring only α-tocopherol and α-tocopherol acetate (IS) at 292nm and retinol acetate (IS) and retinol at 325nm.

Table I. Goodness of fit and lack of fit for Vitamin E (n=12).

<table>
<thead>
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<th>Concentration (µg/ml)</th>
<th>mean</th>
<th>SD</th>
<th>R.S.D. (%)</th>
<th>Bias (%)</th>
</tr>
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<tr>
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<td>1.27</td>
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<td>0.89</td>
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<td>-4.54</td>
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</table>

Table II. Goodness of fit and lack of fit for Vitamin A (n=12).

<table>
<thead>
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<th>Concentration (µg/ml)</th>
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<th>SD</th>
<th>R.S.D. (%)</th>
<th>Bias (%)</th>
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</table>

Conclusions
Quantitative determination of cotinine may reveal which patients are in fact active smokers and which are simply exposed to environmental tobacco smoke.

The advantage of this method is that it measures both compounds in a more rapid, reproducible and accurate manner than in previous HPLC studies [9, 12, 18, 19, 20]. The compounds (vitamin A and E and internal standards) are measured in the same sample at the same time.

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