

THE HPLC PLASMATIC PROFILE OF SOME FAT-SOLUBLE ANTIOXIDANT MICRONUTRIENTS (ALL-TRANS-RETINOL, α -TOCOPHEROL, COENZIME Q10) IN DIABETIC AND DYSLIPIDEMIC PATIENTS

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Abstract

Quantification of lipophilic vitamins in clinical or biological samples is very important from the medical, epidemiological and informational points of view. Adequate dietary intake of nutrients such as vitamin A (retinol) and vitamin E (tocopherol) is essential for normal development and health maintenance [1].

The simultaneous analysis of vitamins A, E and coenzyme Q₁₀ are reported as a profile of fat-soluble vitamins. Decreased concentrations of these principal molecules can reveal the need for an increased dietary intake [2].

Simultaneous determination of these compounds in biological samples (plasma, serum, tissues and plant material) appears to be difficult due to their liability to photooxidation, presence of *cis*- and *trans*-isomers and their diverse polar structures [3].

In this regard, the aim of our study was to assay the plasmatic profile of the fat-soluble (lipophilic) antioxidant micronutrients (all-trans-retinol, α -tocopherol, coenzyme Q₁₀) of diabetic and obese patients using a specific method for their simultaneous quantification, namely HPLC with UV detection.

Rezumat

Determinarea statusului vitaminelor liposolubile din probe clinice sau biologice este importantă din punct de vedere medical, epidemiologic și informațional. O dietă echilibrată ce conține cantități suficiente de vitamină A și E este esențială pentru dezvoltarea armonioasă a organismului. Determinarea simultană a vitaminei A, E și a coenzimei Q₁₀ poate constitui profilul vitaminic liposolubil. O concentrație scăzută a acestor componente poate fi suplinită printr-o alimentație corectă. Analiza simultană a acestor compuși în probe biologice poate fi dificilă datorită fotooxidării moleculelor (sensibilității crescute la lumină), prezenței izomerilor *cis* și *trans* și a polarităților diferite ale acestora. În lucrarea de față, realizată pe probe plasmatiche de pacienți diabetici ne-am propus evaluarea profilului vitaminic liposolubil al unor nutrienți antioxidanți (all-*trans*-retinol, α -tocoferol, coenzimă Q₁₀) folosind o metodă de determinare simultană specifică prin HPLC, cu detecție în UV.

Keywords: all-trans retinol, α -tocopherol, coenzime Q₁₀, type-2 diabetes mellitus, dyslipidemia.

Introduction

The knowledge about the influence of oxidative stress on human health has increased in the last few decades. Oxidative stress, defined as an imbalance between oxygen free radicals formation and their scavenging by antioxidants, is recognised to play an important contributory role in the pathogenesis of numerous degenerative or chronic diseases such as arteriosclerosis, allergy or cancer. Humans along with other aerobic organisms have evolved a variety of mechanisms to protect themselves from the potentially toxic effects of reactive molecules. The so called antioxidation complex includes enzymes such as catalase and superoxide dismutases, repair enzymes such as DNA glycosylases as well as water and lipid soluble vitamins such as ascorbic acid, α -tocopherol, retinol and coenzyme Q₁₀ [4].

The knowledge of the level of antioxidants in human plasma allows estimating the state of health and can help in treatment of serious diseases. There is a hypothesis that an observed decrease of antioxidant intake in developed countries over the past 30 years and changes in dietary pattern can cause the vulnerability of the organism to the oxidative stress [5].

Probably, one of the effects connected with the dietary habits is the increase in the number of chronic diseases.

Quantification of lipophilic vitamins in clinical or biological samples is very important from the medical, epidemiological and informational points of view [6, 7].

Adequate dietary intake of nutrients such as vitamin A (retinol) and vitamin E (tocopherol) is essential for normal development and health maintenance. Vitamin E is the major and most potent lipid soluble antioxidant *in vivo*. Acting as radical scavenger vitamin E (α -tocopherol) has been reported to interrupt the lipid peroxidation chain reaction. Similarly vitamin A reacts indirectly with active oxygen species and inhibits free radical generation by increasing the activities of detoxifying systems. Another major lipid peroxidation inhibitor is coenzyme Q₁₀ (ubiquinone), which also plays an important role in the mitochondrial electron-transfer [8]. Because of their powerful antioxidants potential, retinol, tocopherols and coenzyme Q₁₀ have been linked with prevention of serious diseases, e.g., cardiovascular diseases, arteriosclerosis, bone calcification, neurological disorders (e.g. Parkinson's and Alzheimer's), cancer, cataracts and improvement of child growth. Supplementation with coenzyme Q₁₀ has been studied for its effect on hypertension, some neurodegenerative diseases, in reducing migraine headaches, as a possible marker of oxidative

stress and increased risk of atherosclerosis [9]. Direct measurement of the serum concentration of these important vitamins is essential for the evaluation of nutritional status of a patient. The simultaneous analysis of vitamins A, E and coenzyme Q₁₀ are reported as a profile of fat-soluble vitamins [10]. Decreased concentrations of these principal molecules can reveal the need for increased dietary intake.

Simultaneous determination of these compounds in biological samples (plasma, serum, tissues and plant material) appears to be difficult due to their liability to photooxidation, presence of *cis*- and *trans*-isomers and their diverse polar structures [2].

In this regard, the aim of our study was to assay the plasmatic profile of the fat-soluble (lipophylic) antioxidant micronutrients (all-*trans*-retinol, α -tocopherol, coenzyme Q₁₀) in patients with diabetes mellitus and dyslipidemia, using a specific method for their simultaneous quantification, namely HPLC with UV detection.

Materials and methods

Reagents and chemicals

All reagents used were of highest quality available from various sources. Ubiquinone (coenzyme Q₁₀, CoQ), all-*trans*-retinol (R) and α -tocopherol (α -TP) were purchased from Sigma (USA). Methanol and n-hexane (HPLC/Spectro grade) were obtained from Merck (Germany).

HPLC device and configuration

We used an HPLC reversed-phase system (VARIAN-PROSTAR, USA) with a Chromsep-Inertsil 5 OSD2, 250x4.6mm column. The mobile phase consisted of methanol/n-hexane 72/28 (v/v). The fat-soluble micronutrients were separated and detected simultaneously, using an UV detector, with the following wavelength: 323nm (all-*trans*-retinol), 293nm (α -tocopherol), 270nm (coenzyme Q₁₀).

Standards preparation

Stock solutions of the three internal standards were prepared, as follows: all-*trans*-retinol (R) 1.75 mM in methanol, α -tocopherol (α -TP) 1.15 mM in n-hexane, ubiquinone (CoQ) 0.58 mM in n-hexane. Working solutions of analytes were prepared by dissolving appropriate portions of stock solutions in methanol (R) and n-hexane (α -TP and CoQ).

Patients

The studied group consisted in 40 patients (mean age 70 ± 8 years) hospitalized in „Ana Aslan” Institute of Gerontology and Geriatrics,

Bucharest, Romania. The following clinical and biochemical criteria were used for the selection of patients into the examined group:

- serum levels of glucose ;
- lipidic profile (expressed as plasmatic levels of total cholesterol and tryglicerides).

In this regard the patients were divided into three groups:

- Group 1 – the control group of 13 patients with normal levels of serum glucose (< 110 mg/dL) and normal lipidic profile (total cholesterol < 200 mg/dL, triglycerides < 150 mg/dL);
- Group 2-14 patients with type 2 diabetes mellitus (defined by fasting serum glucose > 120 mg/dL) but with a normal lipidic profile;
- Group 3 – 13 patients with both type 2 diabetes mellitus (defined by fasting serum glucose > 120 mg/dL) and dyslipidemia (total cholesterol > 200 mg/dL, triglycerides > 150 mg/dL).

All patients were not taking any nutritional supplements for six months and gave their informed consent prior to joining this study.

Sample collection

Blood samples were collected from the elbow flexure after overnight fast. The blood samples were taken into test tubes with 0.2 mL of 3.8% sodium citrate solution in the proportion of 9 : 1. Blood samples were centrifugated at 1450 x g at 4°C for 10 minutes, using a Hettich centrifuge, Germany. Plasma was stored at -20°C until the day of the experiment.

Biochemical methods

Serum glucose and lipid metabolism parameters, such as total cholesterol, triglycerides, LDLcholesterol and HDL-cholesterol were measured by enzymatic methods adapted on Olympus 400 Autoanalyzer (Japan), using commercially available kits.

The experimental methodology for evaluating the fat-soluble antioxidant micronutrients (R, α -TP, CoQ) in plasma samples was as follows: 0.25 mL of plasma was pipetted into Eppendorf tube and deproteinised using 0.5 mL methanol. Afterwards, 0.75 mL n-hexane was added. The mixture was vortexed for 5 minutes and centrifuged at 5000 rpm for 15 minutes. Next, the clear hexane layer was transferred to another tube and extraction of plasma vitamins was repeated with a new 0.75 mL portion of n-hexane. The two hexane extracts were mixed and evaporated to dryness under a steam of nitrogen. The dry residue was dissolved in 0.25 mL mobile phase and injected into the HPLC system [2, 11].

The working internal standard solutions were submitted to the same experimental protocol, in order to make the calibration curves for all-trans retinol, α -tocopherol and ubiquinone.

Statistical analysis

All results are expressed as mean \pm standard deviation (SD). Clinical characteristics of patients were compared among the three studied groups of subjects using Student's t test and one-way analysis of variance (ANOVA). For all comparisons, p values < 0.05 were considered as statistically significant.

Results and discussion

The calibration curves for all-trans retinol, α -tocopherol and ubiquinone developed after processing the working internal standard solutions are presented in figure 1a, b, c. The regression equations were as follows:

- for all-trans-retinol: $y = 904711x$ ($R^2 = 0,9992$);
- for alpha-tocopherol $y = 203299x$ ($R^2 = 0,9998$);
- for ubiquinone: $y = 281984x$ ($R^2 = 0,9982$).

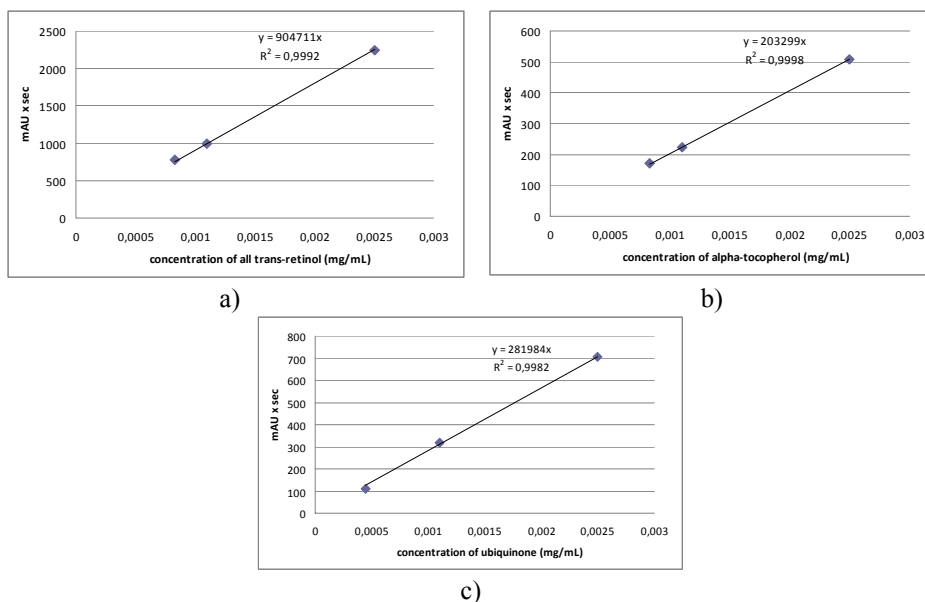


Figure 1

The calibration curve for all-trans-retinol (a), alpha-tocopherol (b) and ubiquinone (c)

The HPLC separation of the three fat-soluble antioxidant micronutrients studied revealed the following chromatographic retention

times: all-trans-retinol \approx 3min.; α -tocopherol \approx 4 min; coenzyme Q10 \approx 8 min (figure 2).

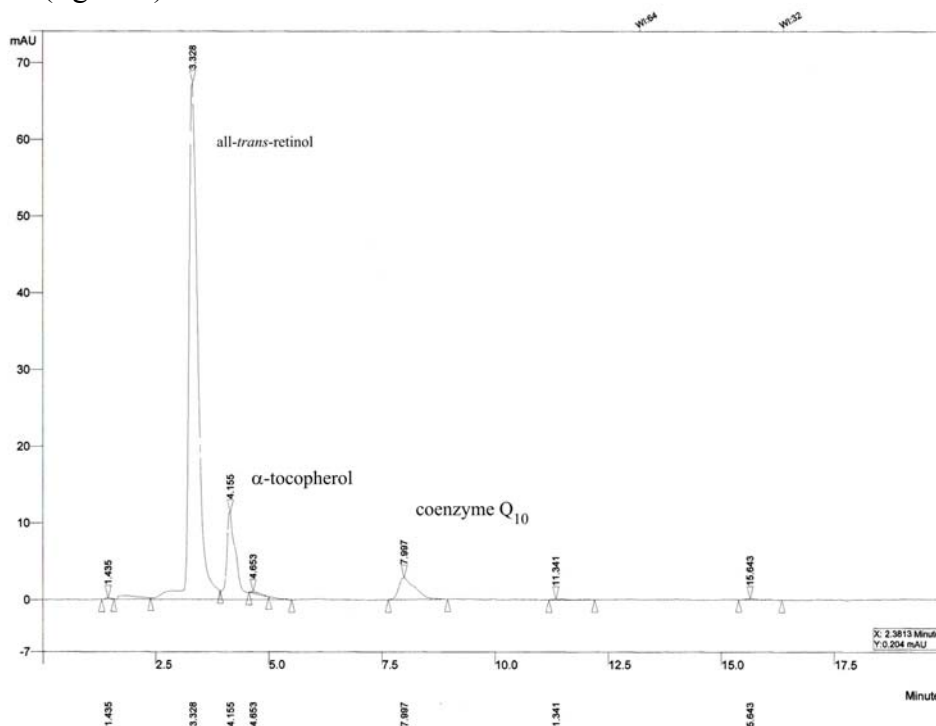


Figure 2

Representative chromatogram for the separation and quantitative assay of all-trans-retinol, α -tocopherol and ubiquinone in human plasma

In order to assess the plasmatic levels of the fat-soluble antioxidant micronutrients studied, we registered, in the first step, the metabolic characteristics (fasting plasma glucose, total cholesterol, tryglicerides) for the three studied clinical groups. These data are presented in table I.

Table I
The blood parameters for the three studied groups

Parameters	Group 1 Control	Group 2 DM 2	Group 3 DM 2+ dyslipidemia
Fasting plasma glucose(mg/dL)	94.80 \pm 13.281	180.85 \pm 24.09	188.90 \pm 36.88
Total cholesterol (CT) (mg/dL)	185.6 \pm 26.03	199.62 \pm 42.38	269.90 \pm 50.06
Tryglicerides (TG) (mg/dL)	110.10 \pm 33.31	115 \pm 32.98	195.81 \pm 52.26

Our experimental data regarding the lipophylic antioxidant (CoQ, R, α -TP) micronutrients in plasma samples of the three studied groups are listed in Table II.

Table II
The experimental results regarding the fat-soluble antioxidant (CoQ, R, α -TP) micronutrients in plasma samples of the three studied groups

Parameters	Group 1 (Control)	Group 2 (DM2)	Group 3 (DM2+dyslipidemia)
Retinol (ng/mL)	63.26 \pm 37.53	76.88 \pm 23.07	79.35 \pm 28.45
α -tocopherol (ng/mL)	708 \pm 53.09	319.81 \pm 66.05**	300.55 \pm 41.03**
Coenzyme Q ₁₀ (ng/mL)	584.66 \pm 42.14	156 \pm 18.18***	40.92 \pm 4.47***

*significantly different from control group ; *** p < 0.001 ; ** p < 0.01

The biochemical parameters investigated showed an interesting dynamics for the three studied groups of patients. There were registered significant differences between plasmatic levels of α -tocopherol (vitamin E) in healthy people and metabolic-imbalanced patients. Our results, depicted in fig 3 and 4 evoke higher levels of α -tocopherol in the control group (studied group1), compared to both group 2 (DM2) and group 3 (DM2 + dyslipidemia) (p<0.01). However, no important difference was noticed between diabetic patients with normal lipidic profile and DM2 patients with dyslipidemia.

Analysing the plasmatic values of ubiquinone for the studied groups of patients, there are important observations to be presented. Coenzyme Q₁₀ developed significantly lower (p<0.001) plasmatic concentrations between the control group and metabolic-disordered ones (studied groups 2 and 3). On the other hand, our data showed that patients enrolled in group 3, diagnosed with both DM2 and an altered lipidic profile, possess the lowest concentration of plasmatic ubiquinone: 40.92 \pm 4.47ng/ml, compared to 156 \pm 18.18 ng/ml (patients diagnosed only with DM2, group 2) and 584.66 \pm 42.14ng/ml.

Interestingly, the assay of all-trans-retinol did not show any significant relevance in regard to the metabolic disorders of the patients investigated (Fig. 3).

On the other hand, significant correlations between biochemical parameters and studied micronutrients were pointed out, as follows:

- plasmatic levels of CoQ: results statistically significant between all groups of patients (p<0.001);
- plasmatic levels of α -TP: group1 vs.group 2 (p<0.01); group1 vs.group 3 (p<0.01).

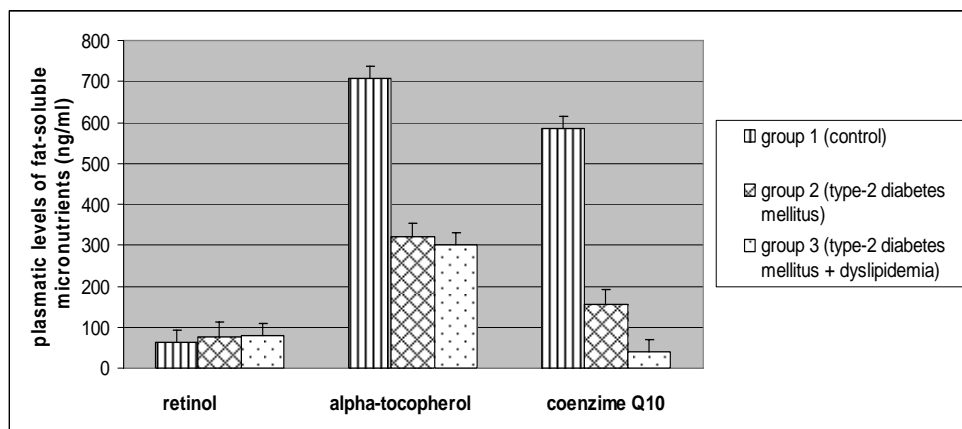


Figure 3

The plasmatic levels of retinol, α -tocopherol and ubiquinone in the three studied groups of patients

We pointed out an inverse correlation between the routine blood parameters (fasting serum glucose, CT, TG) and two of the fat-soluble antioxidant micronutrients investigated: α -TP and CoQ.

Conclusions

In conclusion, our experimental results are in agreement with previous studies and pointed out interesting information regarding the need of appropriate diet and/or an antioxidant supplementation for improving the metabolic balance.

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