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Short communication

Simultaneous determination of serum retinol and α- and γ-tocopherol levels in type II diabetic patients using high-performance liquid chromatography with fluorescence detection

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Abstract

This paper presents a simple reversed-phase high-performance liquid chromatographic method for the simultaneous determination of retinol, and α- and γ-tocopherols in human serum using a fluorescence detector. For chromatographic separation a binary gradient was used: phase A; acetonitrile–butanol (95:5); phase B; water, at a flow-rate of 1.5 ml/min. Serum retinol, and α- and γ-tocopherol levels were measured in patients with non-insulin-dependent diabetes mellitus. Small sample requirement, good reproducibility and sensitivity make this method useful for the determination of the serum levels of these compounds in patients with diabetes mellitus.

Keywords: Vitamins; Retinol; Tocopherol

1. Introduction

Vitamins A (retinol) and E (α-tocopherol) are classified as non-enzymatic antioxidants [1]. Vitamin A ''scavenges'' singlet oxygen and superoxide anions. Apart from direct interaction with active oxygen species, vitamin A is thought to inhibit free radical synthesis via increasing the activity of detoxifying systems [2].

Vitamin E protects unsaturated fatty acids located in both cell and organelle membranes against endo- and exogenous free radicals and active oxygen species, which are involved in the initiation and spread of membrane damage caused by non-enzymatic lipid peroxidation [3,4]. Therefore, both of the vitamins are thought to play an important role in many diseases, such as atherosclerosis, diabetes mellitus and cancer.

The methods of simultaneous determination for these vitamins have been known for many years. In these methods, retinol and tocopherol levels are usually determined using high-performance liquid chromatography (HPLC) in the isocratic [5–10] or gradient reversed-phase [11,12] with a UV detector. Although there are many reports describing the determination of these vitamins, new studies are being undertaken to optimize the conditions of both the chromatographic separation [13–19] and the extraction of these vitamins from biological materials [20].

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The aim of this study was (i) to develop a quick and effective method for the determination of retinol and tocopherols using a fluorescence detector, which enables tocopherols to be determined, and (ii) to answer the question: ''What is the role of these vitamins in the pathogenesis of type II diabetes mellitus?''

2. Materials and methods

2.1. Patients

The study included 24 non-insulin-dependent diabetes mellitus (NIDDM) patients (11 men and 13 women) aged 46–64 years (mean 56±7 years) and 20 healthy patients (9 men and 11 women) aged 42–65 years (mean 54±8 years). All patients had no history of taking vitamin A and E preparations.

2.2. Equipment

In the study, a Beckman chromatography system was used which consisted of a Beckman 126 pump, AI 406 interface (Beckman) and a RF-535 fluorescence detector (Shimadzu). The system was controlled by a Beckman Gold v. 8.01 computer program. A Supelcosil LC-18 chromatographic column, 15 cm×4.6 mm, 3 μm, (Supelco) with an ODS Ultrasphere 100 pre-column, 4.5 cm×4.6 mm, 5 μm (Beckman) was used. Samples were injected using a Rheodyne Model 7725 injector with a 25-μl sample loop.

2.3. Chemicals

HPLC-grade acetonitrile, ethyl acetate, *n*-butanol and *n*-hexane were purchased from Sigma. Water was purified with Ropure and NANOpure systems (Barnstead, USA). Retinol, tocopherols and α-tocopherol acetate were from Sigma. Absolute ethanol and anhydrous sodium sulphate were from POCH (Poland).

2.4. Sample collection and storage

Non-heparinized blood samples were collected into glass tubes wrapped with aluminium foil. One hour after collection, they were centrifuged at 1000 *g* for 10 min (+4°C). The resultant serum was placed in polypropylene tubes wrapped with aluminium foil and stored at −18°C until assayed.

2.5. Sample preparation

Serum samples were prepared according to Lee et al. [7]. Serum (100 μl) and ethanol (100 μl) were pipetted into test tubes wrapped with aluminium foil, and the mixture were shaken vigorously for 30 s. Then, 200 μl of butanol–ethyl acetate (1:1, v/v) was added, and the tubes were shaken again for 1 min. After adding about 20 mg of anhydrous sodium sulphate, the tubes were shaken for 1 min and centrifuged for 10 min (10 000 *g*, +4°C). The supernatant was filtered through a GHP Acrodisc 13 filter, 0.45 μm (Gelman Sciences, USA).

2.6. Chromatographic separation

Two mobile phases were used for chromatographic separation. Phase A consisted of acetonitrile–butanol (95:5, v/v), and phase B was 100% water. The flow of the phases was maintained by two separate pumps at a summaric flow-rate of 1.5 ml/min. The proportion of phase A was changed according to the following protocol: 0–7 min – 90% of phase A, 7–17 min – 100% of phase A, 17–22 min – 90% of phase A. The first change (after 7 min), which lasted for 30 s, was to increase the elution rate of α- and γ-tocopherols. The second change (after 17 min) was to restore the initial proportion before injecting another sample. E_x and E_m for retinol were 300 and 480 nm, respectively, and for α- and γ-tocopherols 285 nm and 325, respectively. In the literature, E_x and E_m values for retinol were 330 and 470, respectively, and for α- and γ-tocopherols 298 and 328, respectively [21]. E_x and E_m values were changed after 9 min. All samples were injected in a 25-μl volume into the HPLC system.

2.7. Linearity, reproducibility, recovery and sensitivity of the method

Retinol and tocopherol standards were dissolved in hexane (1 mg/ml) and served as stock solutions, which were diluted in phase A (acetonitrile–butanol,

95:5, v/v) to obtain a series of working solutions used to calculate calibration curves [6]. Tocopherol acetate, which served as an internal standard (25 μg/ml), was prepared in ethanol from the stock solution (1 mg/ml acetonitrile). The linearity of the response of the detecting system to differential amounts of retinol, and α- and γ-tocopherols was assessed using the following dilutions of the standards: 0.02, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml. The peak area was correlated with the corresponding amounts of standards (μg) injected into the chromatographic column.

To assess the reproducibility of the method, we extracted and analyzed three serum samples containing different concentrations of the compounds. The samples were run three times daily for three consecutive days (Table 1). The intra-assay relative standard deviations (RSDs) for retinol, γ-tocopherol, and α-tocopherol were 3.1, 3.7 and 2.4%, respectively. The inter-assay RSDs for retinol, γ-tocopherol and α-tocopherol were 3.6, 4.2 and 3.6%, respectively.

Analytical recovery was assessed by adding 20-μl aliquots of three standards to 200-μl samples of control serum. The 20-μl aliquots of the following amounts of standards: 0, 0.08, 0.125, or 0.16 μg of retinol (dissolved in ethanol); 0, 0.2, 0.3, or 0.4 μg of γ-tocopherol (dissolved in acetonitrile); and 0, 1.0, 2.0 and 3.0 μg of α-tocopherol (dissolved in acetonitrile). All samples were run in triplicate. The values obtained for pure samples (without standards added) were subtracted from the values obtained for samples containing retinol, α- or γ-tocopherol standards and the differences were expressed as the percentages of retinol, and α- and γ-tocopherols added to the above-mentioned 200-μl samples of control serum.

The sensitivity of the method was estimated by determining a concentration that generates a peak five-fold higher than the baseline noise level. This concentration was expressed in μmol/l serum. Table 2 presents data on the sensitivity, recovery, reproducibility and linearity of the method.

2.8. Statistical analysis

Data were presented as mean±SD and were statistically evaluated by Student's *t*-test. *P* values <0.05 were considered as significant (Table 3).

2.9. Clinical application of the method

Table 3 presents the results of the determination of serum retinol, and α- and γ-tocopherols in NIDDM patients and in healthy controls.

Table 1
Reproducibility of the method

Sample	Compound	Within-day analysis		Between-day analysis	
		Mean (μg/ml)	RSD (%)	Mean (μg/ml)	RSD (%)
1	Retinol	0.24	5.2	0.26	4.2
2		0.36	1.9	0.36	2.7
3		0.47	2.2	0.48	3.9
Mean			3.1		3.6
1	α-Tocopherol	8.94	1.7	8.72	2.9
2		12.55	2.4	12.75	4.1
3		16.43	3.2	16.1	3.7
Mean			2.4		3.6
1	γ-Tocopherol	1.25	4.3	1.28	3.3
2		2.41	3.3	2.55	4.7
3		3.72	3.5	3.69	4.6
Mean			3.7		4.2

Table 2
Sensitivity, recovery, reproducibility and sensitivity of the method

Compound	Sensitivity (μmol/l)	Recovery (%) (n=3)	Reproducibility (RSD, %) (n=3)	Linearity (μmol/l)
Retinol	0.35	96.7±3.1	3.1	0.07–8
α-Tocopherol	0.35	97.3±4.2	2.4	0.07–30
γ-Tocopherol	0.45	100.4±2.3	3.7	0.09–25

3. Results and discussion

The aim of this study was to develop a method for the determination of serum retinol, and α- and γ-tocopherols utilizing the most useful elements of the published procedures. In the study, we utilized a fluorescent detector because the literature data indicate that the sensitivity and selectivity of this method are comparable to or even higher than those of ultraviolet detection [22].

In one of the first studies using a fluorescence detector [23], the concurrent determination of retinol and α-tocopherol levels was performed in an isocratic normal-phase HPLC system with *n*-hexane–ethanol (99:1) as an eluent. In those detection conditions, isomeric tocopherols did not appear. In subsequent studies with a fluorescence detector, retinol [24] and α-tocopherol [13] levels were analyzed separately using an isocratic normal-phase (heptane–2-propanol).

Satomura et al. [25] used an isocratic reversed-phase of 2-propanol–water in order to separate retinol, and α-, β- and γ-tocopherols. However, retention times of about 40–60 min made this method less competitive when compared with others. In two extensive studies performed by Hoffman La Roche, the levels of vitamins A and E were determined in liver tissue [26,21]. In the first study, retinol and α-tocopherol were analyzed separately using two HPLC systems and two isocratic phases,

Table 3
Retinol, and α- and γ-tocopherol levels in controls and NIDDM patients compared with literature data

Compound	Control		NIDDM	
	Our results	Literature data	Our results	Literature data
Retinol				
Mean±SD (μmol/l)	1.82±0.45	1.73±0.30[a]	2.12±0.64	1.96±0.51[a]
Age	54±8	52±9	56±7	57±11
n (men/women)	20 (9/11)	112 (66/46)	24 (11/13)	100 (38/62)
P			<0.05	<0.001
α-Tocopherol				
Mean±SD (μmol/l)	29.4±5.7	20.1±2.3[b]	38.2±9.3	33.4±6.8[b]
Age	54±8	30±7	56±7	38±13
n (men/women)	20 (9/11)	10	24 (11/13)	10
P			<0.02	<0.02
γ-Tocopherol				
Mean±SD (μmol/l)	5.1±2.8	6.1±3.1[c]	6.9±3.9	
Age	54±8	75.1±5.0	56±7	–
n (men/women)	20 (9/11)	230 (men)	24 (11/13)	
P			N.S.	

[a] From Ref. [29].
[b] From Ref. [30] (original value expressed in μg/ml).
[c] From Ref. [32].
The *P* value stands for statistical significance of differences between NIDDM patients and controls.

namely 2.5% propanol in *n*-hexane and 3% 1,4-dioxane in *n*-hexane, respectively. In the second study, retinol and tocopherols were analyzed concurrently using a 25-cm column with an ODS Ultrasphere stationary phase (Beckman) with acetonitrile–tetrahydrofuran–methanol–1% ammonium acetate (684:220:68:28) as a mobile phase. In those conditions, the retention time of retinol was about 2.7 min.

In our study, in which we used the above-mentioned column and mobile phase [21], there were some difficulties in separating retinol, whose peak overlapped with the front of the chromatogram, which prevented us from integrating the peak surface. A similar problem occurred while using shorter reversed-phase columns from Supelco and Knauer (data not shown).

The gradient reversed-phase used in our study was

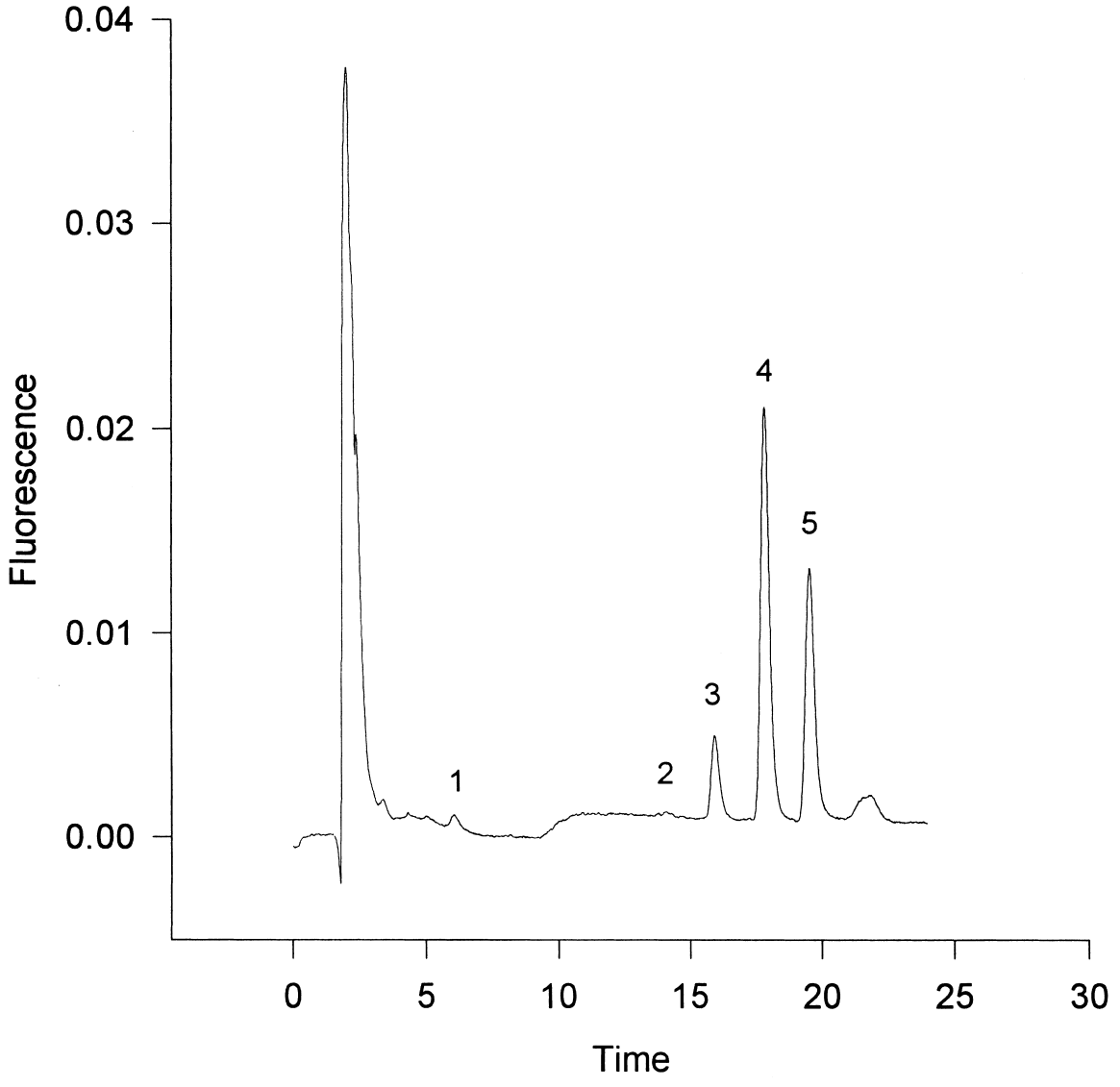

Fig. 1. Chromatogram of serum sample containing retinol (1), δ-tocopherol (2), γ-tocopherol (3) α-tocopherol (4) and tocopherol acetate (5).

90% phase A (acetonitrile–butanol, 95:5) and 10% phase B (water). Fig. 1 presents a typical chromatogram obtained for the samples studied. Under these separation conditions, retention times for retinol, and δ-, and γ- and α-tocopherols were 6.0, 14.1, 15.9 and 17.8 min, respectively. While analyzing these results, we should also discuss the issue of β-tocopherol. Under the conditions described, retention times for β- and γ-tocopherols were almost identical (14.3 and 14.1 min, respectively), which prevented us from separating these tocopherols. Other authors also reported difficulties in separating these compounds [27]. Furthermore, one should bear in mind that the level of serum β-tocopherol is several times lower than that of γ-tocopherol [28]. Under the conditions described in this study, the detection limit of β-tocopherol was almost three-fold higher (1.0 μmol/l) than that of γ-tocopherol (0.45 μmol/l), which allows us to claim that the amounts of β-tocopherol contained in the serum samples studied were below the detection limit. Accordingly, the appropriate peak was considered to reflect the γ-tocopherol content. Nevertheless, there is a need to develop appropriate conditions for the chromatographic separation of β- and γ-tocopherols.

In the samples studied, we found trace amounts of δ-tocopherol which could be quantified only after increasing the concentration of the sample. However, this considerably increases the time of analysis because the sample should be evaporated first and then dissolved in a smaller volume of eluent. It is possible to shorten the time of analysis by decreasing the water content to 5% and by shortening the time of the initial phase gradient from 7 to 3 min. When we decided to use only one phase (acetonitrile–butanol, 95:5), the retention times of α-tocopherol, γ-tocopherol and retinol decreased to 9.9, 9.1 and 3.1 min, respectively. However, the problem of overlapping peaks occurred again.

In the case of retinol, the sensitivity, linearity, recovery and accuracy of our method were comparable to those observed by other authors, while for α- and γ-tocopherols the values of these parameters were very high. We used this method to measure serum retinol, and α-, and γ-tocopherol levels in type II diabetic patients and our results were in agreement with other reports [29–31]. γ-Tocopherol, the levels of which had not been yet determined in NIDDM patients, increased to a lesser degree when compared with α-tocopherol. However, the high standard deviation of γ-tocopherol values and lack of statistical significance suggest that this parameter will not prove useful in type II diabetic patients.

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