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Fast determination of retinol and α-tocopherol in plasma by LC

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Abstract

A fast, selective and economical method for the determination of retinol and α-tocopherol is presented. Both vitamins are separated by high-performance liquid chromatography (HPLC) in less than 4 min using an isocratic elution with methanol. The robustness of the method was checked in real samples, obtaining relative standard deviation lower than 3%. The described method was satisfactorily applied to serum samples proceeding of patients enrolled in a METHADONE maintenance treatment program. The retinol concentrations in the serum of these patients fell into the normal interval of concentrations; however, the serum α-tocopherol contents were higher than the normal values.

Keywords: Retinol; α-Tocopherol; Serum levels; HPLC

1. Introduction

Although the major nutritional emphasis in the understanding the etiologies of cancer has been directed towards establishing links with dietary fat, increasing attention is now being given to dietary micronutrients such as vitamins or trace elements, particularly those that might protect against oxidant damage. Beneficial effects of vegetarian nutritional habits on antioxidative parameters and thus on the reduction of cardiovascular diseases and cancer risk were found [1]. High consumption of vegetables and fruits has consistently associated to reduction in risk of cardiovascular disease and cancer. These findings have stimulated further research aimed at identifying, which compounds present in vegetables and fruits are responsible for reduction in the prevalence of these diseases. There are several micronutrients such as carotenoids, vitamin C, vitamin E, dietary fiber (and its compounds), selenium, magnesium and zinc that can be involved in the prevention of these diseases. However, it is difficult to determine

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whether these nutrients present in vegetable and fruits per se are the sole protective agents or whether other factors associated with foods containing them contribute to the foods' protective effects [2]. Also, other compounds as dithiolthiones, indoles, phenols, protease inhibitors, plant sterols, limonene, and several allium compounds could be related [3,4]. The apparent protection of consuming a diet rich in fruits and vegetables is likely to be the result of a multifactor effect of a number of components of these foods [5].

Micronutrients serum levels has repeatedly been related with risk for cardiovascular disease and cancer. So, serum level of retinol in subjects who died of cancer was lower than in their matched controls [6]. Also, lower serum levels of vitamin E, retinol and β-carotene in future cases of lung cancer as compared with matched control subjects might be markers of pre-clinical disease. Serum level of retinol in subjects who died of cancer were lower than in their matched controls [4]. Therefore, a symptomatic lung cancer that was present at time the blood was drawn might be responsible for the lower levels [7].

Accurate analytical data are essential for studies relating antioxidant vitamins status in serum to health and disease, for establishing appropriate vitamins intake and supplementation guidelines. High-performance liquid chromatography (HPLC) with reversed-phase (RP) is the most analytical technique to determinate retinol and α-tocopherol in biological samples [8–11]. However, methods for determining vitamins require preliminary steps of clean up and pre-concentration to increase the selectivity of the determination. The introduction of diode-array detectors (DADs) in HPLC increases selective and rapid detection of these compounds, allowing to monitor the chromatogram simultaneously at different wavelength, which it has made a more extent use of these detectors, overall in complex samples, as clinical samples.

The objective of this work was developing a routine, fast, cheap and selective method for the determination of retinol and α-tocopherol in short time in serum using a DAD with an isocratic elution. The proposed method was applied to the determination of both vitamins in blood samples from patients enrolled in a METHADONE maintenance treatment program to compare their content with the contents observed in normal people.

2. Experimental

2.1. Materials

2.1.1. Reagents and apparatus

The HPLC system consisted of two isocratic pumps (LC-10AT), an automatic injector (SIL-10) and a photodiode array (SPD-M6A), controlled by software data acquisition (Class-LC-10), all from Shimadzu. Temperature was controlled by the system LKB-2155. The analytical column was a Supelcosil 75 × 4.6 mm I.D. from Supelco with 3-μm particle diameter. Mobile phase consisted of methanol (100%) filtered through 0.45-μm membranes (Millipore) and degassed prior to their use, with a flow rate set at 1.3 ml/min, being injection volume of 20 μl. Detection was carried out at 292 nm for α-tocopherol and 325 nm for retinol using a DAD. Peak identification was effected by interspacing standard and by comparing the UV–Vis spectrum of each one with those of the samples.

HPLC grade methanol, dichloromethane, and hexane were obtained from Merck (Darmstadt, Germany). Ultrapure water from Milli-Q system (Millipore, Bedford, USA) with conductivity of 18 MΩ was used in all cases.

2.2. Preparation of solutions and standards

Retinol, and α-tocopherol were obtained from Roche (Basle, Switzerland). Retinol acetate was used as internal standard. Standards were weighed and solved in a mixture of hexane–dichloromethane (50/50, v/v) containing 0.025% of butylated hydroxy toluene (BHT) to protect vitamins against oxidation. After, this standard solution was diluted in a mixture of dichloromethane–methanol (50/50, v/v) and finally was diluted in methanol (100%) to increase the efficiency of separation. Aliquots of 1 ml were stored under nitrogen atmosphere in the darkness, in order to protect them from light and kept under refrigeration, and used each set of analysis.

Concentrations of standard ranged between 1 and 100 μg/ml were prepared by appropriate dilution of the concentrated stock standard solutions for calibration purposes.

2.3. Collection, storage and preparation of samples

Serum samples were collected of patients enrolled in a METHADONE maintenance treatment program from the Laboratory of Clinic Analysis of the Association of Youth Cooperation 'San Miguel' of Santa Cruz of Tenerife. The blood was extracted and left to spontaneous coagulation. Then the samples were centrifuged at $300 \times g$ for 10 min being excluded hemolyzed samples. The clean serum was frozen (-40 °C) for storage and transportation to the laboratory for analysis. The preparation of samples was made following the proceeding described by Steghens et al. [12] So, at 200 μl of blood centrifuged were added 200 μl of retinol acetate (internal standard) and 200 μl of ethanol and the mixture was shaked for 5 min. At this mixture were added 200 μl of water and 500 μl of hexane and after mixing for 10 min it was centrifuged for 10 min at $2000 \times g$, being extracted 300 μl of the upper organic layer. The rest of solution was extracted again with 300 μl of hexane mixing for 10 min and centrifuged 10 min at $4000 \times g$, being extracted 300 μl of the organic layer. The liquid proceeding of both extractions (600 μl) was evaporated to dryness and solved in 300 μl of methanol–ethanol–hexane (88:10:2, v/v/v) injecting 20 μl into the HPLC system. Three replicates of each sample were injected into HPLC system in order to increase the precision of the results. Recoveries of the extraction procedure were calculated analyzing 10 spiked blood samples in different days, obtaining values above 93% for both vitamins, which is according with the results obtained by Steghens et al. [12].

3. Results and discussion

Most of methods to determinate vitamins utilize semi-aqueous mobile phases consisting of mixture of methanol or acetonitrile–acetic acid–water, using sometimes extremely complex gradient for the separation. Furthermore, these methods have high separation times, including a re-equilibration step and are lower reproducibility than isocratic methods. The introduction of photodiode-array detectors (DADs) has increased the rapid in the identification as UV–Vis spectra provided by these detectors can be compared with

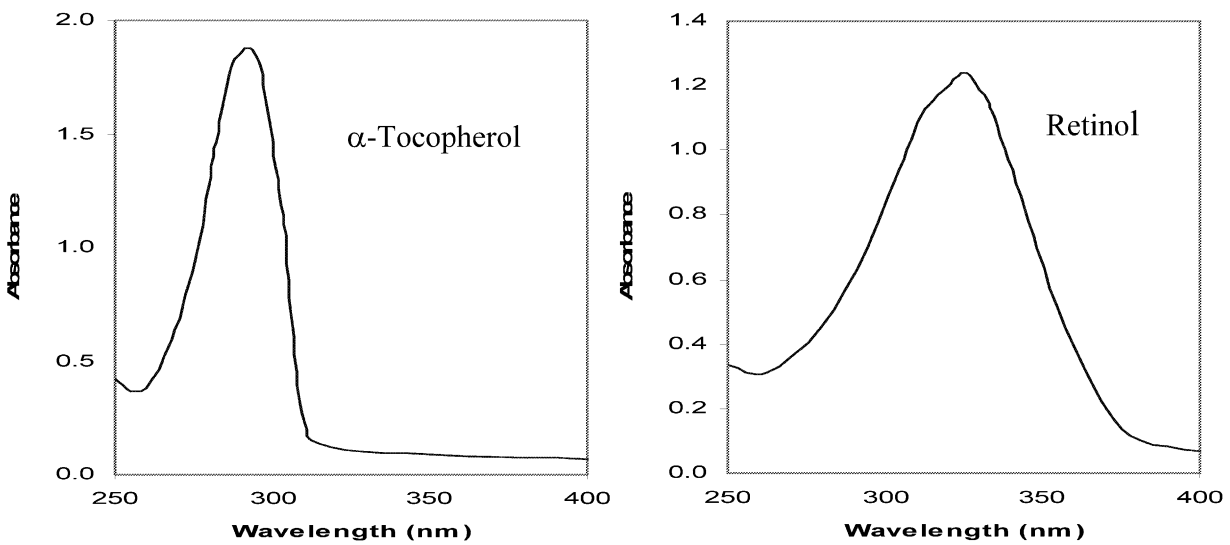

Fig. 1. Absorption spectra of a standard solution of retinol and α-tocopherol in methanol.

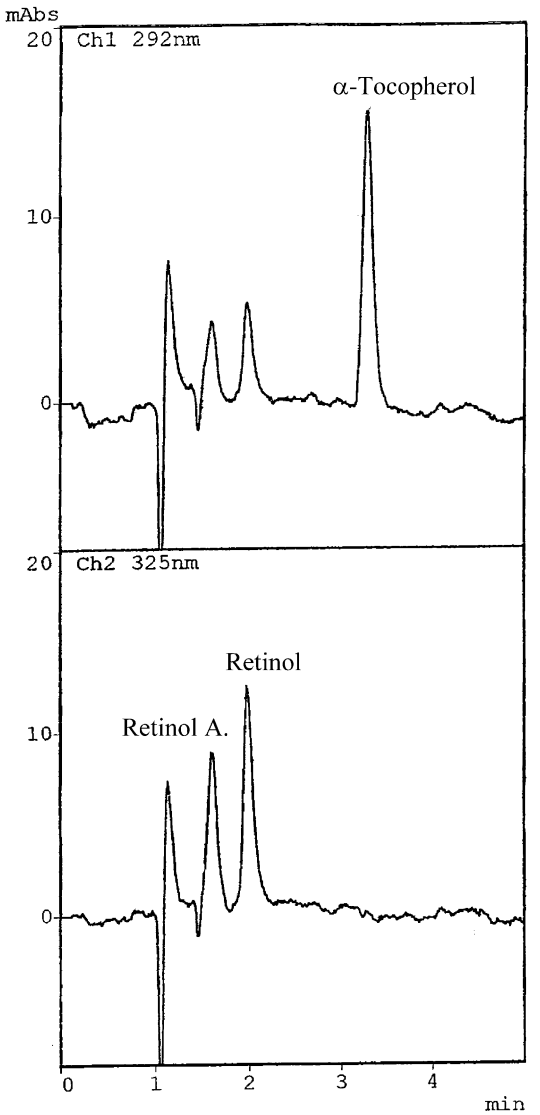

Fig. 2. Chromatograms of a standard mixture of both vitamins spiked with a free vitamins blood sample. Chromatographic conditions and identification are described in Section 2.

standards allowing to check peak purity in complex samples such as clinical samples (blood, urine). The proposed method to determinate retinol and α-tocopherol in blood uses an isocratic separation with only methanol as mobile phase at 1.3 ml/min as flow rate which it allows a fast elution of both vitamins from the column in a short time (approximately 4 min) and do not require preparing complex mobile phase. Methanol was preferred due to several reports show that using acetonitrile the recoveries are lower [13] and this solvent is cheaper and less contaminant. So, as can be seen in Fig. 1 from the spectral characteristic from each standard that retinol can be determinate to $\lambda = 292$ nm while for

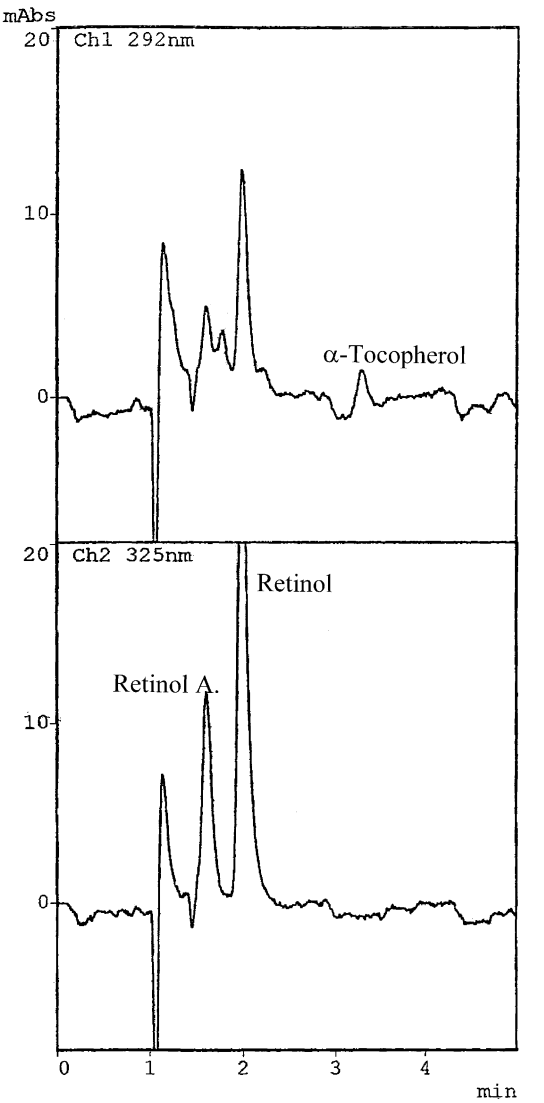

Fig. 3. Matrix effect on the chromatographic profile of a sample of blood of a patient enrolled in a METHADONE program using both wavelengths ($\lambda = 292$ nm for α-tocopherol and $\lambda = 325$ nm for retinol). Chromatographic conditions are the same as for Fig. 2.

Table 1
Figures of merit of the proposed method for the determination of retinol and α-tocopherol

Compound	Equation	r	R	RSD (%)	LOD (μg/ml)
Retinol	$Y = (44\,956 \pm 1827)x - (60\,587 \pm 12\,413)$	0.998	99.67	2.1	0.02
α-Tocopherol	$Y = (27\,972 \pm 216)x + (36\,312 \pm 1099)$	0.999	99.99	2.3	0.04

r, correlation coefficient; R, the curve fitting level (in %) obtained by analysis of variance for the validation of the model; RSD, relative standard deviation; LOD, limit of detection.

α-tocopherol the maximum sensitivity was obtained to $\lambda = 325$ nm, using a DAD as we used along of separations.

Fig. 2 shows a chromatogram of a standard mixture of both vitamins spiked with a free vitamins blood sample to appreciate the influence of the matrix sample on the separation. As can be seen, the resolution of vitamins using a $\lambda = 292$ nm is not so good, as retinol and retinol acetate (internal standard) peaks appear overlapped at this wavelength. This overlapping is solved using another wavelength ($\lambda = 325$ nm) for the determination of both substances, appearing both peaks resolved to baseline and allowing the quantitation using the internal standard, which it is very important in clinical samples where there are appreciable differences in the matrix of blood depending the patient. These facts are better observed as a blood sample of a patient enrolled in METHADONE program is injected into system, Fig. 3. As can be observed the matrix effect obtained at 292 nm can be eliminated measuring only α-tocopherol at 292 nm and quantifying retinol acetate and retinol at 325 nm, increasing the sensitivity of the last compound.

The calibration graphs were realized injecting standard in the range 0.1–19.0 μg/ml for retinol and 0.45–45.0 μg/ml for α-tocopherol using retinol acetate as internal standard and measuring the ratio of the peak areas of analytes to area of the retinol acetate plotting versus the concentration of each standard. So, Table 1 shows the corresponding regression equation and other characteristic parameters for the determination of both vitamins. The correlation coefficients were higher than 0.998 being the curve-fitting level obtained by analysis the variance for the validation of the model higher than 99.60. The limit of detection (LOD) was calculated from the blank value plus three times its standard deviation, being similar values those obtained in the bibliography. The precision of the method was evaluated by measuring repeatability and intermediate precision, analyzing five times a blood sample spiked with 1.00 μg/ml of each standard over different days, obtaining variation coefficients lower than 3% for retinol and 3.2% for α-tocopherol.

3.1. Applicability of method

The proposed method was applied to several samples of heroin patients enrolled in a METHADONE maintenance treatment in order to determine the serum concentrations of retinol and α-tocopherol in these patients, Table 2. Fig. 4 shows the chromatogram of a sample with normal

Table 2
Concentrations of retinol and α-tocopherol in serum of heroin patients

Sample	Retinol (μg/ml)	α-Tocopherol (μg/ml)
1	0.667	13.3
2	0.543	10.9
3	0.689	12.7
4	0.822	10.2
5	1.168	11.7
6	0.532	8.7
7	0.834	25.0
8	0.626	9.0
9	0.781	4.3
10	0.375	7.5
11	0.848	10.7
12	0.724	8.8
13	0.706	6.4
14	0.371	5.6
$X \pm$ SD	0.692 ± 0.206	10.3 ± 5.0

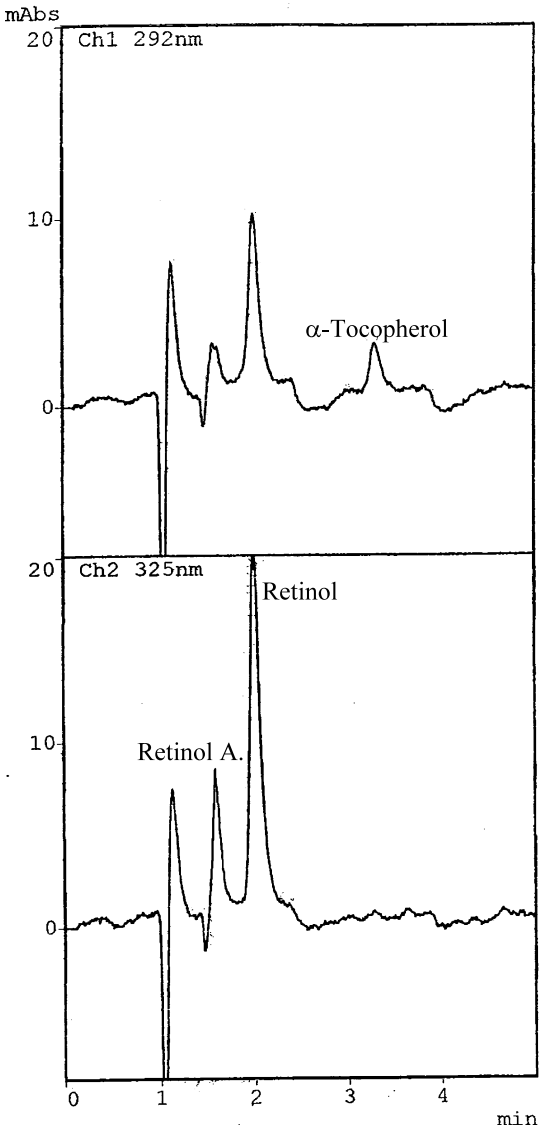

Fig. 4. Chromatogram of patient with normal retinol and α-tocopherol content. Chromatographic conditions are the same as for Fig. 2.

retinol and α-tocopherol content, observing the good separation of peaks and the relative low interferences. The chromatographic profile of the different samples it was similar, which it allowed quantifying both compounds with high accuracy. The serum contents of retinol and α-tocopherol relative to the patients analyzed here were similar than other data published for Canarian population [14]. Individuals within the same range of age than our individuals (between 18 and 49 years), presented a mean concentrations of 0.58 μg/ml for retinol lower than the data reported in this paper. Moreover, most (64%) of our data of retinol are above the reference interval described for retinol (0.30–0.65 μg/ml). This could be a consequence of modifications on the lipid profile observed in these patients. With respect to α-tocopherol, the mean concentration indicated for healthy Canarian population of reference was 14.2 μg/ml [14], which is higher than the mean value found by us. However, most (79%) of our data for α-tocopherol find within the reference interval (5–12 μg/ml) [15]. A weak but significant ($n = 14$; $r^2 = 0.126$; $P < 0.01$) correlation was observed between the serum concentrations of retinol and α-tocopherol, which indicates that the metabolism of both liposoluble vitamins is related. The following line of regression was defined:

$$[\alpha\text{-tocopherol, } \mu g/ml] = 8.55[\text{retinol, } \mu g/ml] + 4.43.$$

4. Conclusions

Using the advantages of a DAD and the use of a pure solvent as a mobile phase, a fast method for the determination of retinol and α-tocopherol is presented allowing to quantify both compounds in less than 4 min. The method present high selective and sensitive and it was used to check the level of both vitamins in patients enrolled in a METHADONE program with good results. Although the sample preparation is the time consuming step, this one can be carried out simultaneously on several samples. Therefore, a sensitive increase of the number of analysis per hour can be developed applying the proposed method.

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