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Short communication

Determination of α-tocopherol in plasma and erythrocytes by high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of α-tocopherol in plasma or erythrocytes with photodiode-array detection is described. Using this detector, information about the spectrum, absorption maxima and purity of the peak is obtained. Tocopherol was separated on a 5-μm Spherisorb ODS-2 column with methanol as element at a flow-rate of 1.0 ml/min. As little as 100 μl of plasma or 150 μl of erythrocytes can be used for accurate analysis with direct extraction without saponification. The speed, specificity, sensitivity and reproducibility of this technique make it particularly suitable for the routine determination of α-tocopherol in plasma or erythrocytes.

1. Introduction

α-Tocopherol is an important antioxidant in biological systems and is probably the most important lipid-soluble chain-breaking antioxidant in vivo. It has been shown to have significant physiological and pharmacological roles in certain disorders of the newborn and is necessary for the maintenance of normal neurological structure and function [1–3]. The newborn infant, especially if premature, is particularly vulnerable to vitamin E deficiency because of inadequate body stores, poor intake, impaired absorption or reduced transport capacity in the blood owing to the low concentrations of low-density lipoproteins in the foetus and infant at birth [4,5].

Nevertheless, some methods for the determination of vitamin E in erythrocytes by high-performance liquid chromatography (HPLC) coupled with ultraviolet detection have been described but need extensive sample preparation as previous saponification is required [4,6–8].

In this paper, a procedure for the determination of α-tocopherol in plasma and red blood cells (RBCs) is reported. Only 150 μl of erythrocyte suspension, without previous saponification, are required, using reversed-phase HPLC with a UV spectrophotometric photodiode-array detector. Concerning the sample size, most workers use a large amount of erythrocytes when a UV–vis spectrophotometric detector is used

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[4,6,7,9,10], and others recommend fluorimetric [11,12] or electrochemical detectors [8,13] when the sample is very small. The present method allows the use of a small sample size and a UV spectrophotometric detector.

2. Experimental

2.1. Reagents and chemicals

α-Tocopherol, α-tocopheryl acetate and pyrogallol (1,2,3-trihydroxybenzene) were purchased from Sigma (St. Louis, MO, USA), EDTA from Fluka (Buchs, Switzerland), sodium chloride from Probus (Badalona, Spain), methanol and ethanol for HPLC from Scharlau (Barcelona, Spain), *n*-hexane for HPLC from SDS (Peypin, France) and diethyl ether for HPLC from Panreac (Barcelona, Spain).

2.2. Subjects

Twenty neonates at the University Hospital of Granada, Spain, participated in this study. Cord blood was obtained through the umbilical vein from separated placenta. The gestation age and birth mass of the neonates were 38 ± 1 weeks and 3000 ± 270 g, respectively. None of the mothers showed complications during pregnancy or delivery. No infant had either delivery complications or evidence of intrauterine malnutrition. The study protocol was approved by the hospital ethics committee and the study was performed with the informed consent of the parents.

2.3. Sample collection and storage

A 1-ml volume of newborn's blood was collected in brown polypropylene tubes containing EDTA K_3 anticoagulant (15% in saline solution). The blood was centrifuged at 3000 *g* for 5 min in a Model ZK centrifuge (Hermle, Gosheim, Germany), the haematocrit was immediately determined for use in calculations and the plasma and buffy layer were removed by aspiration. The RBCs were washed three times in 0.15 *M* NaCl containing 1 m*M* EDTA K_3 at pH 7.4. The washed cells were resuspended in a solution containing 0.15 *M* NaCl, 1 m*M* EDTA K_3 and 0.5% pyrogallol. Plasma and erythrocyte suspension were stored in conical flasks with amber-coloured polypropylene microcentrifuge tubes (Elkay Products, Shrewsbury, MA, USA) in the dark at −70°C for a short period (1–2 weeks). Plasma analytes were stable for at least 28 months [14].

2.4. Preparation and storage of standard solutions

Stock standard solutions of α-tocopherol (9.29 mmol/l) and α-tocopheryl acetate (21.15 mmol/l) were prepared in ethanol. Working standard solutions were prepared from the stock standard solutions by 10-fold (α-tocopherol) or 100-fold (α-tocopheryl acetate) dilutions with ethanol.

All vitamin standard solutions were stored at −20°C in dark bottles for not more than 1 month. α-Tocopherol and α-tocopheryl acetate working standard solutions were prepared weekly and every 2 weeks, respectively.

A calibration graph was prepared just before analysis.

2.5. Plasma sample preparation

A 100-μl aliquot of ethanol containing internal standard (211.54 μmol/l) was added to 100 μl of plasma in an amber-coloured polypropylene microcentrifuge tube. After vortex mixing, the mixture was extracted with 100 μl of *n*-hexane by vortex mixing for 60 s. The mixing must be uniform and sufficiently vigorous to disrupt the precipitated lipoprotein particles. The tubes were centrifuged at 3000 *g* for 5 min. As much of the upper phase as possible (an average volume of 90 μl) was carefully transferred into another polypropylene microcentrifuge tube. The supernatant was evaporated at room temperature under a stream of nitrogen and the residue was reconstituted in 100 μl of methanol–diethyl ether (75:25) according to Bieri et al. [15] and Sharma and Kumar [16]. If many analyses were performed in a single run, the samples were reconstituted in staggered groups so as to mini-

mize the time before sample analysis. A 50-μl aliquot of the solution was injected or stored at −20°C, for not more than 1 day, until HPLC analysis.

2.6. Erythrocyte sample preparation

A 150-μl volume of erythrocyte suspension was added to 1 ml of cold ethanol containing internal standard (211.54 μmol/l) and 1% pyrogallol in a polypropylene tube with a screw-cap, protected by a sheet of aluminium, and the tube was slowly agitated on a vortex mixer. A 1-ml volume of *n*-hexane was then added to the tubes. The tubes were vigorously mixed on a vortex mixer for 60 s, then centrifuged at 3000 *g* for 5 min to separate the phases and the supernatant. An average volume of 950 μl of supernatant was transferred into a 1.5-ml microcentrifuge tube; care was taken so that no protein was taken up. The solvent was evaporated to dryness under a stream of nitrogen. The dry residues were dissolved in 100 μl of methanol–diethyl ether (75:25) and 50 μl were injected into the chromatographic column or stored at −20°C, for not more than 1 day, until HPLC analysis.

2.7. HPLC apparatus and conditions

HPLC separation was carried out using a Hewlett-Packard (Palo Alto, CA, USA) liquid chromatographic system equipped with an HP-1050 pump system and a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a fixed-volume loop of 100 μl. The detector was an HP-1040M photodiode-array detector. The data were stored and processed by an HP-Vectra 486 personal computer (Hewlett-Packard) provided with an HPLC Chemstation (DOS Series) (Hewlett-Packard).

The analytical column used was Spherisorb ODS-2 (250 × 4.6 mm I.D.; 5-μm particle size)(Tracer, Barcelona, Spain), protected by a guard cartridge (C_{18} 5 μm) (Tracer) system and maintained at 50°C. Isocratic elution was performed with methanol at a flow-rate of 1.0 ml/min. A 50-μl aliquot of sample was injected with a 50-μl syringe. Detection was performed at 292 nm. The run time was 10 min.

2.8. Quantification

To determine α-tocopherol in the samples, a working standard solution was always analysed along with the samples and peak-area ratios were used for calculations following the internal standard method. A constant amount of internal standard, 105.77 μmol/l, was added to increasing concentrations (2.32, 4.64, 9.29, 23.22 and 34.83 μmol/l) of α-tocopherol in order to prepare the calibration graph.

3. Results and discussion

3.1. HPLC procedure

Figs. 1 and 2 show typical chromatograms of plasma and erythrocyte suspension. α-Tocopherol was well separated from the internal standard, α-tocopheryl acetate. The determination of α-tocopherol in RBCs required a higher volume as RBCs contain less α-tocopherol than plasma [4,6,7,9].

Various workers [3,6,10,14] have demonstrated that the addition of antioxidants had little

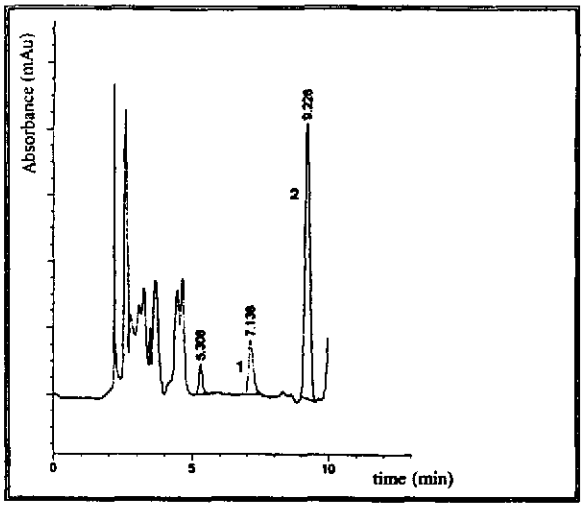

Fig. 1. Typical chromatogram of plasma extract from a newborn. Peaks: 1 = α-tocopherol; 2 = α-tocopheryl acetate.

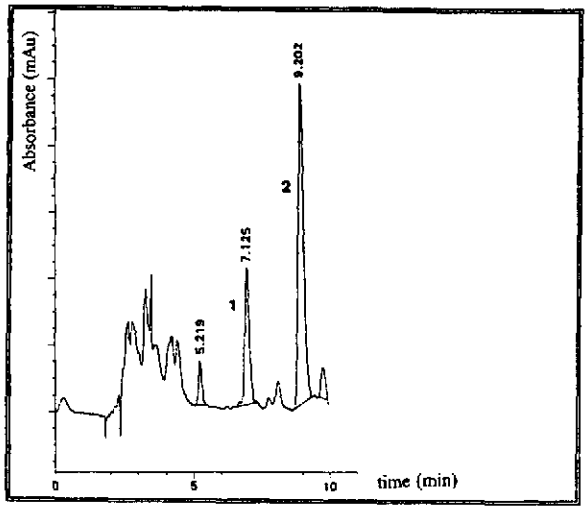

Fig. 2. Typical chromatogram of erythrocyte suspension extract from a newborn. Peaks: 1 = α-tocopherol; 2 = α-tocopheryl acetate.

effect on the recovery of tocopherols from human plasma. In contrast, the use of pyrogallol or ascorbic acid is essential in the analysis of RBCs, to prevent dramatic losses of tocopherol due to interaction with co-extracted iron-containing pigments [12,17]. Stump et al. [17] demonstrated that the prevention of oxidative loss of α-tocopherol during RBC extraction is dependent not only on the antioxidant added, but also on the order of addition of the alcohol and the antioxidant. Insufficient protection against oxidative loss during RBC extraction could lead to 10–50% loss of the membrane α-tocopherol [17].

Saponification is widely used to extract tocopherols from erythrocytes [4,6–8]. The analysis proposed did not require saponification, and was therefore less time consuming.

Peak shapes and retention times were studied using different methanol–water mixtures. The resolution of α-tocopherol and α-tocopheryl acetate was calculated from the equation $R_s = 2[(t_{R_2} - t_{R_1})/(W_2 - W_1)]$, where t_R is the retention time of the compound (in minutes) and W is the band width determined by the intersection of the tangents to the inflection points of the Gaussian peak with the baseline (in minutes) [18,19]. With 100% methanol as eluent, $R_{A/B} = 3.30$, $W_A = 0.52$; with 98%, $R_{A/B} = 4.30$, $W_A = 0.70$; with 96%, $R_{A/B} = 5.90$, $W_A = 0.85$; and with 95%, $R_{A/B} = 6.24$, $W_A = 0.88$; A is α-tocopherol and B is α-tocopheryl acetate. As a result, 100% methanol was adopted in subsequent assays.

For quantitative analysis, the calibration graph was calculated by linear regression. The peak-area ratio of α-tocopherol to α-tocopheryl acetate (y) versus the mass of standard α-tocopherol (x nmol) under these conditions was linear in the range tested, 0.116–1.74 nmol per injection volume. The graph passed close to the origin and the data fitted the equation $y = 0.326x - 0.018$ ($n = 3$) with a correlation coefficient of 0.9999.

With regard to the suitability of the proposed method, a detection limit of 5.18 nmol/l and a quantification limit of 7.56 nmol/l ensured good sensitivity, according to the criteria established by Kateman and Pijpers [20] and by Long and Winefordner [21]. For this reason the initial amount of erythrocyte sample was lower than that applied by other workers using a spectrophotometric detector [3,9,10].

The within-run precision was measured by calculating the standard deviation and coefficient of variation (C.V.) of ten replicate analyses of α-tocopherol in a plasma sample containing 12.53 μmol/l and in an erythrocyte sample containing 8.7 μmol/l. Within the same run, the C.V. for α-tocopherol was 3.85%, in plasma and 3.80% for erythrocytes. To evaluate the between-run precision, ten different analytical runs were carried out on consecutive days. The C.V. for α-tocopherol was 7.8%, in plasma and 7.1% for erythrocytes. The intra- and inter-laboratory precisions were in agreement with the acceptable precision proposed by Horwitz [22] for analyte concentrations of the order of μmol/l.

The standard additions method was used test the accuracy of the method [23]. Standard α-tocopherol was added to three aliquots of a sample at three concentrations levels (12, 23 and 35 μmol/l) in order to calculate the recovery. Each analysis was carried out three times. The mean recoveries for plasma and erythrocyte suspension methods were 92.84% and 94.08%, respectively.

3.2. Spectral analysis

Photodiode-array detection permits peak identification in a complex sample by spectral analysis in real time without stopping the flow. α-Tocopherol and α-tocopheryl acetate show maximum absorbances at 292 and 284 nm, respectively. The contour plot, which is a pseudo-three-dimensional representation of the absorbance, wavelength and time data (isogram) (Fig. 3) allows the selection of the optimum detection wavelength and checks for peak purity using spectral overlay or the ratiogram. It can also substract the sample background. Fig. 3 shows the isogram of the chromatographic separation displayed in Fig. 2.

The spectra recorded between 230 and 400 nm (each tick = 2 nm), for every peak, measured just prior to, at and after their respective maxima, show their purities. The purity factor of α-tocopherol in plasma and red cell samples was 0.999.

3.3. Reference values

The results (mean, median and ranges) ($n = 2$) for the α-tocopherol in red blood cells and plasma, obtained from 20 neonates, were 11.91 μmol/l, 12.03 μmol/l and 13.53–9.75 μmol/l for plasma and 5.73 μmol/l, 5.38 μmol/l and 3.65–8.02 μmol/l for erythrocytes.

In summary, the proposed method is rapid, specific, sensitive, precise and accurate. This method should be of considerable use for the determination of α-tocopherol in plasma and erythrocytes.

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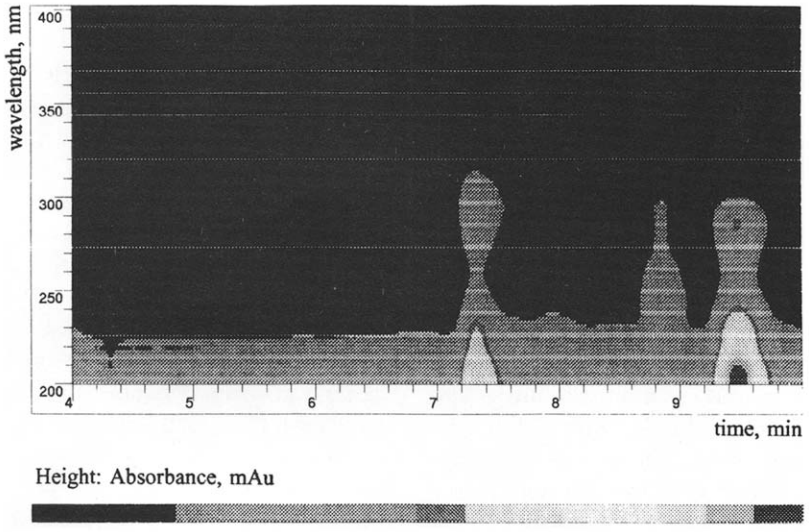

Fig. 3. Isogram of the chromatographic separation displayed in Fig. 2.

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