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Note

Determination of α-tocopherol in human plasma by high-performance liquid chromatography with electrochemical detection

M. VANDEWOUDE

Laboratory of Endocrinology, Metabolism and Nutrition, Faculty of Medicine, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk (Belgium)

M. CLAEYS*

Laboratory of Experimental Pharmacology, Faculty of Medicine, University of Antwerp, Universiteitsplein 1, B-2160 Wilrijk (Belgium)

and

I. DE LEEUW

Laboratory of Endocrinology, Metabolism and Nutrition, Faculty of Medicine, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk (Belgium)

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High-performance liquid chromatography (HPLC) has been employed successfully to determine the naturally occurring vitamin E analogues, α-, β- and γ-tocopherol (TP) in human plasma (Fig. 1). Most recently published procedures make use of ultraviolet (UV) or fluorimetric detection and of reversed-phase methods by which the prevalent form, α-TP, can be separated from its minor co-eluting β- and γ-homologues [1—5].

In this communication we wish to report a rapid, sensitive and reproducible HPLC method with electrochemical detection for routine measurement of α-tocopherol in human plasma. We have selected electrochemical detection, which was first described by Ikenoya et al. [6], for the analysis of tocopherols on the basis of its sensitivity and specificity. Since δ-tocol, which has been employed as internal standard in most HPLC assays based on UV, fluorimetric or electrochemical detection [1,5,6,7], can no longer be commercially obtained, we have evaluated the use of δ-tocopherol. The homologue, δ-TP, which has been demonstrated in some plant oils, e.g. in soybean oil [8], has the advantage that it is a natural readily available form of vitamin E. δ-Tocopherol (Fig. 1) contains a free hydroxyl group at the C-6 position which is responsible

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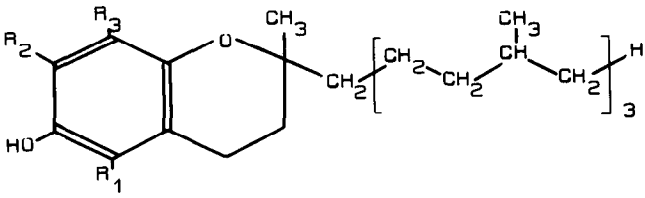

α-TP: $R_1 = R_2 = R_3 = CH_3$

β-TP: $R_1 = R_3 = CH_3$; $R_2 = H$

γ-TP: $R_2 = R_3 = CH_3$; $R_1 = H$

δ-TP: $R_3 = CH_3$; $R_1 = R_2 = H$

Fig. 1. Chemical structures of α-, β-, γ- and δ-tocopherol.

for its electrochemical reactivity and, furthermore, meets the other criteria of an internal standard: (1) it is quantitatively of minor importance in human blood; (2) it is structurally closely related to the compound to be determined so that the use of this homologue can compensate for losses during the sample work-up procedures; and (3) it can be separated from α-TP and its minor β- and γ-homologues during reversed-phase HPLC. The experimental conditions for suitable electrochemical detection of both α- and δ-TP have also been investigated because of the known different reactivity toward singlet oxygen, which correlates with the antioxidant vitamin E activity, α-TP being about ten times more potent than δ-TP [9].

MATERIALS AND METHODS

Chemicals

The solvents and reagents used were obtained from the following companies: ethanol, *n*-hexane, isopropanol and sodium perchlorate from Fluka (Buchs, Switzerland); methanol from Ferak (West-Berlin, F.R.G.); and pyridine from E. Merck (Darmstadt, F.R.G.). All solvents were analytical grade and were used without further purification.

The vitamin E standards, α-, γ-, and δ-TP were purchased from Supelco (Bellefonte, PA, U.S.A.).

High-performance liquid chromatography

The HPLC analyses were performed with a M45 solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), equipped with a U6K universal sample injector (Waters Assoc.) and an electrochemical detector (Model LC5, BioAnalytical Systems, West Lafayette, IN, U.S.A.) with a glassy carbon working electrode. The detector was operated at a potential setting of +0.7 V versus the Ag/AgCl reference electrode. The chromatographic trace was registered by a potentiometer recorder. A reversed-phase LiChrosorb RP-18 column was used in all experiments and was obtained from Chrompack (Middelburg, The Netherlands) (250 × 4.6 mm; 5-μm particles). The eluent consisted of methanol containing 0.1% pyridine and 0.05 *M* sodium perchlorate

as supporting electrolyte, as described by Ikenoya et al. [6] for reversed-phase analysis of tocopherols in combination with electrochemical detection. The eluent was filtered through a Millipore type FH 0.5-μm filter and was degassed before use. The solvent flow-rate was 1 ml/min with a corresponding pressure of 7.9 MPa.

Sample preparation

Blood samples were collected in Li-heparin vacutainers (Becton Dickinson, France) and plasma was separated and frozen until analysed. The plasma was extracted following the procedures described by De Leenheer et al. [5] with minor modifications. In a centrifuge tube, 40 μl of a δ-TP solution in isopropanol (100 μg/ml) and 100 μl of ethanol were added to a plasma sample of 100 μl. After mixing, the sample was extracted with 1.2 ml of *n*-hexane by thoroughly mixing using a vortex-type mixer for 1 min. After centrifugation (5 min, 700 *g*) the organic layer was transferred to a brown conical tube and evaporated under a nitrogen stream. The residue was redissolved in 500 μl of isopropanol and an aliquot of 30 μl was used for HPLC analysis.

Quantification

Known amounts of α-TP, covering the range of 5.80—46.45 μmol/l were added to samples of a plasma pool. The regression curve was made by plotting peak height ratios (α-TP/δ-TP) against the α-TP concentration.

The exact concentration of the α-TP standard solution was determined by measuring the UV absorbance at λ_{max} = 292 nm and by using an ε-value of 3500 l/mol cm [5].

RESULTS AND DISCUSSION

Typical chromatograms of extracts from human plasma, with and without addition of the internal standard δ-TP, are illustrated in Fig. 2. The retention characteristics of the vitamin E homologues are given in Table I. The total elution of the vitamin E homologues takes about 13 min. Because δ-TP is a natural vitamin E homologue, the plasma of 40 healthy individuals was checked for the possible occurrence of δ-TP. In only 8 out of 40 samples could traces of endogenous δ-TP be detected (0.82 ± 0.65 μmol/l) at the lower detection limit of δ-TP (signal-to-noise ratio = 3). The finding that δ-TP is quantitatively of minor importance in human plasma justifies its use as internal standard and is in agreement with data published by Kato et al. [7], who reported the total absence of δ-TP in human serum for a group of 19 individuals. In order to minimize the possible error in the determination of peak height ratios (α-TP/δ-TP) due to the contribution of traces of endogenous δ-TP, a 120-fold excess of the expected δ-TP amount was added as internal standard.

In a series of experiments we have also investigated the experimental conditions for suitable electrochemical oxidation of both α- and δ-TP. α- and δ-TP have been demonstrated to display a different reactivity toward singlet oxygen, which correlates with the antioxidant vitamin E activity, α-TP being about ten times more potent than δ-TP [9]. Consequently, different half-wave potentials for the electrochemical oxidation of α-TP ($E_{1/2}$ = 0.45 V versus Ag/AgCl) and of

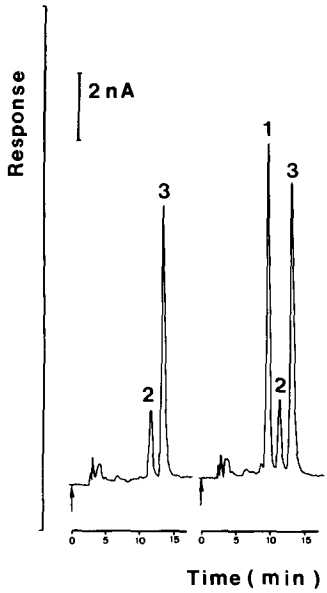

Fig. 2. High-performance liquid chromatogram of a plasma extract without and with addition of the internal standard, δ-TP. Column: 25 cm × 4.6 mm I.D., packed with 5-μm LiChrosorb RP-18. Eluent: methanol containing 0.1% pyridine and 0.05 *M* sodium perchlorate; flow-rate 1 ml/min. Detection: electrochemical oxidation at a potential of +0.7 V. 1 = δ-TP; 2 = β- + γ-TP; 3 = α-TP.

TABLE I

RETENTION CHARACTERISTICS OF TOCOPHEROLS

	Capacity ratio (k')	Retention time (min)
δ-TP	2.8	9.2
β-TP/γ-TP	3.5	11.0
α-TP	4.2	12.6

δ-TP ($E_{1/2}$ = 0.62 V) have been found [6]. Mixtures of α- and δ-TP, containing 5.8 and 7.5 ng, respectively, were subjected to HPLC analysis and the oxidizing potential of the glassy carbon working electrode was varied from +0.50 to +0.90 V versus the Ag/AgCl reference electrode in order to select a reasonably low oxidizing potential for δ-TP in its plateau region. Potentials higher than +0.90 V were not applied because of serious baseline instability, probably due to impurities in the eluent which started to oxidize. Fig. 3 shows the relationship between the peak response (in nA) and the applied potential. As can be seen from Fig. 3, a plateau value is reached for α-TP at approximately +0.80 V but not for δ-TP at the highest potential evaluated, i.e. +0.90 V. Because of baseline instability, which already started to occur at +0.80 V, and because sensitivity was not a limiting factor (detection limit 0.6 ng; signal-to-noise ratio = 10), we finally decided to use an oxidizing potential of +0.70 V for routine determinations of α-TP. A linear relationship between peak height ratios (α-TP/δ-TP) and the α-TP concentration was found (Y = 0.7005X + 0.7866; r = 0.99985; n = 6).

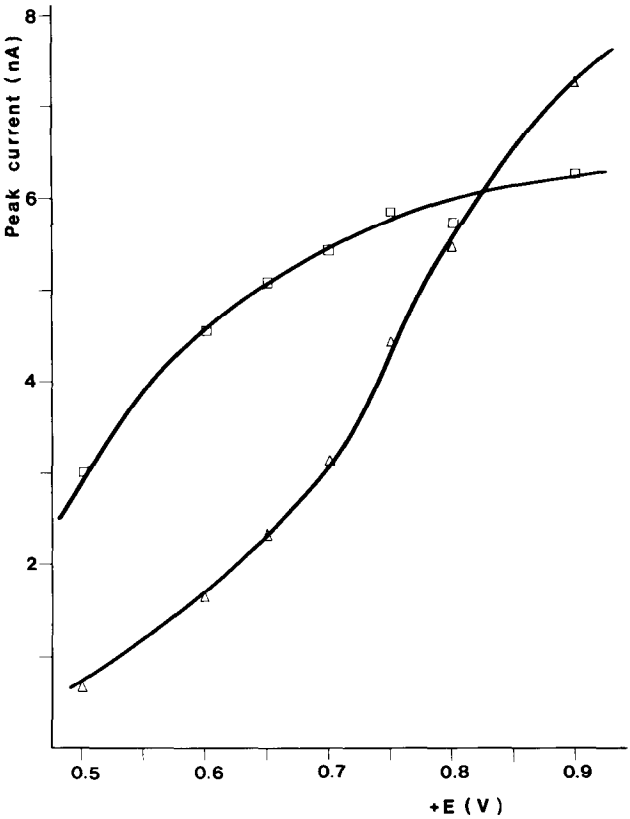

Fig. 3. Voltammograms for α-TP (□) and δ-TP (△) obtained under the conditions of Fig. 2. Injected amounts: 5.8 ng of α-TP; 7.5 ng of δ-TP.

For the estimation of the unknown concentrations, the procedure described by De Leenheer et al. [5] is applied. Briefly, the calibration curve is used after subtraction of the intercept, which represents the endogenous level of α-TP in the plasma pool, and the concentration in unknown plasma samples is determined after calculation of the peak height ratios. Analysis of nine samples of a plasma pool revealed a within-day precision (coefficient of variation, C.V.) of 2.5% (mean ± S.D. = 18.05 ± 0.46 μmol/l). The day-to-day precision (C.V.) as measured over a period of ten days was 4.3% at the concentration level of 26.89 mol/l (S.D. = 1.16 μmol/l). All these values are within an acceptable 5% limit. The detection limit of the procedure was established using aqueous standards and was shown to be 0.23 μmol/l (signal-to-noise ratio = 10), which corresponds to concentrations about 100 times lower than those normally found in human plasma.

As a test of the applicability of the method, plasma from 25 human donors was analysed. Data are given in Table II and are in good agreement with literature data [5,10].

The HPLC method in combination with electrochemical detection for the estimation of α-TP is as simple and as short (total analysis time approximately 30 min) as the UV method described by De Leenheer et al. [5] and has a comparable precision: the C.V. for the UV method was 3% against 4% for our

TABLE II

PLASMA α-TOCOPHEROL CONCENTRATIONS FOR A GROUP OF ADULT HUMANS

Age (years)	Sex*	α-TP concentration (μmol/l)
20	M	15.79
20	F	23.46
22	F	21.83
22	M	22.53
25	M	16.49
25	F	24.15
26	F	23.46
27	F	26.70
28	M	25.08
29	M	33.44
30	F	31.12
30	F	21.60
31	M	50.39
31	M	29.96
31	M	22.76
32	F	27.64
32	F	25.78
36	F	34.60
36	F	26.70
36	M	20.44
39	F	22.29
51	F	58.52
62	M	36.00
63	F	22.53
66	M	36.23

Mean ± S.D.: 28.05 ± 9.48 μmol/l
Range: 15.79—58.52 μmol/l

*M = male; F = female.

method. The very clean chromatograms, which are totally free from interferences, indicate that the specificity of electrochemical detection, in which a relatively low oxidizing potential of +0.70 V is employed, is high. This is in contrast with methods based on direct fluorimetry and UV spectrophotometry, in which co-extracted compounds interfere in the analysis [3,11]. The HPLC method proposed is extremely sensitive as only 100 μl of plasma are utilized and only 1/16 of the final extract is employed for HPLC analysis. The method may therefore have a particular value in cases where the sample size is a limiting factor, e.g. in white blood cell analysis [1].

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