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Note

Quantitation of serum tocopherols by high-performance liquid chromatography with fluorescence detection

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d-α-Tocopherol is considered to have the highest biological activity of the naturally occurring forms of vitamin E [1]. Therefore, vitamin E status in human populations is usually assessed in terms of the α-tocopherol level in blood. The other tocopherols (β-, γ- and δ-tocopherol), however, are an important part of the daily vitamin E intake [2]. The significance of the non-α-tocopherols in human nutrition is not known. This depends to some extent on the lack of simple and rapid methods for their determination in serum samples. The chromatographic methods hitherto used to quantify the various tocopherols in serum are based on either gas chromatography (GC) [3] or thin-layer chromatography (TLC) [4]. Our previously described high-performance liquid chromatography (HPLC) method with UV detection [5] was inadequate for resolving the various tocopherols in serum from other endogenous compounds, but in a recent paper [6] it is indicated that HPLC combined with fluorescence detection can be used to separate the tocopherols in vegetable oils.

This paper describes a refined method, using *dl*-tocol as an internal standard, to quantify the various tocopherols in serum samples. The method has been applied to serum samples from healthy individuals and from mothers and their infants (cord blood).

EXPERIMENTAL

Reagents and chemicals

n-Hexane (analytical-reagent grade), redistilled once before use, was purchased from Rathburn Chemicals, Walkerburn, Great Britain. Diisopropyl

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ether and isopropanol (spectroscopic grade) were purchased from BDH, Poole, Great Britain. α-Tocopherol was obtained from Merck, Darmstadt, G.F.R. β-, γ- and ∂-tocopherol was a gift from Dr. K. Abe, Eisai Research Labs., Bunkyo-ku, Tokyo, Japan. *dl*-Tocol was obtained from Koch-Light, Colnbrook, Great Britain.

Instrumentation

Normal-phase HPLC was performed utilizing a Waters Model ALC/GPC 204 liquid chromatograph equipped with a U6K loop injector and a μPorasil column (10 μm particle size; Waters Assoc., Milford, Mass., U.S.A.). The column was eluted with *n*-hexane—diisopropyl ether (92:8) at a flow-rate of 2.5 ml/min. The fluorescence intensity of the column eluent was monitored continuously using a Schoeffel variable-wavelength spectrofluorimeter (Schoeffel Instrument Corp., Westwood, N.J., U.S.A.) equipped with a deuterium lamp. The attenuation was in the range 0.1—0.2. The excitation wavelength was set at 295 nm. The instrument was equipped with a cut-off emission filter (370 nm). For chromatographic comparison we also recorded the absorbance of the eluent at 280 nm with a Waters Model 440 UV detector.

Mass spectra were obtained on a JEOL JMS D-300 instrument equipped with a combined electron impact—chemical ionization ion source and a direct inlet probe. The mass spectrometer was coupled to a JMA 2000 mass data analysis system.

Preparation of standard solutions

Four standard mixtures with increasing concentrations of α-, β-, γ- and ∂-tocopherol and a constant concentration of the *dl*-tocol internal standard (21.5 μmol/l) were prepared and analysed by HPLC. Peak areas were determined by triangulation (peak base width × peak height). The fluorescence peak-area ratios of the standard α-, β-, γ- or ∂-tocopherol and the internal reference compound were plotted against the corresponding concentration ratios. The graphs showed good linearity in the following concentration ranges: α-tocopherol, 5—46 μmol/l; β-tocopherol, 0.5—3 μmol/l; γ-tocopherol, 1—10 μmol/l; ∂-tocopherol, 0.1—1 μmol/l. The coefficients in the equation (k = slope; l = intercept) and regression coefficients (r) are as follows: α-tocopherol, $k = 0.887$, $l = 0.024$, $r = 1.000$; β-tocopherol, $k = 1.001$, $l = 0.039$, $r = 0.999$; γ-tocopherol, $k = 0.983$, $l = 0.017$, $r = 1.000$; and ∂-tocopherol, $k = 1.142$, $l = 0.060$, $r = 0.999$.

Preparation of samples

Blood samples were obtained by venipuncture from ten healthy individuals (five males and five females), from fifteen mothers immediately after delivery and from the cord blood of their infants. The serum was removed, frozen and stored at −20° until taken for analysis. To each serum sample (aliquots of 500 μl) were added 500 μl of 99.5% ethanol containing 10.75 nmol of *dl*-tocol. After the addition of 1 ml of *n*-hexane and Vortex-mixing for 30 sec the samples were centrifuged for 5 min at 30,000 *g*. The organic layer was removed by pipette and evaporated to dryness in a stream of nitrogen. When completely dry the residue was redissolved in 50 μl of *n*-hexane. A 10—20-μl volume of

the extract was injected into the column. The same procedure was also applied to smaller serum samples (down to 100 μl).

RESULTS AND DISCUSSION

Chromatogram

Fig. 1a shows a typical chromatogram of an extract from a serum sample with *dl*-tocol as internal standard, in which α-, β- and γ-tocopherol can easily be identified. Their relative retention times are 0.37, 0.51 and 0.59, respectively. ∂-Tocopherol is not observed. It is important to note that the true retention time varied by at most 5% from day to day.

A chromatogram of an extract from the serum of an infant given a lipid emulsion intravenously (Intalipid; Vitrum, Stockholm, Sweden) is shown in Fig. 1b. Intralipid is derived from fractionated soy-bean oil, which is rich in γ- and ∂-tocopherol. In this chromatogram γ-tocopherol is the predominant vitamin E form. The presence of ∂-tocopherol in the chromatogram of this serum extract is of interest.

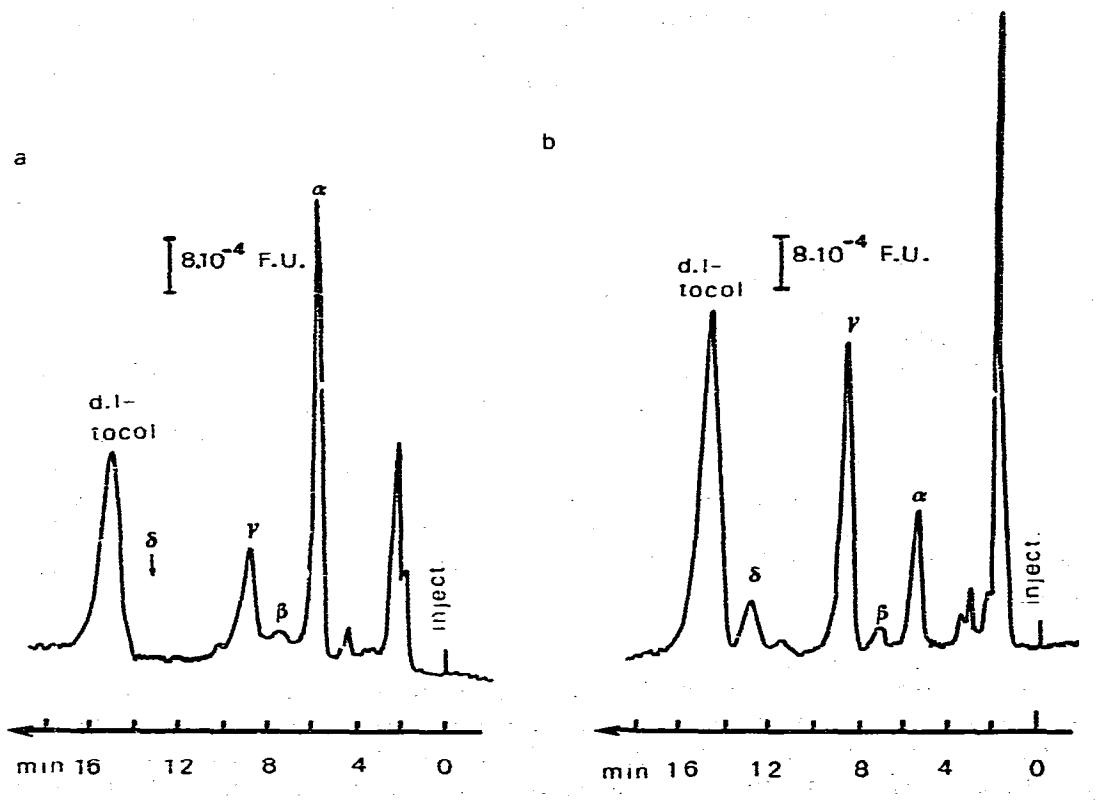

Fig. 1. HPLC separation of tocopherols in serum. (a) Normal serum; (b) serum from an infant given Intralipid. Conditions: column, μPorasil; eluent, *n*-hexane—diisopropyl ether (92:8); flow-rate, 2.5 ml/min (1000 p.s.i.); temperature, ambient; fluorescence detection; internal standard, *dl*-tocol.

Precision, sensitivity and selectivity

The recoveries of added α-, β-, γ- and ∂-tocopherol to a serum sample were found to be 92% for ∂-tocopherol and 100% for the others. The precision and reproducibility of the method were tested by analysing extracts from two different serum pools twice a day over a period of 5 days. The coefficients of variation (mean for the two serum samples) were determined to be 1.5%, 22% and 7% for α-, β- and γ-tocopherol, respectively. The high coefficient of variation for β-tocopherol is probably due to the small amount present in serum. The minimal detectable amount of injected pure α-tocopherol was 21 pmol, which corresponds to twice the noise level. The detectable amount varied only slightly for the other tocopherols, as the responses were almost identical at a given concentration.

Another chromatographic system was also tested in order to ensure that no peaks in the system described above are hidden under the tocopherol peaks. The column was exchanged for a more polar column (μ-NH_2-Bondapak, 5 μm particle size; Waters Assoc.) which was eluted with *n*-hexane—isopropanol (98:2). With this system we obtained a good separation of α- and β-tocopherol in a serum extract, but γ-tocopherol was separated less well from a new peak not observed in the other system (see Fig. 2). However, an estimation of

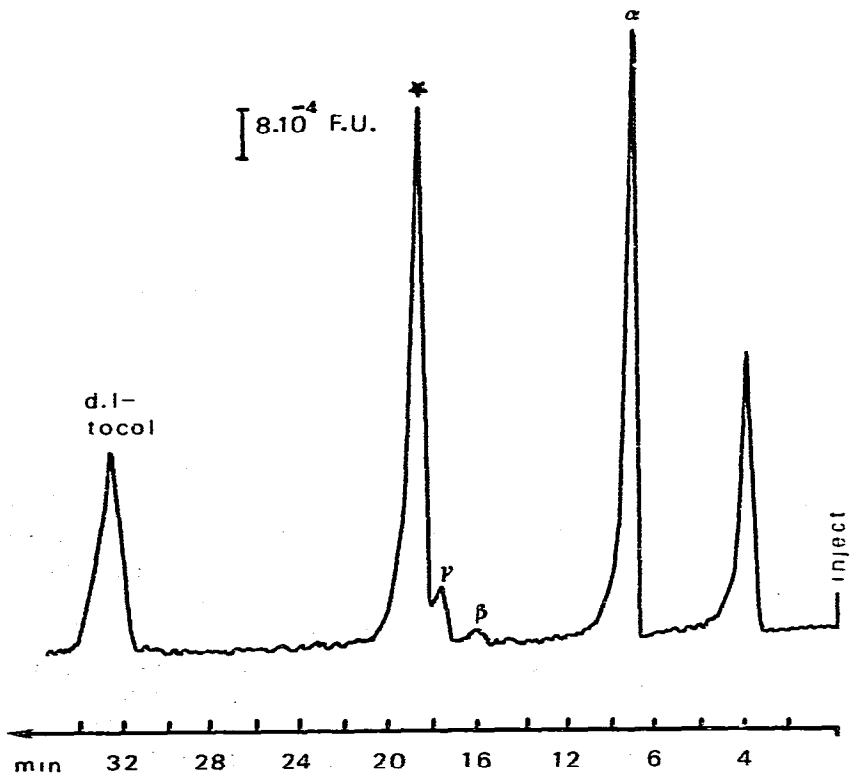

Fig. 2. HPLC separation of tocopherols in normal serum. Conditions: column, μ-NH_2-Bondapak; eluent, *n*-hexane—isopropanol (98:2); flow-rate, 1.5 ml/min (800 p.s.i.); temperature, ambient; fluorescence detection; internal standard, *dl*-tocol. The peak marked with an asterisk is not identified.

the γ-tocopherol content in a sample agreed within 10% with the result obtained on the same sample with the system described above. A fraction of the new peak in this chromatographic systems was collected and analysed by electron-impact and chemical-ionization mass spectrometry. A molar peak at $m/e = 368$ was found. The structure of the compound was not investigated further.

A quantitative evaluation of the α-tocopherol content in serum samples using UV detection showed agreement to within 3% with fluorescence detection.

Tocopherols in serum

Concentrations of the tocopherols in different serum extracts are presented in Table I. In normal serum α-tocopherol accounted for 87% (range 82.6—93.3%), β-tocopherol for 1.2% (range 0—2.3%) and γ-tocopherol for 11.8% (range 6.7—15.7%) of the total amount of tocopherols. δ-Tocopherol could be detected in the chromatogram of a normal serum if the amount of the extract injected was increased approximately 20-fold (<0.024 μmol/l). The values found in this study are higher than those previously reported [2, 4] using TLC separation with spectrophotometric determination of the tocopherols. The reason for this is not necessarily related to the precision and selectivity of the earlier method [2], as these differences can also be attributed to different dietary conditions of the individuals investigated.

TABLE I

α-, β- AND γ-TOCOPHEROL LEVELS IN DIFFERENT SERA

Type of serum	No. of samples	Mean* (μmol/l)		
		α	β	γ
Normal	16	30.6 (16.3—48.4)	0.4 (0.0—0.7)	4.1 (1.9—6.0)
Maternal	15	37.4 (20.0—62.5)	0.2 (0.0—0.7)	5.8 (1.2—10.5)
Cord	15	6.0 (1.39—12.1)	—	0.2 (0.0—1.2)

*Range in parentheses.

Maternal and cord blood was also studied (Table I). The total tocopherol concentrations found in these sera are in good agreement with those obtained in earlier studies showing high maternal and low cord blood tocopherol values [7]. This may be attributed to differences in transport capacity, as the level of plasma β-lipoprotein (the principal plasma carrier of vitamin E) has been found to correlate well with the vitamin E level [8]. However, in previous studies the various tocopherols were not separated and determined individually. In the maternal serum investigated by us α-tocopherol accounted for 86% (range 73.5—96%), β-tocopherol for 0.5% (range 0—4%) and γ-tocopherol for 13.5% (range 4—24%). In cord blood serum the α-tocopherol concentration was approximately one sixth of the maternal serum level. β-Tocopherol could not be detected, but γ-tocopherol was observed in some of the serum extracts.

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REFERENCES

1 M.K. Horwitt, Amer. J. Clin. Nutr., 29 (1976) 569.
2 C.K. Chow, Amer. J. Clin. Nutr., 28 (1975) 756.
3 J. Lehman and H.T. Slover, Lipids, 6 (1971) 35.
4 J.G. Bieri and E.L. Prival, Proc. Soc. Exp. Biol. Med., 120 (1965) 554.
5 B. Nilsson, B. Johansson, L. Jansson and L. Holmberg, J. Chromatogr., 145 (1978) 169.
6 K. Abe, Y. Yuguchi and G. Katusi, J. Nutr. Sci. Vitaminol., 21 (1975) 183.
7 M. Mino and H. Nishino, J. Nutr. Sci. Vitaminol., 19 (1973) 475.
8 P. Hågå and G. Lunde, Acta Paediatr. Scand., 67 (1978) 735.