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Note

The estimation of α-tocopherol in biological material by gas chromatography

GÖREL ÖSTERLÖF* and AUD NYHEIM

Department of Analytical Chemistry, Astra Läkemedel AB, S-151 85 Södertälje (Sweden)

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α-Tocopherol, the biologically most active of the E-vitamins, is a natural scavenger of free radicals and protects the organism from non-enzymatic attack by molecular oxygen on poly-unsaturated fatty acids. The normal range for free vitamin E concentrations in human plasma is 5—20 μg/ml. According to Horwitt [1] levels of at least 5 μg/ml are essential to prevent peroxide-induced hemolysis of red blood cells. Levels lower than 5 μg/ml are considered to indicate vitamin E deficiency.

The connection between α-tocopherol and cardiac toxicity of iron in a case with extremely low levels of α-tocopherol in serum has been investigated by Lindvall [2]. For this study a specific and sensitive gas chromatographic (GC) method for determination of α-tocopherol in plasma was required.

A large number of methods already exists for determination of tocopherols. A review by Bunnell [3] summarizes the different types of methods reported up to 1971. Many of these methods are based on indirect measurements of α-tocopherol and do not differentiate between the tocopherol isomers, and other reducing substances may also interfere.

Sheppard et al. [4] reviews GC assays for vitamin E in plants, animal tissues and food materials up to 1972. In recent years some gas and liquid chromatographic methods for estimation of plasma levels of α-tocopherol have been described [5—11]. The main problem to be overcome in the GC determination is interference by cholesterol present in plasma. Prior separation of α-tocopherol and cholesterol using techniques such as thin-layer chromatography [5,8] or digitonide precipitation of cholesterol [12] is necessary and this may be time-consuming. Even if the separation can be performed directly on the GC column it will take a rather long time [6,7].

The method given below is a fast and accurate GC method to determine non-esterified α-tocopherol in plasma. Even now the cholesterol must be removed in advance, but this is rapidly and easily done on a small digitonin—celite column, a modification of a method described by Christie et al. [12]. The α-tocopherol is chromatographed as tocopheryl acetate and is eluted with a retention

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time of about 6 min. The method requires 0.1 to 1.0 ml of plasma and the detection limit of tocopherol is about 0.2 μg/ml.

EXPERIMENTAL

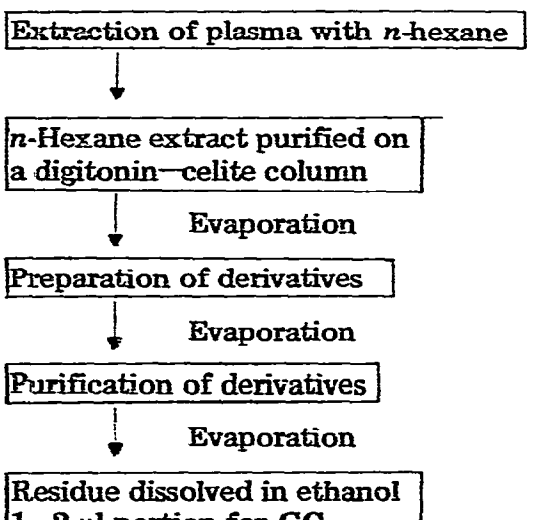
Reagents

α-Tocopherol and digitonin were obtained from Merck (Darmstadt, G.F.R.). *n*-Hexane, ethanol, pyridine and acetic anhydride were all of analytical grade quality and used without further purification. Celite 45 was standardized kieselguhr with a particle size of 20—45 μm. Octacosane, also analytical grade, was used as internal standard.

Instrumental conditions

A Perkin-Elmer gas chromatograph Model F 11 equipped with a flame ionization detector was used. The electrometer was connected to a Perkin-Elmer 159 1-mV recorder and the chart speed was 60 cm/h. The column used was a silanized glass column (180 cm × 2 mm I.D.) packed with 3% JXR (methyl silicone, Applied Science Europe, Oud-Beijerland, The Netherlands) on Gas-Chrom Q (100—120 mesh). The temperature of the oven was 260°C and that of the injector and detector 280°C. The flow-rate of the carrier gas (nitrogen) was 30 ml/min.

General procedure



Extraction

A 0.1—1.0-ml plasma sample (if less than 1.0 ml plasma, add distilled water up to 1.0 ml) was mixed with 1.0 ml absolute ethanol in a 10-ml test tube. *n*-Hexane (2.0 ml) containing the internal standard octacosane (4 μg/ml) was added and the tube was shaken mechanically in a rotary mixer for 15 min. After centrifugation for about 10 min at 3000 *g* as much as possible of the

organic phase was transferred to the digitonin—celite 545 column described below in order to purify the extract from cholesterol.

Removal of cholesterol

Digitonin (300 mg) was dissolved by heating in 5 ml of water. The solution was well mixed with 10 g celite 545, which had been dried overnight at 110°C [12]. About 250—300 mg of the mixture was dry packed in a pasteur pipette containing a glass wool plug. The hexane extract was added to the column and allowed to pass at a rate of approximately 1 drop/sec. About 2 ml *n*-hexane was added to wash the column. The eluate was then evaporated to dryness in a 3-ml test-tube at a temperature of 50°C under a stream of nitrogen.

Acylation and purification of derivatives

To the residue 20 μl dry pyridine and 100 μl acetic anhydride were added. The tube was stoppered and incubated in a heating block at 50°C for 15 min. The excess of reagents was evaporated with a stream of nitrogen at a temperature of 50°C. The residue was dissolved in about 1.5 ml of *n*-hexane; 1.0 ml of water was added and the tube was shaken vigorously on a whirlimixer for 30 sec. After centrifugation for 5 min, the *n*-hexane extract was evaporated to dryness in the same way as above. The residue was dissolved in 200 μl absolute ethanol and 1—2 μl was injected into the chromatograph.

Calibration curve

Ethanolic solutions of α-tocopherol ranging from about 0.4 to 17.0 μg/ml were used as standards. From each of these solutions 1.0 ml was evaporated to dryness together with the internal standard and acylated as above without the purification steps. A calibration curve was plotted from the ratio of the peak heights of α-tocopherol and octacosane against the α-tocopherol concentration (Fig. 3B).

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1A. The retention time for α-tocopherol as tocopheryl acetate was about 5.5 min and for octacosane about 2.5 min. Low levels of δ-, β- and γ-tocopheryl acetates were also shown by the chromatogram (retention time for δ-tocopheryl acetate about 3.5 min, for β- and γ-tocopheryl acetate about 4.5 min). No attempt was made to determine these tocopherols.

Cholesterol is completely removed by a digitonin—celite mixture, easily packed as a very small column (about 2 cm high) into a pasteur pipette. Only 250—300 mg of the mixture was necessary. Such a small column usually gave a drop rate of about 1 drop/sec which means that the whole purification is performed in a few minutes. Fig. 1 shows the effect of such a purification step (chromatogram A) while chromatogram B was run without this treatment.

It is also necessary to purify the derivatives after acylation, otherwise unidentified interferences occur as in the chromatogram in Fig. 2 (note the large asymmetrical peak between the internal standard and tocopheryl acetate). This purification involves an evaporation to remove the derivatisation reagents and

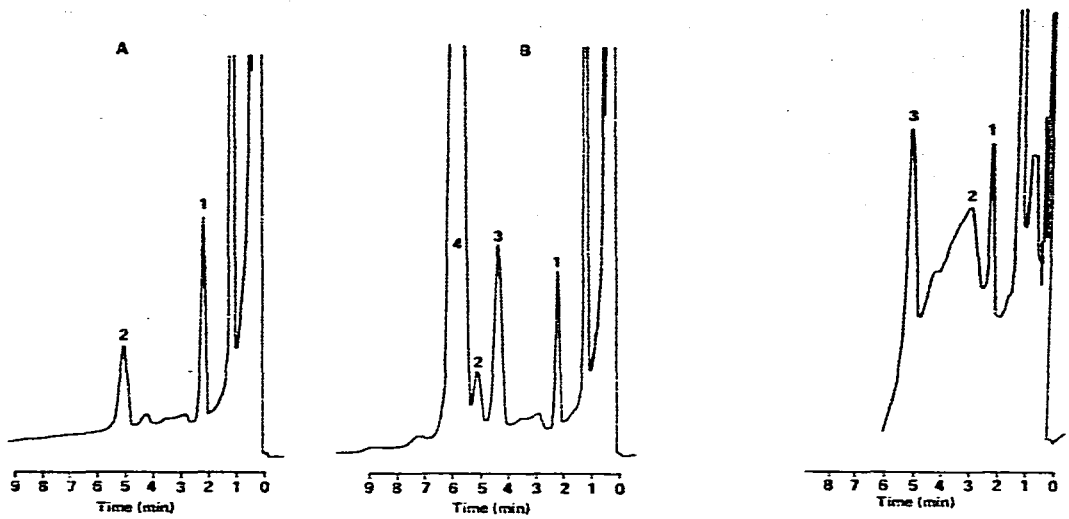


Fig. 1. Chromatograms of an extract of normal plasma (A) analyzed according to the method and (B) not treated on the digitonin—celite column. Peaks: 1, octacosane; 2, α-tocopheryl acetate; 3, cholesterol and 4, cholesteryl acetate.

Fig. 2. Chromatogram of a normal plasma extract analyzed according to the method but without purification of the derivatives. Peaks: 1, octacosane; 2, unidentified impurities and 3, α-tocopheryl acetate.

an aqueous extraction of the hexane solution of the residue, both steps being very important for getting a clean extract. Without this purification the injection part of the column as well as the detector are rapidly contaminated making further chromatography impossible.

Fig. 3 shows two calibration curves where the ratio of the peak heights of α-tocopherol and octacosane is plotted against the α-tocopherol concentration. Curve A is obtained after analysis of plasma samples spiked with known amounts of α-tocopherol (0, 0.81, 4.25 and 17.0 μg/ml). In curve B ethanolic solutions of α-tocopherol in a concentration ranging from 0.4 to 17.0 μg/ml were used. The two curves are parallel with correlation coefficients of 0.999 in both cases; therefore ethanolic solutions can be used as standards for determination of plasma levels of α-tocopherol. The parallel displacement between the two curves is due to the basic level of α-tocopherol in the pooled human plasma used.

The reproducibility of the method was tested with pooled human plasma analyzed with regard to the basic α-tocopherol level. For a series of five analyses of the same sample the mean value was 6.81 μg/ml with a coefficient of variation of 3.2%. The validity of this value was confirmed by gas chromatographic—mass spectrometric analysis showing that the registered peak contained only α-tocopheryl acetate. The recovery and reproducibility were also investigated with spiked plasma at three different levels (0.81, 4.25 and 17.0 μg/ml). Five samples at each level were analyzed according to the procedure described. The results are given in Table I. The recovery ranged from 100 to 104%, with a mean value of 101%.

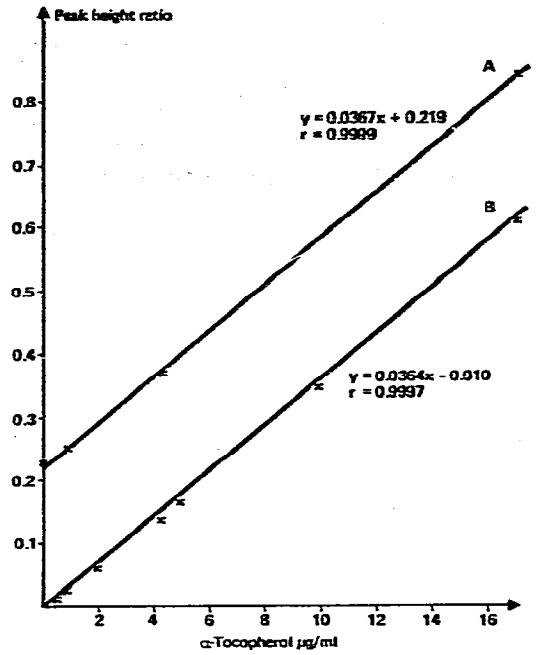


Fig. 3. Calibration curves. (A) Plasma solutions supplemented with known amounts of α-tocopherol. Each point is the mean of the analysis of five samples. (B) Ethanolic solutions of α-tocopherol.

TABLE I

REPRODUCIBILITY AND RECOVERY OF α-TOCOPHEROL ADDED TO PLASMA CONTAINING A BASIC α-TOCOPHEROL LEVEL OF 6.81 µg/ml

Each level is analyzed five times.

Initial concn. (µg/ml)	Added (µg/ml)	Total level (µg/ml)	Found (µg/ml) (Mean, C.V.%)		Recovery (%)
6.81	0.85	7.66	7.72	1.2	100.8
6.81	4.25	11.06	11.03	3.2	99.7
6.81	17.02	23.83	24.67	1.3	103.5
					Mean 101.3 ± 2.7 (S.D.)

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