Journal of Chromatography, 147 (1978) 481–484

CHROM. 10,205

Note

Determination of sub-microgram levels of α-tocopherol in serum by gas–liquid chromatography with solid injection

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(First received March 15th, 1977; revised manuscript received May 16th, 1977)

In connection with our studies on the chronic toxicity of cannabinoids[1,2], a specific and sensitive method for determining α-tocopherol in serum was required. Methods already described are mostly based on spectrophotometric[3–5] and gas chromatographic techniques[3,6–8]. The former methods, which require a very accurate separation from interfering substances as they are based on the reducing property of α-tocopherol, lack specificity, while the latter methods are more sensitive and specific, although in some circumstances the amount of α-tocopherol can be too low to be determined with normal apparatus and techniques. Recently, the simultaneous determination of α-tocopherol and free fatty acids in human plasma by gas chromatography with glass open-tubular capillary columns was described; by this method, levels of 0.5–0.6 mg/100 ml of tocopherol in plasma can be determined without analytical risk[9,10].

We describe here a method based on the solid injection of the sample[11], which permits the determination of lower levels of α-tocopherol in serum.

MATERIALS AND METHODS

All reagents were of analytical grade and were re-distilled before use.

Thin-layer chromatography was carried out on activated silica gel G plates containing 0.03% Rhodamine 6G. The solvent system cyclohexane–diethyl ether (8:2) was used for separating α-tocopherol from cholesterol and other interfering compounds[3].

Gas chromatographic analysis was performed with a Carlo Erba Fractovap 2300 apparatus, equipped with a flame-ionization detector and with a device for the introduction of solid samples. A 2 m × 4 mm I.D. glass column packed with silanized Gas-Chrom Q (100–120 mesh) impregnated with 3% SE-30 was used. The temperature of the oven was 242°, the temperature of the injector was 275° and the flow-rate of the carrier gas (nitrogen) was 70 ml/min. Under very similar conditions, it has been shown that α-tocopherol is separated well from other tocopherols[12].

One volume (usually 0.5 ml) of human or rabbit serum was added to two volumes of a 3% ethanolic solution of pyrogallol in glass-stoppered centrifuge tubes. After heating at 70° in a water-bath for 5 min, 0.3 ml of saturated potassium hydroxide solution was added and the tubes were shaken vigorously. A flow of nitrogen was gently passed into the tubes, which were then immediately stoppered. The mixture was heated again at 70° for 30 min, cooled, one volume of distilled water was added and the mixture was extracted twice with a total volume of 5 ml of *n*-hexane. The extraction was carried out in centrifuge tubes in melting ice. After centrifugation, the organic layer was transferred into conical tubes and concentrated to a small volume under nitrogen.

The extract was purified by thin-layer chromatography and the area corresponding to α-tocopherol ($R_F = 0.40$) was scraped off and eluted with chloroform. This solution was evaporated under nitrogen and the residue was re-dissolved in 0.1 ml of dichloroethane.

Samples of 0.01–0.05 ml were transferred quantitatively onto stainless-steel gauzes placed on a PTFE plate and the solvent was evaporated. This operation was carried out in darkness and under nitrogen. The gauzes were then placed for 5 h in an atmosphere saturated with pyridine and acetic anydride. Alternatively, for practical purposes, this stage could be extended overnight. Finally, the gauzes were introduced into the gas chromatographic device for solid samples and dropped one by one into the column.

Quantitation of α-tocopherol was carried out by the peak-height method using a dose–response calibration graph constructed by using pure α-tocopherol (E. Merck Darmstadt, G.F.R.) processed together with the sample (Fig. 1).

RESULTS AND DISCUSSION

The proposed method was used to determine sub-microgram levels of α-tocopherol in small samples of blood serum. The technical difficulties of dosing very small amounts of such a degradable substance were mainly overcome by gas–liquid chromatography with solid injection, which permits virtually the whole α-tocopherol content of the sample to be concentrated on to gauzes, and by the use of a particularly sensitive thin-layer chromatographic indicator. Great care was always taken to avoid losses of α-tocopherol by oxidation, because oxidation by air proceeds rapidly, especially when α-tocopherol is in alkaline solution, in daylight and fluorescent light.

The thin-layer chromatographic technique allowed the rapid location of α-tocopherol with a blue fluorescence at $R_F = 0.40$. For this purpose, Rhodamine 6G proved to be more sensitive than Rhodamine B, and had the advantage over the usual fluorescence indicators of enabling one to avoid, in most instances, exposure of the plates to UV light, provided that the amount of α-tocopherol was at least 1 μg. This technique also allowed a good separation from cholesterol, which appeared with yellow fluorescence at $R_F = 0.25$, and from other lipids, most of which migrated with the front.

The quantitative transfer on to metallic gauzes could be ensured only if the surface of the PTFE plate was perfectly smooth and the evaporation of the solvent was not disturbed.

The acetylation performed directly on the gauzes protected the highly reactive

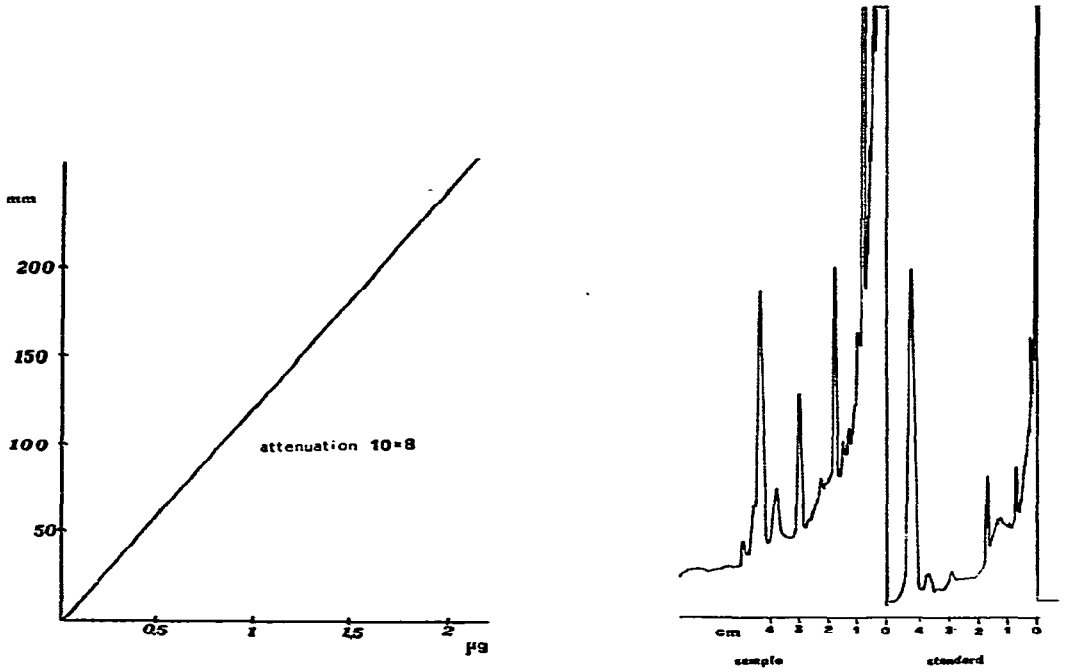

Fig. 1. Dose–response proportionality graph constructed with pure α-tocopherol processed together with the sample. Ordinate, height of the gaschromatographic peak (mm); abscissa, amount of α-tocopherol (µg).

Fig. 2. Gas chromatographic analysis of a sample of rabbit serum (half of the total unsaponifiable extract) by comparison with pure α-tocopherol processed together with the sample.

hydroxyl group of α-tocopherol and permitted manipulations such as repeated heating in alkaline solution, which are necessary if the acetylation is carried out in the usual way, to be avoided. Impurities in the reagents were also avoided and better chromatographic responses were obtained. The amount of α-tocopherol that reacted was 87% after 5–12 h in an atmosphere saturated with pyridine and acetic anydride, as determined by comparison with pure α-tocopherol acetate (E. Merck).

As this procedure provided very clean extracts, the α-tocopherol peak was symmetrical and well separated from the background at the retention time of 21.5 min (Fig. 2). The gas chromatographic analysis was also carried out using an 3% OV-17 column, and similar results were obtained, but the SE-30 packed column was preferred, due to more rapid resolution at lower temperature.

The recovery of α-tocopherol through the entire procedure was measured with unlabelled and ^{3}H-labelled α-tocopherol (Radiochemical Centre, Amersham, Great Britain) added to samples. The mean recovery was 80% with unlabelled tocopherol and 70% with labelled tocopherol.

The reproducibility of the method was tested by processing simultaneously eight aliquots of a sample of human and a sample of rabbit serum. The mean value was 1.40 ± 0.03 (S.E.) mg/100 ml for human serum and 0.189 ± 0.04 (S.E.) mg/100 ml for rabbit serum.

TABLE I

α-TOCOPHEROL CONTENT OF HUMAN AND RABBIT SERUM

Rabbits were fed with a normal diet containing 14 mg/kg of α-tocopherol.

α-Tocopherol content (mg/100 ml)	
Human serum	*Rabbit serum*
1.40	0.133
0.98	0.114
1.01	0.114
1.00	0.189
0.90	0.168
Mean: 1.058 ± 0.083 (S.E.)	0.096
	0.120
	0.053
	0.160
	0.280
	0.167
	0.161
	Mean: 0.146 ± 0.0141 (S.E.)

The results of the analysis of 12 samples of rabbit serum and of 5 samples of human serum are given in Table I. These results are comparable to those obtained by other workers[6] for human plasma with a preliminary oxidation of α-tocopherol to α-tocopherylquinone followed by the determination of the latter by gas–liquid chromatography, and to those obtained by Lin and Horning[9,10] by open-tubular capillary column gas chromatography. The latter method allows the determination of α-tocopherol without preliminary saponification of the sample and without the thin-layer chromatographic purification, but the risk of the analysis, when very small amounts of α-tocopherol are to be determined, is relatively high and was stated to be about 0.5–0.6 mg/100 ml. This sophisticated technique seems to be very useful in most circumstances in order to achieve high resolution, and is rapid, but when the level of α-tocopherol is very low, for example in some experimental animals and in humans in a deficient state, the solid injection method should be preferred, as it permits the determination of lower levels of α-tocopherol without analytical risk if the whole unsaponifiable extract is used.

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