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Short communication

Simple high-performance liquid chromatographic method for the determination of tocotrienols in human plasma

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Abstract

A simple high-performance liquid chromatographic method using fluorescence detection was developed for the determination of vitamin E especially δ-, γ- and α-tocotrienols in human plasma. The method entailed direct injection of plasma sample after deproteinization using a 3:2 mixture of acetonitrile–tetrahydrofuran. The mobile phase comprised 0.5% (v/v) of distilled water in methanol. Analyses were run at a flow-rate of 1.5 ml/min with the detector operating at an excitation wavelength of 296 nm and emission wavelength of 330 nm. This method is specific and sensitive, with a quantification limit of approximately 40, 34 and 16 ng/ml for α-, γ- and δ-tocotrienol, respectively. The mean absolute recovery values were about 98% while the within-day and between-day relative standard deviation and percent error values of the assay method were all less than 12.0% for α-, γ- and δ-tocotrienol. The calibration curve was linear over a concentration range of 40–2500, 30–4000 and 16–1000 ng/ml for α-, γ- and δ-tocotrienol, respectively. Application of the method in a bioavailability study for determination of the above compounds was also demonstrated.

Keywords: Vitamins; Tocotrienols

1. Introduction

Vitamin E is a collective name for naturally occurring tocopherols and tocotrienols in plants. They possess general structural features of an aromatic chromanol head and a 16-carbon hydrocarbon tail. The α-, β-, γ- and δ-homologues are determined by the amount and position of methyl substituents in the chromanol nucleus. The only difference in tocopherols and tocotrienols is that tocotrienols have three unsaturated (double) bonds in the tail, whereas tocopherols have a saturated tail [1]. In recent years, there is a growing interest in the tocotrienols for their cholesterol lowering effect [2,3], anticancer and tumor suppressive activities [4,5], antioxidant properties [6,7] and anti-aggregation of blood platelets [8]. This in turn has created a need to optimize further HPLC separation systems for quantification of the individual components of vitamin E. Various high-performance liquid chromatography (HPLC) methods using UV, fluorescence and electrochemical detection have been developed for the determination of tocopherols and tocotrienols in biological samples [9–17]. Most of these methods [9–15], require deproteinization with ethanol and extraction with hexane, being tedious and time consuming. For

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example, Sommerburg et al. [10] reported the use of saponification with ethanolic KOH and enzyme mixture of lipase and cholesterol esterase during extraction; but the alkaline condition might lead to oxidation and degradation of tocopherols and tocotrienols. Qureshi et al. [11] reported a combination of extracting the serum with hexane before using methanol to extract the remaining aqueous layer of the serum aliquot to estimate total serum tocols. However, the aqueous layer needs to be heated to dryness in a vacuum oven (2 p.s.i.) and held at 180°C for 1 h (1 p.s.i.=6894.76 Pa). In another method, Podda et al. [12] reported a HPLC gradient system with in-line ultraviolet and electrochemical detection for measuring α- and γ-tocopherols as well as different tocotrienols but the method has a relatively long run time of about 20 min. Two direct injection methods have been reported by Cooper et al. [16] and Julianto et al. [19], respectively, but both methods only target analysis of tocopherols and not tocotrienols.

In this paper, we report a relatively simple, specific and sensitive HPLC method using fluorescence detection for the simultaneous determination of three homologues of tocotrienols, namely α-, γ- and δ-tocotrienol in human plasma. We also demonstrated the applicability of this method in a bioavailability study.

2. Experimental

2.1. Materials

Tocomin 50%, which contains a minimum of 50.0% of phyto-tocotrienol/tocopherol complex, was obtained from Carotech (Ipoh, Malaysia). A tocotrienol standard kit was purchased from Merck (Darmstadt, Germany). All of the other solvents used were either of analytical reagent grade or of HPLC grade and were purchased from Merck and Ajax (Auburn, Australia).

2.2. Instrumentation

The HPLC system comprised a Jasco PU-980 pump (Jasco, Hachioji City, Tokyo, Japan), a Jasco 821-FP fluorescence detector (Jasco) and a Hitachi D-2500 chromato-integrator (Hitachi, Tokyo, Japan). The detector was operated using an excitation wavelength of 296 nm and an emission wavelength of 330 nm, gain ×10, attenuation 32. A Metaphase Crestpak C18S (5 μm, 250×4.6 mm I.D.), fitted with a refillable guard column (Upchurch Scientific, Oak Harbor, WA, USA), packed with Perisorb RP-18 (30–40 μm, pellicular, Upchurch Scientific), was used for the chromatographic separation. The mobile phase comprised 0.5% (v/v) distilled water in methanol. Analyses were run at a flow-rate of 1.5 ml/min and the samples were quantified using peak height.

2.3. Sample preparation

A 250-μl aliquot of plasma sample was measured into an Eppendorf microcentrifuge tube and deproteinized by adding 500-μl of a mixture of acetonitrile–tetrahydrofuran (3:2, v/v). The mixture was vortex-mixed for 2.5 min using a vortex mixer and then centrifuged at 12 800 *g* for 20 min. A 100-μl aliquot of the supernatant was then injected onto the column.

2.4. Assay validation

Standard calibration curves were constructed by spiking pooled plasma with a known amount of α-, γ- and δ-tocotrienol at a concentration range of 40–2580, 34–4340 and 16–1040 ng/ml, respectively. The standards were stored at −20°C in amber bottles and were stable for more than three months. The plasma standards were also used to determine the within-day and between-day precision and accuracy (n=6) of the method. In addition, the absolute recovery (n=6) was estimated by comparison with directly injected solutions of the tocotrienols in methanol of corresponding concentrations after similar treatment as for the plasma sample.

3. Results and discussion

Chromatograms obtained with blank plasma and plasma spiked with tocotrienols are shown in Fig. 1A and B, while that of a human volunteer 4 h after dosing with 200 mg tocotrienols soft gelatin capsules are shown in Fig. 1C. It can be seen that the α-, γ-

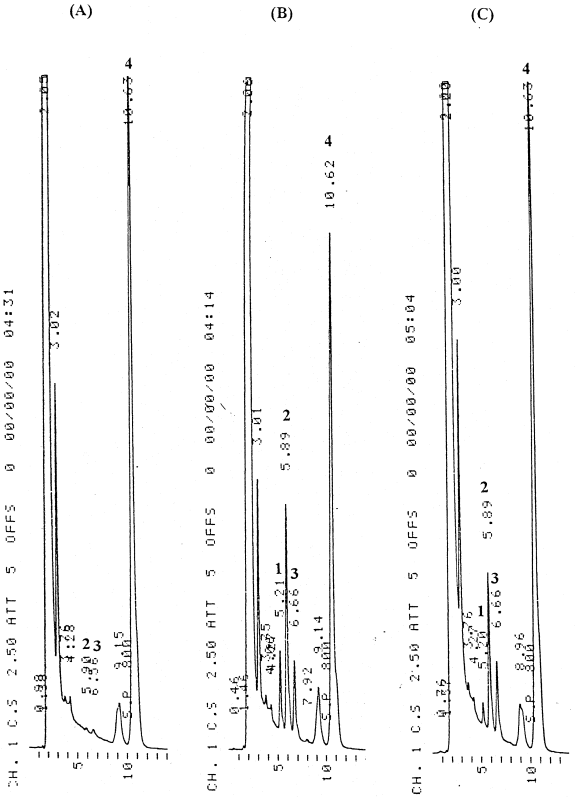

Fig. 1. Chromatograms for the analysis of α-, γ- and δ-tocotirenols and tocopherol in plasma. (A) Blank plasma. (B) Plasma spiked with 260, 645 and 1085 ng/ml δ-, α- and γ-tocotrienols, respectively. (C) A volunteer plasma containing 89.60, 690.37 and 625.42 ng/ml of δ-, γ- and α-tocotirenols 4 h after oral administration of 200 mg phyto-tocotrienol/tocopherol soft gelatin capsules. (*y*-Axis: attenuation=5, *x*-axis: chart speed=2.5 mm/min. Peaks: 1=δ-tocotrienol, 2=γ-tocotrienol, 3=α-tocotrienol and 4=α-tocopherol).

and δ-tocotrienol peaks, with retention times of approximately 5.2, 5.9 and 6.6 min, respectively, were well resolved, while α-tocopherol has a retention time of approximately 10.6 min. β-Tocotrienol was not detectable but endogenous plasma α-, γ- and δ-tocotrienols were found to be present at approximately 45–112, 30–80 and 0–20 ng/ml, respectively. These values were estimated by extrapolation of the standard curve according to the procedure of the standard addition method [18]. Therefore, it was necessary to subtract the endogenous levels of tocotrienols to present a more accurate profile of the absorbed tocotrienols following oral administration to the volunteers. The total run time for each injection/sample was only 12 min. At the excitation and emission wavelengths of 296 nm and 330 nm, respectively, all three tocotrienols, namely α-, γ- and δ-tocotrienol could be determined at a satisfactory limit of quantification, namely 40, 34 and 16 ng/ml, respectively. These values were the lowest concentrations used to construct the standard curve for each homologue, and possessed satisfactory between-day and within-day accuracy and precision. On the other hand, the method is also suitable for simultaneous determination of α-tocopherol and has a quantification limit of approximately 1 μg/ml – comparable to a few reported methods [15,17]. The sensitivity could be further improved by using a gain of ×100 at the fluorescence detector.

The absolute recovery, within-day and between-day accuracy and precision values for α-, γ- and δ-tocotrienol are presented in Tables 1–3. All the relative standard deviations (RSDs) and percent error values of both the within-day and between-day

Table 1
Absolute recovery, within-day and between-day precision and accuracy (n=6) for α-tocotrienol

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	RSD (%)	Precision (RSD, %)	Accuracy (% of true value)	Precision (RSD, %)	Accuracy (% of true value)
40.31	98.28	6.71	8.07	111.65	11.37	106.07
80.63	101.31	3.14	11.41	100.70	7.85	97.16
161.25	99.90	2.79	3.17	96.89	3.12	102.39
322.50	93.68	4.56	3.98	96.24	3.76	102.04
645.00	98.12	5.97	1.38	98.42	2.06	100.87
1290.00	97.26	2.71	2.88	97.68	2.09	99.80
2580.00	97.83	2.16	2.84	101.56	4.88	101.77

Table 2
Absolute recovery, within-day and between-day precision and accuracy ($n=6$) for γ-tocotrienol

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	RSD (%)	Precision (RSD, %)	Accuracy (% of true value)	Precision (RSD, %)	Accuracy (% of true value)
33.91	94.45	3.96	3.12	91.98	7.77	89.54
67.81	97.27	2.38	3.27	107.21	3.94	97.86
135.63	97.81	1.26	4.84	100.42	2.99	100.70
271.25	100.00	1.18	2.88	101.57	1.20	100.66
542.50	97.74	1.58	5.07	96.70	2.26	99.79
1085.00	100.95	4.20	1.39	100.50	1.58	98.44
2170.00	95.16	4.30	2.27	98.52	1.65	100.40
4340.00	97.05	2.75	2.70	100.33	6.80	100.65

precision and accuracy were less than 12% at the concentrations used. The standard curve ($n=6$) was found to be linear over the concentration range used with a correlation coefficient of 0.9993, 0.9999 and 0.9999 for α-, γ- and δ-tocotrienol, respectively. The mean absolute recoveries of α-, γ- and δ-tocotrienol were 98.09%, 97.63% and 97.49%, respectively. Since an extraction procedure was not involved in this method, an internal standard was found to be unnecessary.

Water was added to the mobile phase to achieve a better resolution of the three peaks, namely α-, γ- and δ-tocotrienol. The deproteinizing solution that consisted of acetonitrile–tetrahydrofuran (3:2) is similar to the one we have used previously in the assay of α-tocopherol [19] and was critical to achieve a satisfactory recovery for all three isomers. Like tocopherols, tocotrienols are highly lipophilic and are bound to lipoproteins in the plasma [11]. Therefore, it is important that the deproteinizing agent can completely deproteinize the sample to release the tocotrienols from the plasma. Moreover, the polarity of the resulting mixture of solutions must be optimized to dissolve the tocotrienols. Tetrahydrofuran alone is a weak deproteinizing agent, and could not induce complete deproteinization of the plasma samples. In comparison, acetonitrile is a better deproteinizing agent than tetrahydrofuran, but tocotrienols do not dissolve well in acetonitrile, which resulted in a recovery of 75 to 80% for the three tocotrienols. Besides, the amount of deproteinizing agent used, which is two parts to one part of plasma, is also critical to assure complete deproteinization and release of protein-bound tocotrienols. A ratio of (1:1) of acetonitrile–tetrahydrofuran to plasma sample yields a low recovery of 50% for the three tocotrienols.

The use of other deproteinizing agents such as ethanol, methanol and perchloric acid was also investigated. It was found that the original combina-

Table 3
Absolute recovery, within-day and between-day precision and accuracy ($n=6$) for δ-tocotrienol

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	RSD (%)	Precision (RSD, %)	Accuracy (% of true value)	Precision (RSD, %)	Accuracy (% of true value)
16.25	96.49	3.40	11.68	98.99	2.27	102.27
32.50	98.03	2.32	5.19	94.22	3.77	96.23
65.00	98.72	3.39	4.50	99.23	0.50	99.50
130.00	99.36	1.99	3.18	98.97	1.51	98.49
260.00	95.09	3.25	1.26	100.36	0.68	99.32
520.00	97.24	4.91	2.36	97.68	0.24	99.76
1040.00	97.40	1.80	2.87	100.38	3.89	105.24

tion yields the highest recovery as reported above. The recovery of the three tocotrienols using ethanol alone, as a deproteinizing agent was about 90%. On the other hand, methanol alone yielded a low recovery of about 25 to 40%. Although perchloric acid is a very good deproteinizing agent, it is not able to solubilize tocotrienols and hence gave poor recovery. Moreover, the combination of perchloric acid and tetrahydrofuran only yielded a poor recovery of approximately 40%. This was probably due to the high acidity of perchloric acid which might have resulted in the tocotrienols being oxidized.

The present method was applied to analyze plasma samples of eight healthy adult male volunteers who had participated in a comparative bioavailability study of two different tocotrienol preparations, namely the phyto-tocopherol/tocotrienol soft gelatin capsule and phyto-tocotrienol/tocopherol in a powder formulation. Fig. 2 shows the plasma concentration–time profiles for α-, γ- and δ-tocotrienol of one of the volunteers who had participated in the bioavailability study. Unlike α-tocopherol [20], peak concentrations of all three homologues of tocotrienols were achieved rapidly at approximately 4 to 5 h and the decrease was more rapid.

In conclusion, the present HPLC method was simple, specific, sensitive and suitable to be used for determination of plasma concentration of tocotrienols and tocopherols for pharmacokinetic/bioavailability studies.

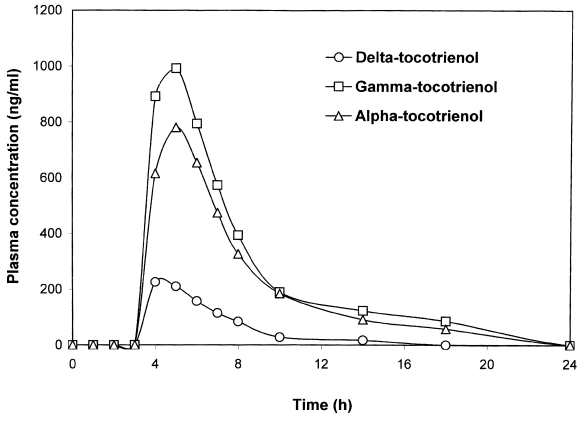

Fig. 2. Plasma concentration versus time profiles of δ-, γ- and α-tocotrienols from a volunteer after oral administration of 200 mg phyto-tocotrienol/tocopherol complex in a powder formulation.

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