

Oral α -Tocopherol Supplements Decrease Plasma γ -Tocopherol Levels in Humans¹

GARRY J. HANDELMAN, LAWRENCE J. MACHLIN,*
KARLOTTA FITCH,† JOHN J. WEITER† AND
EDWARD A. DRATZ

*Division of Natural Sciences, University of California, Santa Cruz, CA 95064, and *Department of Clinical Nutrition, Hoffmann-La Roche Inc., Nutley, NJ 07110 and †The Retina Foundation, The Eye Research Institute, Boston, MA 02114*

ABSTRACT In a cross-sectional survey of 86 elderly persons, it was observed that subjects with elevated plasma α -tocopherol levels had depressed plasma γ -tocopherol. Tocopherols were measured by both reverse-phase and normal-phase high performance liquid chromatography (HPLC). When eight human volunteers (age range 30–60) were given 1200 IU of all-*rac*- α -tocopherol daily for 8 wk, plasma γ -tocopherol and β -tocopherol decreased in all subjects. After supplementation, γ -tocopherol values were typically 30–50% of initial values, and α -tocopherol values were typically 200–400% of initial values. These results suggest that intestinal uptake and/or plasma transport make more efficient use of α -tocopherol than of γ - or β -tocopherol. Moreover, the results indicate that the ratio of γ - to α -tocopherol in plasma would be a more satisfactory index to measure compliance in trials involving supplementation with α -tocopherol. *J. Nutr.* 115: 807–813, 1985.

INDEXING KEY WORDS α -tocopherol • γ -tocopherol • nutrient interaction • dietary supplements • HPLC

Most American diets are now two- to threefold richer in γ -tocopherol than α -tocopherol (1). However, numerous reports (2–4) indicate that plasma α -tocopherol is about 10-fold higher than γ -tocopherol. These observations suggest that α -tocopherol is preferentially absorbed, transported and/or retained by tissues.

Pearson and Barnes (5) found the order of uptake from rat intestinal loops to be α - and γ - > β - > δ -. Generally, α -tocopherol is stored in tissues more avidly than γ -, β -, or δ -tocopherol (6). Disappearance curves from the blood of rats indicate that the tocopherols are retained in the order of α - > γ - > β - > δ - (7). Peake and Bieri (8) demonstrated that although rat tissues take up α - and γ -tocopherol to approximately the same extent, there is a more rapid dis-

appearance of γ -tocopherol.

Behrens and Madere (9) present exhaustive data in the rat suggesting that the mechanisms regulating the absorption, transport and tissue uptake of vitamin E are so specific for α -tocopherol, that relatively small amounts will displace γ -tocopherol. A similar effect in humans could account for the observation of lower plasma γ -tocopherol levels in spite of high dietary intakes.

In the present study, the relationship of α - to γ -tocopherol in plasma was evaluated in

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a cross-sectional study of an elderly population. In addition, the effect of supplementation of α -tocopherol on plasma levels of α -, γ - and β -tocopherol was investigated.

METHODS

Two separate groups of people were studied in the course of this investigation. The first was a group of 86 elderly subjects from the Boston, MA area (49 women and 37 men, all >50 yr of age, mean age 71.8 ± 6.4 yr). This group was undergoing nutritional assessment as part of an epidemiological study. Blood samples were collected at a single time point, and this set of data is referred to as the cross-sectional study group.

The second study group consisted of eight healthy adult middle-aged male subjects (aged 30–60 yr) selected from a general medical practice in Nutley, NJ. These subjects underwent a 2-mo regimen of oral supplementation with all-*rac*- α -tocopherol, 3×400 IU daily, with each meal. These subjects were instructed to adhere to their customary diet throughout the period of the study, and to abstain from nutritional supplements during this period. Blood samples were collected from this group before and after the 2-mo period of supplementation. This set of data is referred to as the α -tocopherol supplementation study group.

Blood samples were collected from the antecubital vein into a heparinized tube. Plasma was separated, stored at -80°C , and shipped on dry ice to Santa Cruz, CA, for analysis.

Tocopherols were analyzed with reverse-phase high performance liquid chromatography (HPLC) by a method adapted from Hatam and Kayden (4). Tocopherols were also analyzed with normal-phase HPLC on silica by a method adapted from Tangney et al. (10).

Distilled water was deionized, passed over charcoal, and filtered through a $0.45\text{-}\mu\text{m}$ Millipore filter (Millipore Corp., Bedford, MA). Ethanol was 200 proof, USP grade (Publicker Industries, Linfield, PA), and redistilled. Other solvents were redistilled, reagent grade.

Plasma extraction was carried out under subdued incandescent light. For extraction

of tocopherols, $50\ \mu\text{l}$ of plasma and $150\ \mu\text{l}$ of distilled water were mixed in a 4-ml vial (Kimble, No. 60910-L, Kimble Div., Owens-Illinois Inc., Toledo, OH) with a Teflon-lined cap (Thomas No. 2390-H22, Arthur H. Thomas Co., Philadelphia, PA). Redistilled ethanol ($200\ \mu\text{l}$) was added, and the sample was mixed vigorously for 60 s with a Vari-Whirl vortexer (VWR, San Francisco, CA). One milliliter of hexane, containing $0.5\ \mu\text{g}$ of tocol for internal standard, was added, and the sample was mixed vigorously for 3 min in a special clamped holder. This vigorous mixing with a vortex apparatus is necessary for complete extraction of tocopherols from plasma samples that have been frozen and thawed. The sample was centrifuged at $2000 \times g$ for 1 min, and the supernatant was filtered through a $0.2\text{-}\mu\text{m}$ Teflon filter in a Swinex adapter (Millipore Corp.). The hexane was evaporated under nitrogen at 40°C , and the residue was dissolved in $40\ \mu\text{l}$ of methanol for reverse-phase HPLC. For normal-phase HPLC, the residue was dissolved in $20\ \mu\text{l}$ of hexane.

The HPLC consisted of an Altex 110 pump, Altex 210 injection valve (Altex Scientific, div. Beckman Instruments, Berkeley, CA) with $20\text{-}\mu\text{l}$ injection loop (except for normal-phase chromatography, where a $10\text{-}\mu\text{l}$ loop was used), Perkin-Elmer 650-LC fluorescence detector (Perkin-Elmer Corp., Norwalk, CT) with $20\ \mu\text{l}$ flow cell, Spectra-Physics Minigrator integrator (Spectra Physics, Mountain View, CA) and Hewlett-Packard 7132 chart recorder. The fluorescence detector was set at excitation 295 nm, 10 nm slit, emission 340 nm, 20 nm slit. A LiChrosorb $10\text{-}\mu\text{-C18}$ column, 25×0.46 cm (Altex Scientific), with precolumn, was used for reverse-phase separation, eluting with 97% methanol/3% H_2O at 2.0 ml/min. An Alltech Solvent Miser $5\ \mu$ silica column, $25\ \text{cm} \times 0.21\ \text{cm}$ (Alltech Associates, Deerfield, IL), with precolumn, was used for the normal-phase separations, eluting with 99.75% hexane/0.25% isopropanol at 0.8 ml/min.

Peak height ratios between unknowns and internal standards were used for quantitation. The integrator was used to monitor retention times of unknowns versus standards and to ensure day-to-day stability of the chromatographic system.

Linearity of the method was confirmed over the range of 0.01–2.0 μg α -tocopherol per run. Calibration on a day-to-day basis was done with a secondary standard plasma pool. Numerous aliquots of this pool were prepared and frozen at -80°C . The pool was calibrated against primary standards of α - and γ -tocopherol. A fresh aliquot of the plasma pool was used for each day's work. The day-to-day precision of measurement was equal to or better than $\pm 2\%$.

α -Tocopherol was obtained from Sigma Chemical Co. (St. Louis, MO), and γ -tocopherol was from Eastman Kodak (Rochester, NY). Tocol was kindly supplied by Hoffmann-La Roche, Nutley, NJ. The concentration of each standard was determined by using a molar extinction coefficient (in hexane) of 3170 for α -tocopherol at 292 nm (11), and 3870 for γ -tocopherol at 298 nm (11). The tocol solution was made by direct weight of the material.

β -Tocopherol was isolated from wheat germ oil (Viobin, Monticello, IL) and used for calibration for the normal-phase HPLC. One gram of wheat germ oil was saponified at 70°C in ethanol with 3% KOH and 1% ascorbic acid. The hexane extract was purified on the normal-phase column. The purified β -tocopherol ran as a single peak on normal phase, and its UV spectrum was identical to published spectra (11). The β -tocopherol was quantified by its absorbance at 297 nm, with a molar extinction (in hexane) of 3650 (11).

Data from the cross-sectional study of α -tocopherol and γ -tocopherol levels was analyzed with Pearson's product-moment correlation (12). Data from the longitudinal dosing study was analyzed by the paired t -test (12).

RESULTS

In a nutritional assessment of elderly subjects in Boston, MA, we noticed a marked association between elevated plasma α -tocopherol and depressed plasma γ -tocopherol (see tocopherol structures, fig. 1). Figure 2 is a graph of the results from the Boston cross-sectional study group. This study is a tocopherol survey of 86 elderly men and women. The plasma α -tocopherol

and γ -tocopherol levels are inversely correlated, $r = -0.45$ ($P < 0.001$).

The association we observed between elevated plasma α -tocopherol and depressed γ -tocopherol led us to test for a cause-effect relationship in an α -tocopherol supplementation study carried out on a group of middle-aged subjects. Figure 3A shows the reverse-phase HPLC of plasma tocopherols from a typical subject in the supplementation study before α -tocopherol supplementation, and figure 3B shows the plasma tocopherols from the same subject after supplementation. The tocol peak in these chromatograms is the internal standard. The plasma γ -tocopherol is substantially decreased while the plasma α -tocopherol is substantially increased by oral α -tocopherol supplementation.

It has been reported that β -tocopherol and γ -tocopherol elute together on reverse-phase HPLC (4, 13). We attempted their separation on an Ultrasphere C18- 5μ 25×0.46 cm column (Altex Scientific), which developed more than 10,000 theoretical plates in our system. We could not resolve the β -tocopherol and γ -tocopherol by reverse-phase HPLC even with this high resolution column. It was important to know how large a contribution the β -tocopherol made to the predominantly γ -tocopherol peak observed on reverse-phase HPLC and whether the effect of α -tocopherol supplementation showed any selectivity for β -tocopherol or γ -tocopherol.

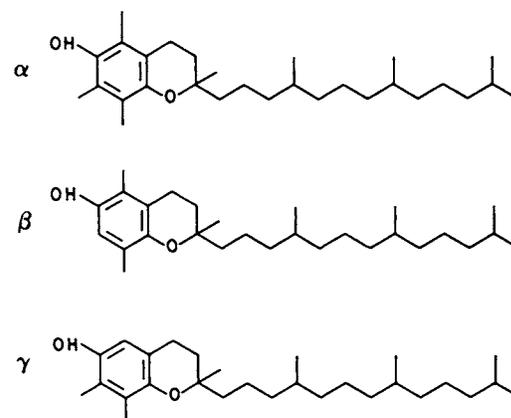


Fig. 1 Structures of α -, β - and γ -tocopherol.

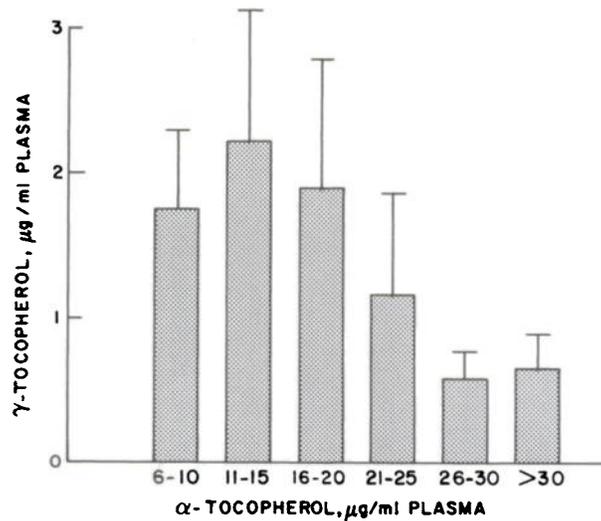


Fig. 2 Relation between plasma α - and γ -tocopherol levels observed in 86 elderly subjects, in Boston, MA, 1981-1982. γ -Tocopherol is given as mean, T-bars, \pm SD.

Therefore, we analyzed the tocopherols by normal-phase HPLC, which is a well-established method for resolving all the tocopherols (10, 14).

The normal-phase chromatograms of plasma tocopherols presented in figure 4 are from the same samples shown in figure 3

(reverse-phase). Figure 4A is before supplementation and figure 4B is after supplementation. The plasma β -tocopherol was about 10% of the γ -tocopherol.

Several points are important in comparing the reverse-phase and normal-phase chromatograms:

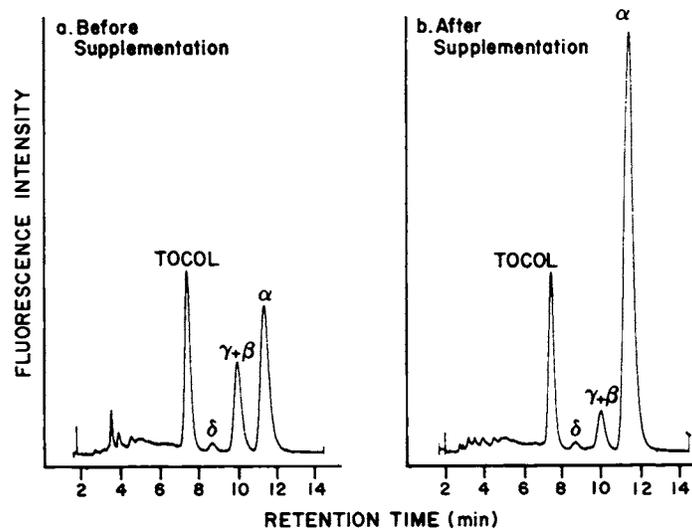


Fig. 3 Reverse-phase HPLC analysis of tocopherols in plasma of subject 5, before and after α -tocopherol supplementation. Column: C18 LiChrosorb, 10 μ , 25 \times 0.46 cm. Mobile phase, 97% MeOH/3% H₂O, 2.0 ml/min. Fluorescence detection, excitation 295 nm, emission 340 nm.

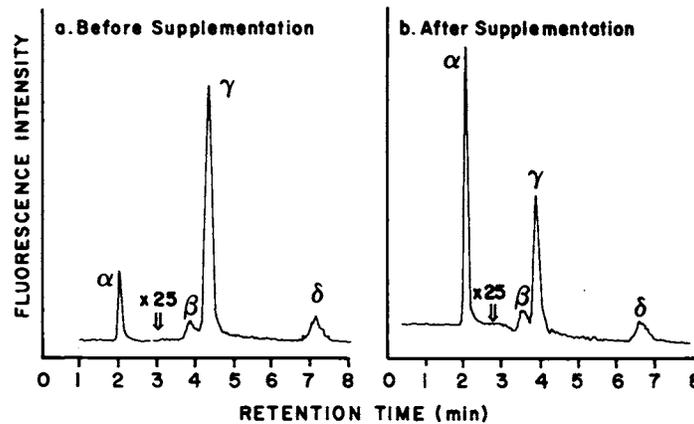


Fig. 4 Normal-phase HPLC analysis of plasma tocopherols from the same subject as in fig. 3. Column: Alltech Solvent Miser silica, 5 μ , 25 \times 0.21 cm. Mobile phase: 99.75% hexane, 0.25% isopropanol, 0.8 ml/min. Fluorescence detection, as figure 3. Both γ - and β -tocopherol are resolved with this technique.

1. The fluorescent yield of γ -tocopherol in methanol mobile phase is about four times that of α -tocopherol (4) (varying somewhat with the water content of the mobile phase, and the wavelength settings of the fluorescence detector). In hexane mobile phase, the fluorescent yield of γ -tocopherol is about one-and-one-half times that of α -tocopherol.

2. The tocopherols elute in the opposite order in normal phase and in reverse phase. Later peaks are wider and lower than earlier peaks.

3. The chart expansion for the normal-phase chromatogram is increased 25-fold for the β - and γ -peaks, to allow accurate quantitation of these two tocopherols.

Table 1 summarizes the normal-phase results for β - and γ -tocopherol and compares these to the (γ + β)-tocopherol peak in reverse phase. The sum of the β -tocopherol and γ -tocopherol peaks determined by normal phase is generally similar to the (γ + β)-tocopherol determined by reverse-phase. The β -tocopherol was $9 \pm 3\%$ of the

TABLE 1

Plasma γ - and β -tocopherol values by reverse-phase and normal-phase HPLC before and after α -tocopherol supplementation¹

Subject	Tocopherol values from HPLC							
	Before supplement				After supplement			
	Reverse phase	Normal phase			Reverse phase	Normal phase		
	(γ + β)	γ	β	(γ + β)	(γ + β)	γ	β	(γ + β)
	$\mu\text{g/ml plasma}$							
1	2.07	2.21	0.20	2.41	1.37	1.40	<0.01	1.40
2	1.42	1.20	0.08	1.28	0.45	0.14	0.02	0.16
3	1.91	1.77	0.19	1.96	0.57	0.22	0.07	0.29
4	2.42	2.70	0.27	2.97	0.78	0.72	0.20	0.92
5	3.33	3.30	0.16	3.46	1.43	1.40	0.14	1.54
6	1.96	1.81	0.16	1.97	0.61	0.45	0.10	0.55
7	1.29	1.30	0.16	1.46	0.72	0.50	<0.01	0.50
8	1.89	2.58	0.28	2.86	0.40	0.16	0.08	0.24

¹Supplement: 3 \times 400 IU/day, orally, for 8 wk.

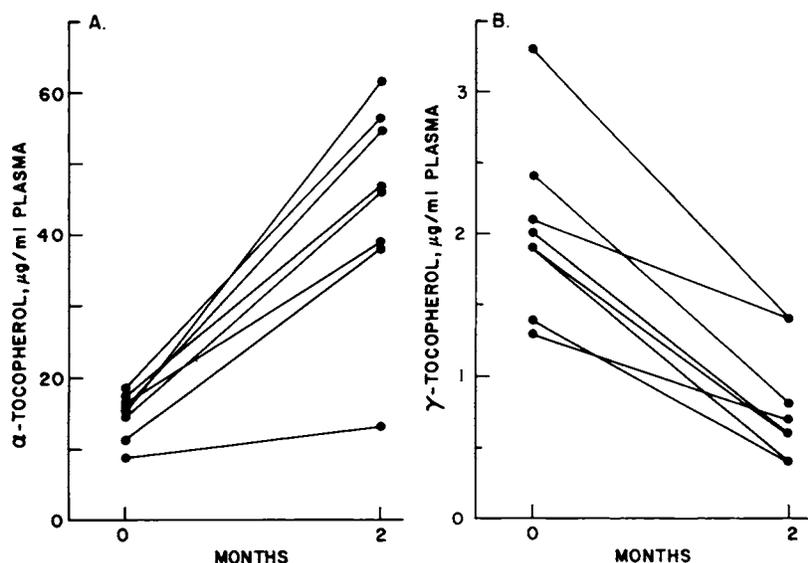


Fig. 5 Individual values of plasma α - and γ -tocopherol for each subject, before and after oral α -tocopherol supplementation.

γ -tocopherol before supplementation which is in good agreement with other published values as determined by HPLC (2, 3). The β -tocopherol was $20 \pm 6\%$ of the γ -tocopherol after the supplementation. For most purposes the reverse phase ($\gamma + \beta$)-tocopherol peak provides an adequate estimation of γ -tocopherol. This is a useful conclusion for many studies since reverse phase is much more convenient to use than normal phase.

Figure 5 graphically presents the levels of plasma α -tocopherol (fig. 5a) and ($\gamma + \beta$)-tocopherol (fig. 5b, shown as γ -tocopherol) before and after the period of α -tocopherol supplementation. The α -tocopherol concentration was $13.4 \pm 4.0 \mu\text{g/ml}$ plasma (mean \pm SD) before supplementation and $43.5 \pm 18.0 \mu\text{g/ml}$ after supplementation. The ($\gamma + \beta$)-tocopherol was $2.04 \pm 0.63 \mu\text{g/ml}$ plasma before α -tocopherol supplementation and $0.79 \pm 0.39 \mu\text{g/ml}$ after supplementation. The changes in both α -tocopherol and ($\gamma + \beta$)-tocopherol were significant at $P < 0.001$ (paired t -test).

DISCUSSION

Normal-phase HPLC shows that the level of β -tocopherol is negligible relative to γ - and α -tocopherol unless γ -tocopherol levels

are greatly depressed by high α -tocopherol intake. Reverse-phase HPLC, with a high sensitivity fluorescence detector,² has many advantages for the study of α -tocopherol- γ -tocopherol interactions (so long as characterization of the minor component β -tocopherol is not required). Fluorescent detection provides excellent sensitivity for tocopherols. Reverse-phase systems benefit from superior day-to-day stability and predictable chromatographic behavior, long column life, and low mobile phase flammability hazard, when compared to normal phase. Finally, in methanol mobile phase, the fluorescence yield of γ -tocopherol is substantially enhanced (4), further improving the sensitivity for the detection of γ -tocopherol, which is especially important when plasma γ -tocopherol is suppressed to low levels.

Competition for intestinal absorption is observed among many fat-soluble nutrients (reviewed by Weber, ref. 15). Our data suggest to us the hypothesis that α -tocopherol and γ -tocopherol compete for a common uptake mechanism. The American diet is an abundant source of γ -tocopherol, and we

²The Perkin-Elmer 650-LC (Norwalk, CT) and the Schoeffel FS-970 (Westwood, NJ) are suitable fluorescence detectors.

have shown that large oral doses of α -tocopherol reduce the uptake of γ -tocopherol. Elevated α -tocopherol also decreases the efficiency of α -tocopherol absorption, and plasma and tissue levels increase approximately as the log of the oral dose in animal studies (16). Fecal balance studies in humans indicate that increased α -tocopherol intake decreases the efficiency of uptake from the intestine (17).

Kitabchi and Wimalasena (18) investigated the binding of α - and γ -tocopherol to human erythrocyte membranes, and found that the erythrocyte membrane receptors had much higher affinity for α -tocopherol than for γ -tocopherol. The relative affinities of the different tocopherols for plasma lipoproteins and membranes from other cell types should be investigated.

The depression in γ -tocopherol evidently can occur during large dietary α -tocopherol intake even without a large absolute increase in α -tocopherol in the plasma: subject 6 showed a rather small α -tocopherol increase from 8.7 to 13.8 $\mu\text{g/ml}$, but a large γ -tocopherol decrease from 1.96 to 0.61 $\mu\text{g/ml}$. Thus, a decrease in γ -tocopherol and particularly in the ratio of γ - to α -tocopherol may provide a much more reliable index of compliance to α -tocopherol supplementation than simply measuring total α -tocopherol or total tocopherols.

To adequately determine the usefulness and sensitivity of plasma γ -tocopherol for monitoring compliance to α -tocopherol supplementation it would be useful to know the kinetics of plasma γ -tocopherol decrease or increase after starting or discontinuing α -tocopherol supplementation, and the magnitude of the decrease in plasma γ -tocopherol levels obtained with various dosage levels of α -tocopherol.

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