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Gas chromatography–mass spectrometry microanalysis of alpha- and gamma-tocopherol in plasma and whole blood

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ABSTRACT

Background: Assessing vitamin E status in humans is critical for nutritional evaluation and verification of clinical and biological compliance of supplemented subjects. An accurate analytical method for measuring the two main vitamin E isoforms, i.e. α- and γ-tocopherol (α- and γ-TOH) in small volumes of plasma can facilitate the application of this analysis to clinical trials and in situations where a limited amount of sample is available.
Methods: We have developed a micro method, which uses only 5 μL plasma, based on isotope dilution, trimethylsilation and GC–MS. The method was validated according to the guidelines of the International Conference on Harmonization of analytical procedures. The method was also applied to 5 μL of whole blood for the potential use in conditions were the availability of specimens is limited.
Results: Accurate quantitation of α-TOH and γ-TOH was achieved at levels ≥0.417 μM and ≥0.007 μM, respectively. Within-day coefficient of variation was 1.31% and 4.70% for α-TOH and γ-TOH, respectively. Between-day coefficient of variation was 1.32% and 2.88% for α-TOH and γ-TOH, respectively. Recovery, assessed at three concentration levels, ranged 98–103% and 100–102% for α-TOH and γ-TOH, respectively. The method allowed the detection of α-TOH and γ-TOH in 5 μL whole blood and in membranes of red blood cells washed from 5 μL of blood as well.
The analytical performance was assessed in plasma from a cohort of Italian healthy subjects ($n = 205$). The mean plasma concentrations were 28.01 ± 6.31 and 0.68 ± 0.48 μM (mean ± SD) for α-TOH and γ-TOH, respectively. Alpha-TOH correlated with total cholesterol ($r = 0.617$, $p < 0.0001$) and triglycerides ($r = 0.420$, $p < 0.0001$) while γ-TOH correlated modestly with total cholesterol ($r = 0.213$, $p < 0.0001$) but not with triglycerides. γ-TOH, but not α-TOH, was significantly lower in smokers than in non-smokers (0.72 ± 0.50 vs. 0.56 ± 0.37, μM, mean ± SD, $p = 0.017$). Given the high sensitivity, the method allowed to be applied to 5 μM whole blood without specific modification.
Conclusions: This micro-method represents an analytical advancement in α- and γ-TOH assay that is available to accurately verify the nutritional status and compliance after supplementation in large-scale settings, and to measure the two vitamers in conditions where sample availability is limited.

1. Introduction

The term "vitamin E" includes four naturally occurring vitamers with close structural homology, identified with the Greek letters α, β, γ, and δ. These vitamers differ from each other in the number and position of methyl groups in the chroman ring. The four isomers (Fig. 1) are present in biological fluids in a wide relative concentration, having an approximate rank order of α ≫ γ ≫ β ≈ δ [1].

From the time when Evans and Bishop first identified an unknown factor associated with animal fertility in plant organisms [2], and even more after the identification of a role as fat-soluble antioxidant, a number of favorable biological properties have been found in support of beneficial effects of vitamin E on human health [3–5]. As a consequence, and with the support of several observational and nutritional epidemiology reports, the main form of vitamin E, i.e. α-tocopherol (α-TOH), has been investigated in primary and secondary prevention trials of chronic and age-related conditions and particularly in coronary heart disease (CHD) [5]. Disappointing results, however, have been obtained in the majority of these attempts [6]. Despite this, the interest in vitamin E to prevent chronic diseases remains intense and several groups are investigating methodological and clinical aspects that may explain disappointing clinical findings so far reported in vitamin E

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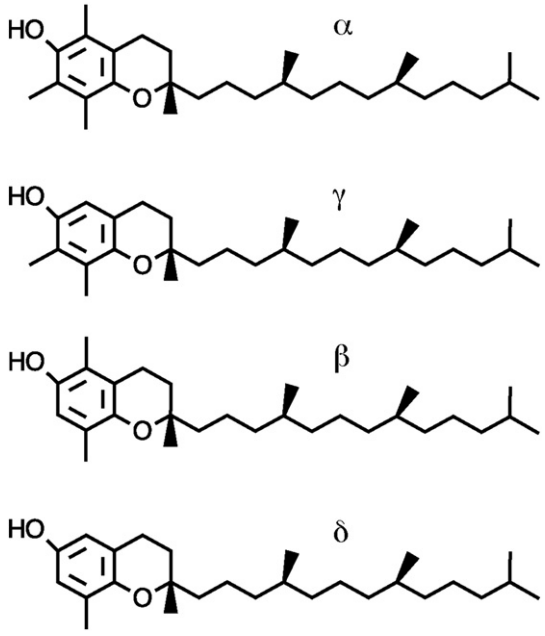

Fig. 1. Structure of tocopherol isoforms.

therapy of CHD and oxidative stress-related diseases [5,7]. Apart from formulation and dosage of vitamin E used as a supplement and patient selection criteria, the main bias present in clinical studies was the absence of verification for the compliance to the nutritional intervention [7], which can be accurately assessed only by laboratory methods. Vitamin E status in the study population should be appropriately performed by serial blood profiling of vitamin E starting at enrollment and up to clinical endpoint verification [7]. In addition most studies focused on α-TOH, the most abundant form in blood and tissues. Its concentration in plasma is in the low micromolar level, usually ranging 15–35 μM, and reaching steady state concentrations of 60–90 μM after chronic supplementation [8]. Gamma-TOH, whose plasma concentrations are almost one order of magnitude lower than α-TOH [1], has been tested in few studies notwithstanding its structure carries potential, potent, physiological actions not covered by α-TOH [6,9], including peroxynitrite-dependent damage [3]. In fact, γ-TOH has also been associated with the risk of coronary heart disease [10,11] and prostate cancer [12]. γ-TOH levels in blood being one order of magnitude lower than that of α-TOH, the simultaneous determination of both vitamers represents an analytical challenge that can be accomplished only with the support of specialized laboratories. Analytical methods for vitamin E and its metabolites can be performed by HPLC coupled with UV, fluorescence and electrochemical detectors, and more recently with mass spectrometric detection [13–16]. HPLC with UV detection is the common method for measurement of vitamin E [16] and fluorescence and ECD detection are not recommended in the last guidelines [17].

While α-TOH analysis is straightforward, assessing minor forms can be challenging even in experienced labs and there is a paucity of data regarding the reference concentration of γ-TOH in plasma [8], which may result from either analytical or biological reasons. Indeed, poor sensitivity and differential response of detection methods, usually tailored to α-TOH analysis (i.e. UV detectors that are often used in routine HPLC analyses or some mass spectrometry detectors), can limit the precision of γ-TOH determination in plasma. In addition, reference values are influenced by the geographic distribution of study populations that differs as regards to dietary patterns and other so far unexplored aspects that may include genetic and metabolic factors. Gamma-TOH intake in the US and Asian populations is notoriously higher than that of European subjects, thus leading to markedly different blood and tissue levels of this form of vitamin E [18]. Gamma-tocopherol in the diet greatly influences its plasma levels [19] and, on the other hand, sustained intake of α-TOH by supplements can increase metabolic processing of γ-TOH [9] potentially lowering its blood level to the limit of detection of most analytical procedures.

Herein, we report on the simultaneous quantitative determination of α-TOH and γ-TOH in a very small volume of plasma, 5 μL. The analytical development proposed in this study provides a high throughput and a sensitive analysis platform to assess vitamin E status in routine nutritional monitoring and in large-population studies. Structural similarity combined with very different concentrations of the two main vitamers in plasma are the analytical limits addressed in this GC/MS micro-method for vitamin E analysis in biological samples.

2. Materials and methods

2.1. Chemicals and analytical standards

(2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydro-2H-1-benzopyran-6-ol (α-TOH) and 2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-1-benzopyran-6-ol (γ-TOH) were obtained from Sigma-Aldrich (Milan, Italy). [$^2H_3$]α-TOH was from (Cognis Deutschland GmbH, Germany), [$^2H_2$]γ-TOH was synthesized as previously described [20]. Sylon (hexamethyldisilazane, trimethylchlorosilane, pyridine, (HTP, 3:1:9) from Supelcho (Bellefonte, PA)). Organic solvents and other reagents were of the highest grade available by Sigma-Aldrich (Milan, Italy).

2.2. Blood samples

To validate the method, assays were carried out in pooled samples of plasma. Specimens were obtained as an aliquot of blood samples collected for the routine clinical chemistry. Samplings were carried out in vacutainer tubes (Beckton Dickinson, Milan, Italy), between 8:00 and 9:00 a.m. after fasting overnight. Serum or EDTA-anticoagulated plasma were prepared immediately after blood drawings and were stored at −80 °C until assay. To assess α-TOH and γ-TOH concentrations in plasma of healthy subjects, plasma specimens were obtained from volunteers of the AVIS blood donors association at Latina (Italy). Exclusion criteria were age > 70 years and the use of antioxidant supplements for at least 3 months before blood donation. The study procedure was developed according to the guidelines of the Ethics Committee, which approved the protocol, and the Helsinki Declaration of 1975 as revised in 2008. All subjects signed written informed consent.

2.3. Preparation of standard curves and plasma assay

Tocopherol standard curves were prepared by the internal standard ratio method.

Stock solutions (250 μM) of α-TOH and γ-TOH in ethanol were accurately prepared by spectrophotometric calibration using $^{M}\varepsilon_{292} = 3260$ and $^{M}\varepsilon_{298} = 3810$ for α-TOH and γ-TOH, respectively [1].

The stock solution was used to prepare a series of standard ethanolic solutions for α-TOH (10–250 μM) and for γ-TOH (0.250–5 μM), which also contained a fixed amount of the respective deuterated isotopomer, i.e. 400 ng [$^2H_3$]α-TOH and 5 ng [$^2H_2$]γ-TOH. The standards were then derivatized, as follows. The solvent was evaporated under a stream of N_2, 130 μL derivatizing solution (hexamethyldisilazane, trimethyldichlorosilane, pyridine; 3:1:9) added and incubated for 30 min at 60 °C. At the end of incubation time, the solvent was evaporated under a stream of N_2, the derivatized compounds taken with 50 μL hexane and transferred to an autosampler vial.

For plasma vitamin E assay, plasma (5 μL) was mixed with 10 μL [$^2H_3$]α-TOH (4 ng/mL in ethanol), 5 μL [$^2H_2$]γ-TOH (1 ng/mL in ethanol), 100 μL ethanol, and 95 μL saline. Next, one mL hexane was added and the sample was extensively vortexed and then centrifuged