J Basic Clin Physiol Pharmacol 2012;23(1):45–48 © 2012 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/jbcpp-2011-0033

A modified simple method for determination of serum α-tocopherol (vitamin E)

Jameel G. Jargar[1], Shaheenkousar H. Hattiwale[1], Swastika Das[2], Salim A. Dhundasi[1] and Kusal K. Das[1,*]

[1] Department of Physiology, Environmental Health Research Unit, Al-Ameen Medical College, Bijapur, Karnataka, India

[2] Department of Engineering Chemistry, B.L.D.E.A's V.P. Dr. P.G. Halakatti College of Engineering and Technology, Bijapur, Karnataka, India

Abstract

Background: Vitamin E is one of the important antioxidants linked to regulate various diseases, such as atherosclerosis, hypertension, and male infertility. A relatively simple and economic biochemical modified method has been developed to determine serum α-tocopherol concentration.

Methods: The current modified method is based on previous Baker and Frank method and the method of Martinek by using 2,2′-bipyridyl, ferric chloride, and xylene. The complex of ferrous ions generated in this reaction with 2,2′-bipyridyl is determined by using a plain enzyme-linked immunosorbent assay microplate (non-antibody coated) at 492 nm.

Results: The standard curve of this new modified method shows a linearity with correlation r=0.997 (concentration vs. absorbance). The absorbance of this color complex is directly proportional to the α-tocopherol concentration. The sensitivity of this new modified method has been compared and correlated with Baker and Frank method by using 15 human samples (r=0.99, p<0.0001).

Conclusions: This simple and economic method may be routinely used to analyze α-tocopherol concentration in serum.

Keywords: data analysis; modified method; serum α-tocopherol assay.

Introduction

Vitamin E proved to be effective in preventing lipid peroxidation (LPO) and other radical-driven oxidative events (1, 2). Vitamin E is classified into eight forms which are referred to as tocopherols. Among these forms, α-tocopherol is found to be the most studied because of its maximum bioavailability (3). Vitamin E functions as a chain-breaking antioxidant which prevents the propagation of free radical reactions (4, 5). Vitamin E is also a recognized exogenous, lipophilic antioxidant of the semen and it plays a key role in male reproduction by protecting sperm membrane polyunsaturated fatty acid against oxidation by reactive oxygen species (6). New evidences suggest that α-tocopherol suppresses LPO in sperm as assessed by reduction of malondialdehyde concentration and improvement in sperm motility (7). Vitamin E has been related to reduction in cardiovascular disease risk (8), diabetic complications (9), certain cancers (10), and cataracts (11).

In view of its clinical importance, estimation of serum vitamin E concentration becomes necessary to evaluate the blood antioxidant status. Hence, several biochemical and assay methods to estimate this antioxidant vitamin are developed in this direction. However, certain drawbacks and difficulties are observed among the present available methods to estimate serum vitamin E concentration. Some methods need costly chemicals, kits, and instruments, such as high-performance liquid chromatography (12, 13), whereas some methods are very lengthy or time consuming and tedious, such as enzyme-linked immunosorbent assay (ELISA) kit methods (14), paper chromatography methods (15), or thin layer chromatography methods (16). Some methods need a large quantity of serum (17, 18) which may be a hindrance for estimating rat or mice serum vitamin E, as the quantity of rats or mice serum collected is relatively less. Some methods are also found to have lower sensitivity and accuracy (19) or need correction factor calculations (17, 19). Keeping these in mind, a suitable, less tedious, economic, rapid, and new micro method, which utilizes a small quantity of serum, has been investigated and described in this research article to determine α-tocopherol (vitamin E) in serum.

Materials and methods

All glasswares required for this experiment were cleaned with 0.1 N HCl followed by distilled water and kept in an oven to dry overnight. DL α-tocopherol acetate (Merck, Mumbai, India), ethanol (aldehyde free) (Merck, Darmstadt, Germany), xylene (extra pure) (Merck, Mumbai, India), 2,2′-bipyridyl (Loba Chemie, Mumbai, India), n-propanol (Merck, Mumbai, India), ferric chloride (Qualigens, Mumbai, India), and triple distilled water were used in this experiment.

Preparation of reagents

Stock standard of α-tocopherol (0.27%, w/v): 270 mg of α-tocopherol acetate diluted in 100 mL ethanol (aldehyde free) and mixed thoroughly.

*Corresponding author: Prof. Kusal K. Das, Department of Physiology, Environmental Health Research Unit, Al-Ameen Medical College, Bijapur-586108, Karnataka, India
Phone: (W) +91-8352-272502, +91-8352-271257,
Fax: +91-8352-270184, E-mail: kusaldas@yahoo.com
Received October 14, 2011; accepted December 10, 2011; previously published online January 19, 2012

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2,2′-Bipyridyl (0.12%, w/v): 120 mg 2,2′-bipyridyl dissolved and made the volume up to 100 mL with n-propanol and kept in a brown bottle. Ferric chloride (0.12%, w/v): 120 mg $FeCl_3 \cdot 6H_2O$ dissolved in 100 mL ethanol (aldehyde free) and also kept in a brown bottle. All these solutions are stable at room temperature (24°C±2°C).

Preparation of standard curve

Working standard of α-tocopherol (27 µg/mL): 1 mL of stock standard solution was taken and made the volume up to 100 mL with ethanol (aldehyde free) to obtain a concentration of 27 µg/mL. This solution is stable at room temperature.

Six centrifuge tubes were taken and labeled as B (blank) and S_1, S_2, S_3, S_4, and S_5 for standard solution. In these tubes, 0 (zero), 150, 300, 450, 600, and 750 µL of working standard solution (27 µg/mL) were added, respectively, and made the volume up to 750 µL by adding ethanol (aldehyde free). These solutions (S_1–S_5) are equivalent to 4, 8, 12, 14, 16, and 20 µg/mL of α-tocopherol concentration, respectively. The entire protocol is shown in Table 1. The absorbance was measured by using 200 µL of each of the above-mentioned solutions (including blank) put on a plain ELISA microplate (non-antibody coated) and read in an ELISA reader (ERBA-Lisa Scan II) at 492 nm. A standard curve was plotted with absorbance vs. α-tocopherol (µg/mL) concentration. A typical standard curve is shown in Figure 1.

Analysis of α-tocopherol in serum

Fifteen serum samples from healthy volunteers (age 25–60 years) were obtained after collecting informed consent from them. We performed two sets of experiments, i.e., Baker and Frank method (17) and our current experimental method for confirmation of validity.

Blood samples were collected in plain centrifuge tubes without using any anticoagulants. The tubes were centrifuged at 3000 revolutions per minute (rpm) for 15 min to get the serum. Serum for the analysis of α-tocopherol should be protected from sunlight as α-tocopherol darkens on exposure to light and is slowly oxidized by atmospheric oxygen. α-Tocopherol was found to be stable in separated serum at 25°C for 1 day, at 4°C for 2 weeks, and at –20°C for 2 months (19). Special precaution was taken to prevent hemolysis as it may influence the result. The following steps were followed.

Step 1 Centrifuge tubes labeled as T and B (i.e., sample/test and blank) were taken. Ethanol (aldehyde free) (750 µL) and serum (750 µL) were added in these sample tubes. Serum should be added slowly with mild shaking to obtain a finely divided protein precipitate. The blank was prepared by adding 750 µL of distilled water and 750 µL of ethanol (aldehyde free). All the tubes were covered tightly by wrap paper and shaken vigorously for at least 30 s. Xylene (750 µL) was then added in all these tubes. Furthermore, all the tubes were covered tightly by wrap paper and shaken vigorously for at least another 30 s and then centrifuged for 10 min at 3000 rpm.

Step 2 Xylene layer (supernatant) (500 µL) was transferred into properly labeled, clean, and small-size test tubes (5 mL volume). To each tube, 500 µL of 2,2′-bipyridyl solution was added followed by 100 µL of ferric chloride solution and waited for 2 min.

Step 3 The solutions (200 µL) from each of these tubes were transferred to a plain ELISA microplate (non-antibody coated). The primary wavelength was set at 492 nm and the absorbance of all the samples (including blank) were measured. The ELISA reader was set in ‘rapid measure’ mode. The serum α-tocopherol concentration of each of the sample was obtained by using the standard curve prepared earlier (Figure 1). These concentrations of serum α-tocopherol (vitamin E) estimated by our current experimental method were

Table 1 Preparation of standard curve by our modified method.

SN	Working standard, µL		Distilled water, µL	Xylene, µL		Xylene layer, µL	2,2′-Bipyridyl, µL	Ferric chloride, µL		Absorbance in ELISA reader at 492 nm	Concentration of α-tocopherol, µg/mL
	α-Tocopherol	Ethanol (aldehyde free)									
B	0	750	750	750		500	500	100		0.442	0
S_1	150	600	750	750		500	500	100		0.474	4
S_2	300	450	750	750	Centrifuge for	500	500	100	After 2 min	0.511	8
S_3	450	300	750	750	10 min at	500	500	100	absorbance at	0.536	12
S_4	600	150	750	750	3000 rpm	500	500	100	492 nm	0.571	16
S_5	750	0	750	750		500	500	100		0.606	20

B, blank; S_1–S_5, solutions of α-tocopherol with different concentrations; SN, serial numbers.

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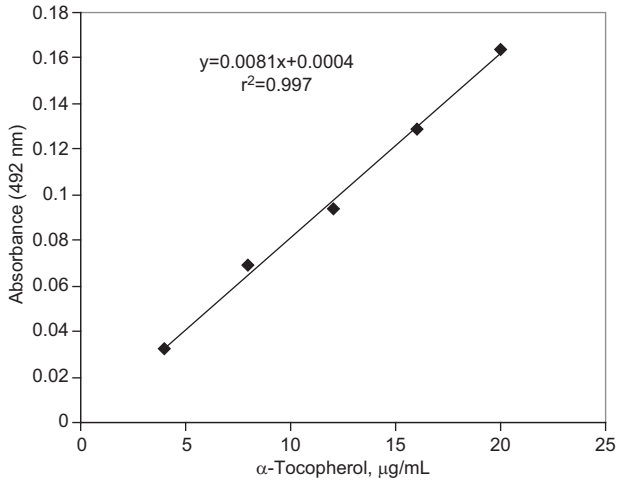

Figure 1 Standard curve of α-tocopherol (vitamin E) at 492 nm by our modified method.

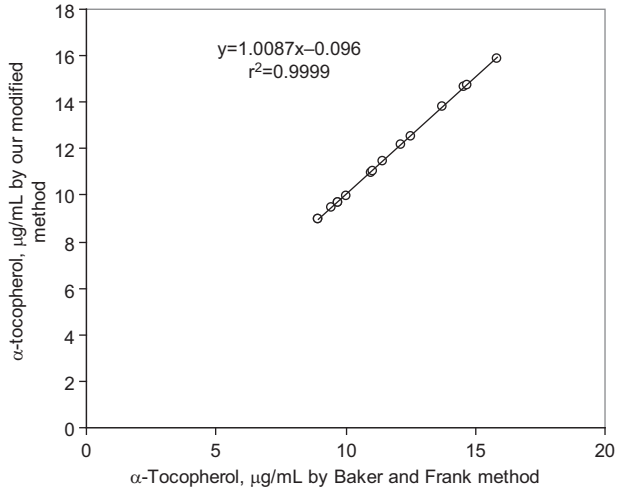

Figure 2 Correlation between Baker and Frank method and our modified method.
Correlation (r) is 0.99, n=15, p<0.0001.

compared and analyzed with Baker and Frank method (17) (Table 2). The percent change difference between our method and Baker and Frank method was calculated (Table 2) and the correlation between them was also evaluated (Figure 2). The mean±SD values of the serum α-tocopherol of both methods were compared using Student's unpaired t-test.

The entire research protocol was approved by the Institutional Ethical Committee as per the guidelines of Declaration of Helsinki (1964) (20).

Results and discussion

Table 1 shows the detailed procedure of preparation of α-tocopherol standard curve and reading of absorbance in the ELISA reader. Figure 1 depicts a typical standard curve of α-tocopherol at 492 nm. The standard curve has been plotted against the concentration of α-tocopherol (μg/mL) with the absorbance at 492 nm. The curve clearly shows its linearity with correlation value of r at 0.997. Based on this standard curve, the α-tocopherol concentration in serum has been calculated for 15 samples and compared with Baker and Frank method by using a spectrophotometer. The percent change difference between these two methods in each of the samples has also been noted down. Results clearly show very negligible percent change difference between the individual results of both methods (Table 2). An unpaired Student's t-test between the two methods also shows a non-significant statistical difference. A significant correlation (r=0.99; p<0.00001) between the method of Baker and Frank and our modified

Table 2 A comparative analysis of α-tocopherol in serum.

Sample	(I) Baker and Frank method by using spectrophotometer at 460 and 520 nm α-Tocopherol[a] (μg/mL)	(II) Our modified method by using ELISA reader at 492 nm α-Tocopherol (μg/mL)	Percent change difference (I vs. II)
T_1	13.71	13.77	0.43
T_2	12.16	12.17	0.08
T_3	10.96	10.94	0.27
T_4	14.60	14.64	0.27
T_5	8.95	8.96	0.11
T_6	15.86	15.87	0.06
T_7	10.00	9.95	0.05
T_8	14.72	14.76	0.27
T_9	9.71	9.70	0.10
T_{10}	9.48	9.46	0.21
T_{11}	8.95	8.96	0.11
T_{12}	11.04	11.06	0.18
T_{13}	11.45	11.43	0.17
T_{14}	9.74	9.70	0.41
T_{15}	12.50	12.54	0.32
Mean±SD	11.58±2.25	11.59±2.28	

t-value=0.0112; SE difference=0.830 (non-significant). [a]As per Baker and Frank method, the calculation is serum tocopherol (μg/mL)=(A' of unknown/A' of standard)×10, where $A'=A_{520}-0.29\times A_{460}$.

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method has also been observed, which reflects the validity of our current modified method.

In our modified method, the blank, standard, and sample followed a uniform procedure; hence, time-dependent alteration of color development remained the same. The use of 2,2′-bipyridyl and xylene in our modified method clearly reflects distinct advantage as these chemicals have lower cost but with equal sensitivity with other equivalent chemicals, such as 2,4,6-tripyridyl-s-triazine and hexane. The mean value of α-tocopherol (μg/mL) concentration in 15 samples was found to be 11.58±2.23, which is very much within the normal range (8–15 μg/mL) of α-tocopherol (vitamin E) concentration in humans (19).

Hence, our modified method may be considered as a rapid diagnostic test to estimate serum vitamin E for biochemical analysis.

Acknowledgments

The authors greatly acknowledge the Director of the Defence Institute of Physiology and Allied Sciences, Delhi, Defence Research and Development Organisation, Ministry of Defence, Government of India for providing financial assistance to the corresponding author Prof. Kusal K. Das.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

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