

# A SIMPLE MICRO METHOD FOR DETERMINATION OF PLASMA LEVELS OF ALPHA TOCOPHEROL (VITAMIN E) IN PAKISTANI NORMAL ADULTS

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## ABSTRACT

A relatively simple spectrophotometric method has been developed for the determination of  $\alpha$  tocopherol in human plasma. Method is modification of a previous micromethod base on oxidation of  $\alpha$  tocopherol by ferric chloride. The complex of ferrous ions (generated in this reaction) with bathophenanthroline is determined spectrophotometrically at 536 nm. The absorbance of this colored complex is directly proportional to concentration of  $\alpha$  tocopherol. The method involves extraction of  $\alpha$  tocopherol from human plasma using n-hexane which is relatively less toxic than xylene. The assay is sensitive enough to detect as little as 0.2  $\mu\text{g}$  of vitamin. The recovery of  $\alpha$  tocopherol from the plasma using n-hexane was in the range of 75% - 100%. The mean values of intra-assay and interassay coefficient of variation were found to be 5.3% and 13%, respectively. The assay was used to monitor  $\alpha$  tocopherol levels in plasma samples of 81 normal healthy adults. Mean concentration of plasma  $\alpha$  tocopherol in these normal healthy adults was found to be  $9.45 \pm 2.64 \mu\text{g/ml}$ . Sixteen percent of adults had low levels of  $\alpha$  tocopherol. The method is rapid, convenient, reproducible and relatively less hazardous compared to methods using xylene for the extraction of vitamin E. It can be routinely used to analyze as many as 20 plasma samples in about 2 hours time.

**Keywords:**  $\alpha$  tocopherol, vitamin E, spectrophotometric assay, Pakistani adults.

## INTRODUCTION

Therapeutic use of antioxidants has gained considerable interest during the last decade. Vitamin E has received considerable attention in the treatment and/or prevention of human diseases, such as, atherosclerosis, preeclampsia or hypertension (Rodrigo *et al.*, 2007). The most active component of the vitamin E complex is  $\alpha$  tocopherol. This organic substance is one of the most powerful antioxidants in the lipid phase of the human body (Wang and Quinn, 2000). It protects against free radical damage which has been the underlying mechanism of injury in a number of life threatening human diseases, such as atherosclerosis, diabetes, cancer and aging (Bonneyfoy *et al.*, 2002; Huang *et al.*, 2006; Flora, 2007; Munteanu and Zingg, 2007).

Plasma levels of  $\alpha$  tocopherol have been known to decrease in these disease conditions (Bonneyfoy *et al.*, 2002; Haung *et al.*, 2006; Munteanu and Zingg, 2007). To monitor very low levels of vitamin E in biological samples, simple, rapid and reliable micromethods are required for diagnostic and prognostic purposes.

Several procedures based on chemical and biological assay methods have been developed (Nair and Magar, 1955; Kayden *et al.*, 1973; Nirungsan and Thongnopnua, 2006; Demirkaya and Kadioglu, 2007). Some are lacking in sensitivity and specificity, while others require sophisticated and expensive instrumentation (Nirungsan

and Thongnopnua, 2006; Demirkaya and Kadioglu, 2007). The aim of this study is to develop a simple, rapid and inexpensive micro-procedure for the determination of plasma levels of  $\alpha$  tocopherol in Pakistani adults.

## MATERIALS AND METHODS

Absolute ethanol, n-hexane (analytic reagent grade), 4, 7-diphenyl-1,10-phenanthroline (bathophenanthroline), ferric chlorohydrate hexahydrate, orthophosphoric acid (85%) and  $\alpha$  tocopherol were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### *Preparation of standard curve*

A 0.2% solution of  $\alpha$  tocopherol in absolute ethanol is prepared and kept at 4°C. For working solution, 1 ml of stock solution is diluted to 100 ml with purified ethanol to obtain a concentration of 20  $\mu\text{g/ml}$ . This working solution is used to construct a standard curve ranging 0.5  $\mu\text{g}$ -4  $\mu\text{g}$  of  $\alpha$  tocopherol.

### *Procedure*

The determination of  $\alpha$  tocopherol was carried out using a modification of the method by Fabianek *et al.*, (1967). Frozen plasma was thawed and 0.4 ml of this sample was transferred into 12x75 mm glass tube and marked as test sample. For the standard curve, 0.4 ml of each standard solution of  $\alpha$  tocopherol (0.5  $\mu\text{g}$ -4  $\mu\text{g}$ ) and 0.4 ml of distilled water (for blank) were taken in 12x75 mm glass tubes and marked as standard and blank, respectively. An equal volume of (0.4 ml) absolute ethanol was added to the test sample and blank, while 0.4 ml of distilled water

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**Table 1:** Intra-assay precision.

Mean  $\pm$  SD

Sample No.	Concentration of $\alpha$ tocopherol ( $\mu\text{g/ml}$ )	Coefficient of Variation (%)	Replicates (n)
1	5.6 $\pm$ 0.38	6.8	5
2	9.74 $\pm$ .11	1.13	5
3	6.52 $\pm$ 0.52	7.97	5

Mean coefficient of variation = 5.3%

**Table 2:** Inter-assay precision

Sample No.	Alpha tocopherol ( $\mu\text{g/ml}$ ) assayed on day					Mean $\pm$ SD ( $\mu\text{g/ml}$ )	Coefficient of Variation (%)
	1	2	3	4	5		
1	6.8	6.6	5.6	6.6	6.05	6.33 $\pm$ 0.49	7.7
2	11.52	9.74	10.3	8.0	10.9	10.1 $\pm$ 1.34	13
3	6.52	5.7	7.8	9.3	8.4	7.54 $\pm$ 1.44	19

Mean coefficient of variation = 13%

**Table 3:** Cumulative percentage of normal adults with their corresponding values of  $\alpha$  tocopherol.

Alpha tocopherol ( $\mu\text{g/ml}$ )	Frequency (f)	Percentage (%)	Cumulative frequency (%)
0.1 – 2.50	0	-	-
2.51 – 5.00	4	4.9	4.9
5.01 – 7.50	17	21.0	25.9
7.51 – 10.00	26	32.1	58.0
10.01 – 12.50	24	29.6	87.7
12.51 – 15.00	9	11.1	98.8
>15.00	1	1.2	100

Mean $\pm$ SD: 9.45 $\pm$ 2.64  $\mu\text{g/ml}$

was added to the standard samples. The contents were mixed for 30 seconds and 0.4 ml of n-hexane was added to each reaction. The contents were again mixed for 30 seconds and subjected to centrifugation for 10 minutes at 1500g at 4°C. After centrifugation, the upper hexane layer from each tube, which contained the extracted tocopherol, was collected with a pasture pipette and transferred to another glass tube and covered with Parafilm to avoid evaporation. These supernatant fractions were analyzed for  $\alpha$  tocopherol. Bathophenanthroline, ferric chloride hexahydrate, orthophosphoric acid (85%) were prepared just before the assay.

For analysis of  $\alpha$  tocopherol, all the subsequent reactions were run in eppendorf microfuge tubes. A typical reaction mixture for standard curve in a total volume of 500  $\mu\text{l}$  contained 200  $\mu\text{l}$  of supernatant solution of  $\alpha$  tocopherol (0.5-4  $\mu\text{g}$ ), 50  $\mu\text{l}$  of 0.4% bathophenanthroline, 150  $\mu\text{l}$  of 0.06% ferric chloride and 100  $\mu\text{l}$  of 0.5%, orthophosphoric acid (85%). For test reactions to determine concentration of  $\alpha$  tocopherol in plasma, 200  $\mu\text{l}$  of hexane supernatant solution from test sample would be added in place of hexane solution containing standard  $\alpha$  tocopherol. For blank reactions, 200  $\mu\text{l}$  of plain n-hexane

from the upper layer of blank extraction tube will be added in place of supernatant containing  $\alpha$  tocopherol.

The order of reagent addition was critical. The absorbance in standard and test reactions was measured at 536 nm after setting the instrument to zero absorbance with the blank reaction and using 0.5 ml cuvette in DU-730 spectrometer (Beckman Coulter, Palo Alto, CA, USA).

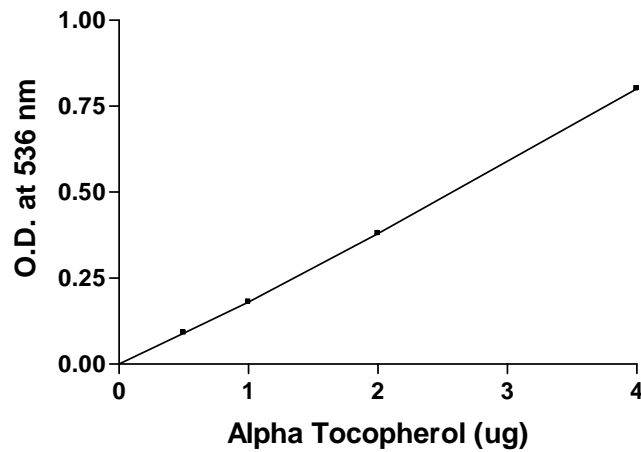
A standard curve was obtained by plotting absorbance vs. amount of  $\alpha$  tocopherol. The levels of  $\alpha$  tocopherol in the test (unknown) sample was obtained from this standard curve.

#### **Recovery studies**

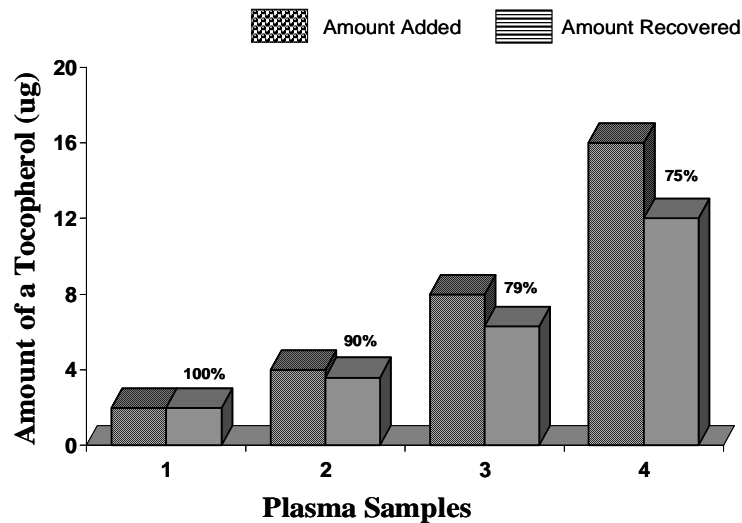
In order to assess the validity of the modified assay procedure, a known amount of  $\alpha$  tocopherol (2-16  $\mu\text{g}$ ) was added to human plasma. Using the modified procedure, its recovery was assessed in each sample.

#### **Plasma samples for determination of $\alpha$ tocopherol**

Eighty one normal healthy adults (58 males and 23 females; aged 20-65 years) were randomly selected from the personnel of the Aga Khan University. None of them



**Fig. 1:** Dose-response curve resulting from plotting absorbance at 536 nm versus amount of  $\alpha$  tocopherol in the reaction.



**Fig. 2:** Recovery of  $\alpha$  tocopherol from human plasma. A known amount of vitamin was added to plasma and assayed by the spectrophotometric procedure reported here.

had taken vitamin E supplementation during the past 6 months. Ten ml blood was obtained with prior consent in tubes containing sodium heparin. Plasma was immediately removed by centrifugation and stored frozen at  $-70^{\circ}\text{C}$  until analysis. The study had the approval of the Research Ethics Committee of the institution.

### STATISTICAL ANALYSIS

The mean values have been presented as means $\pm$ SD. Intrassay precision and inter-assay precision were calculated as described by Chard (1978). Statistical analyses were done with the help of an SPSS® (Statistical Package for Social Sciences) software version 13.0 for Windows®.

### RESULTS

Fig. 1 shows a typical dose-response curve obtained by plotting the amount of  $\alpha$  tocopherol ( $\mu\text{g}$ ) vs. the absorbance at 536 nm. The curve is linear and the sensitivity of the procedure is such that as low as  $0.2 \mu\text{g}$  of  $\alpha$  tocopherol can be easily detected.

The validity of the assay system was assessed by adding known amounts of  $\alpha$  tocopherol to human plasma and then recovering it by the modified assay procedure. Fig. 2 shows the recovery of  $\alpha$  tocopherol in 4 different samples. The recovery was from 75%-100% of the added vitamin. Intra-assay coefficient of variation was 5.3% (table 1), while inter-assay coefficient of variation was found to be

13% when the same samples were measured in assays run on different dates (table 2). These values are quite acceptable for the assay of any compound in biological sample.

Since there have been hardly any reports regarding plasma levels of  $\alpha$  tocopherol in Pakistani population, this method was employed to investigate the endogenous levels of  $\alpha$  tocopherol in plasma samples of 81 normal healthy adults (58 males and 23 females; age ranging from 20 years to 65 years). The mean $\pm$ SD plasma concentration of  $\alpha$  tocopherol was found to be 9.45 $\pm$ 2.64  $\mu$ g/ml (table 3).

## DISCUSSION

The assay is a modification of the procedure by Fabianek *et al.* (1968) to measure  $\alpha$  tocopherol in human blood samples. The assay is based on the principle that  $\alpha$  tocopherol being a reducing agent can convert ferric iron into ferrous iron which can then be coupled with a chelating agent, such as 4, 7-diphenyl-1, 10-phenanthroline (bathophenanthroline; BA) to produce the pink ferrous-BA colored complex. The absorbance of this colored complex at 536 nm is proportional to the concentration of  $\alpha$  tocopherol in the reaction. The modified assay involves extraction of  $\alpha$  tocopherol in n-hexane rather than xylene which in terms of contact with the skin and chronic exposure is less hazardous compared to xylene (MSDS, Material Safety Data Sheet, MSDS Website). Moreover, assay reactions following extraction can be run in eppendorf microfuge tubes which are considerably less expensive than the borosilicate or pyrex glass tubes normally used for such reactions. Treatment of standard samples with n-hexane along with test (unknown) samples has improved the precision of the assay.

The assay is specific for  $\alpha$  tocopherol. Addition of orthophosphoric acid in the assay reaction diminishes the interference from reductones, creatinine, acetol, reductic acid, glutathione and cysteine in the plasma (Washko *et al.*, 1992) and also reduces interference of carotene (Fabianek *et al.*, 1968).

In terms of mean recovery of 86% of added vitamin E, our method is superior to the spectrophotometric methods reported by Fabianek *et al.*, (1968) and Kayden *et al.*, (1973). This spectrophotometric assay is also superior to many other methods which are laborious or require expensive instrumentation based on High Performance Liquid Chromatography (Nirungsan and Thongnopnue, 2006), gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry (Mottier *et al.*, 2002), and, therefore, more suitable in a developing country with limited resources, such as Pakistan. The method would be of interest especially to laboratories involved in research on nutrition and vitamins.

With the exception of one small study (Jilani *et al.*, 2008), there is hardly any report in the literature regarding plasma levels of  $\alpha$  tocopherol in Pakistani population. Therefore, we employed this assay to monitor levels of this vitamin in 81 normal healthy subjects. We found that most of the values fell within the reported normal ranges of plasma  $\alpha$  tocopherol in Western populations (Fabianek *et al.*, 1968; Kayden *et al.*, 1973). The mean $\pm$ SD value of  $\alpha$  tocopherol in Pakistani normal healthy adults (9.45 $\pm$ 2.6  $\mu$ g/ml) is very close to the mean $\pm$ SE value of 7.9 $\pm$ 0.4  $\mu$ g/ml reported by Farrell *et al.*, (1978) in a USA population. On the basis of a number of studies, human subjects with plasma levels of  $\alpha$  tocopherol less than 5  $\mu$ g/ml are considered to be vitamin E deficient (Vitamin E or alpha tocopherol. [www.health-herbal.com/html/vite.html](http://www.health-herbal.com/html/vite.html)). Applying this criterion, 4.9% of subjects monitored in this study were found to be vitamin E deficient (Table 3), while 16% were more than 1 standard deviation below the mean. This suggests that vitamin E deficiency could be quite common in Pakistani normal adults. This is despite the fact that we consume a lot of Palm oil which is a rich source of vitamin E (Vitamins and mineral requirements in human nutrition, 2004).

The major biological function of vitamin E is to protect polyunsaturated fatty acids and other components of cell membrane and low density lipoprotein from oxidation by free radicals. Therefore, damage to the cell membranes is one of the major pathologies associated with vitamin E deficiency (Vitamins and mineral requirements in human nutrition, 2004). Therefore, diseases generally associated with free radical damage, such as cardiovascular disease, neurological problems and anemia would be expected to be quite common in Pakistani population, and subnormal levels of vitamin E could be contributing to the high prevalence of these diseases in this country. Smoking has been shown to significantly decrease serum levels of  $\alpha$  tocopherol (Faure *et al.*, 2006). In Pakistan where smoking among the young adults is still on the rise, a sizeable proportion of the young population could be having subnormal levels of vitamin E. Therefore, large scale "community-based" studies would be required to ascertain the prevalence of deficiency of vitamin E in Pakistani population.

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