THE DETERMINATION OF VITAMIN E IN THE PLASMA OF SICKLE CELL PATIENTS BY HPLC

by

George G. Gentsy Jr.

Submitted in Partial Fulfillment of the Requirements for the

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ABSTRACT

THE DETERMINATION OF VITAMIN E IN THE PLASMA OF SICKLE CELL PATIENTS BY HPLC

George G. Gentsy jr.

Master of Science

Youngstown State University, 1987

A High Performance Liquid Chromatography (HPLC) method using a C_{18} column with W detection set at 280 nm and methanol/water as the mobile phase was employed to quantitatively determine mg/L of a-tocopherol and γ-tocopherol in the plasma of sickle cell and normal individuals. The method involved the use of a-tocopherol acetate as an internal standard. The plasma sample was denatured with ethanol and extracted with n-hexane. The hexane layer was evaporated and the residue dissolved in a mixture of isopropanol and methanol. The solvent mixture containing the tocopherols was injected into the chromatograph and separation was accomplished in fifteen minutes at a flow rate of 2 mL/min. The height of the resulting peaks was employed to quantitatively determine the amount of a-tocopherol and γ-tocopherol present.

The clinical study involved the determination of the amount of a-tocopherol and γ-tocopherol in the plasma of

eleven sickle cell patients and eleven normal individuals. For the sickle cell patients, ten of the eleven patients studied had a-tocopherol levels of less than 5 mg/L. For the normal individuals, ten of the eleven individuals had a-tocopherol levels of more than 5 mg/L. For γ-tocopherol, the plasma levels for the sickle cell patients ranged from 1.23 to 4.31 mg/L. For the plasma of the normal patients, the γ-tocopherol levels varied from 1.00 to 2.79 mg/L.

A statistical t test was performed to compare the means of the a-tocopherol and γ-tocopherol plasma results for the sickle cell and normal individuals. The mean a-tocopherol value for the sickle cell patients' plasma was 4.71 ± 1.16 (mean + standard deviation). The mean a-tocopherol value for the normal patients' plasma was 6.72 ± 1.46. The mean γ-tocopherol value for the sickle cell patients' plasma was 2.31 ± 0.989 and the mean γ-tocopherol value for the normal patients' plasma was 1.64 ± 0.571. A significant statistical difference was found between the means of the plasma a-tocopherol levels for the sickle cell patients and the normal individuals. No significant statistical difference was found between the means of the γ-tocopherol plasma levels for the sickle cell and normal individuals. However the γ-tocopherol plasma level in sickle cell patients plasma comprised a larger percentage of total plasma tocopherols when compared to γ-tocopherol plasma levels in normal individuals.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND DEFINITIONS

AUFS	**Absorbance units full scale**
C_{18} – –	**Octadecylsilane bonded packing material**
Flow rate	**Milliliters of mobile phase pumped through the column in a given time frame.**
GLC	**Gas-Liquid Chromatography**
HPLC – –	**High Performance Liquid Chromatography**
ISC'S	**Irreversibly sickled cells**
IU	**International Units**
Mobile phase – – – –	**Solvent pumped through the column in which the components to be separated are soluble .**
Pellicular support	**Support having a hard central**

	core and a porous surface.
Reverse phase chromatography –	A form of partition chromatography where the mobile phase is more polar than the stationary phase.
SCA	Sickle cell anemia
Stationary phase –	Packing material inside the column which can interact with the solute components.
TLC	Thin-Layer Chromatography
Vaso-occlusive –	The blockage of a blood vessel

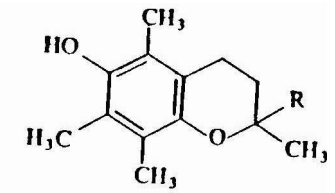
CHAPTER 1

INTRODUCTION

Structure of Vitamin E

Vitamin E, a lipid soluble vitamin, was discovered in the 1920's and was later given the generic name tocopherol. There are eight vitamin E isomers and they are listed in figure 1. The four most important vitamin E isomers occuring naturally are a-, β-, γ-, and δ-tocopherol. These tocopherols vary in amount and biological activity (ability to prevent oxidation of red blood cell membranes) in the bloodstream of humans. The predominant isomer in human blood is a-tocopherol which is the most biologically active tocopherol (1).

Animals and humans cannot synthesize vitamin E and must rely on food sources for their supply. Foods containing tocopherols include vegetable oils, fresh leafy vegetables, legumes, margarine, and egg yolks (1). In human plasma, 88% of total vitamin E by weight is in the d-alpha-tocopherol form while d-gamma-tocopherol is 10%, and d-beta-tocopherol is 2%. No detectable amount of d-delta-tocopherol is usually found in human blood. The tocotrienols are found

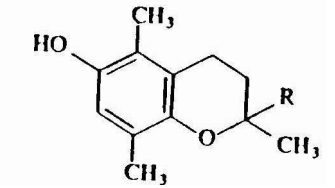


R = —(CH₂—CH₂—CH(CH₃)—CH₂)₃H

Alpha-tocopherol

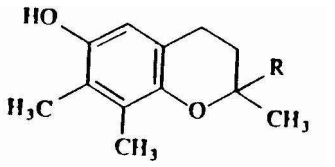
R = —(CH₂—CH=C(CH₃)—CH₂)₃H

Alpha-tocotrienol



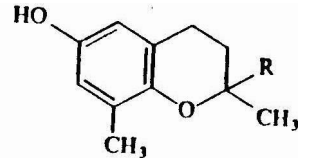
Beta-tocopherol

Beta-tocotrienol



Gamma-tocopherol

Gamma-tocotrienol



Delta-tocopherol

Delta-tocotrienol

Figure Structure of vitamin isomers

only in plants and not in mammals (2).

Function of Vitamin E

The function of vitamin E is to protect unsaturated lipids in the red blood cell membrane from peroxidation. Peroxidation is a chemical process in which oxygen addition occurs across the double bond of an unsaturated site, resulting in cleavage of the fatty acid and the formation of free radicals (3). The presence of peroxidized lipids in human erythrocytes has resulted in increased susceptability of the red cells to hydrogen peroxide induced hemolysis (1).

The exact process whereby vitamin E protects the erythrocyte membrane from oxidative damage is not known. Plasma concentrations of a-tocopherol below 6 mg/L can result in increased erythrocyte hemolysis by hydrogen peroxide attack on the red cell membrane. The condition described above is considered a nutritional deficiency of vitamin E in the blood (4). Vitamin E circulates in blood attached to β-lipoproteins. A strong correlation between plasma a-tocopherol concentration and plasma lipid concentration exists, suggesting that vitamin E concentrations should be interpreted relative to plasma lipid levels (5,6). A concentration of 0.8 mg of tocopherol per gram of total plasma lipids is considered adequate for

protection. Elevation of plasma total lipids above 15 g/L can apparently shift erythrocyte tocopherol to plasma, resulting in erythrocyte susceptibility to oxidation. The susceptibility exists even if plasma concentrations of tocopherol in hyperlipidemic states are adequate (5,6). Other suggested roles for vitamin E include the prevention of retrolental fibroplasia and bronchopulmonary dysplasia of neonates in infants (3). Vitamin E is also reported to act as an agent in slowing the aging process , preventing heart disease , and increasing sexual endurance in humans (3).

Absorption of Vitamin E

Absorption of vitamin E into the body is believed to be associated with intestinal fat absorption. Approximately 40% of ingested tocopherol is absorbed through the gastro -intestinal tract, and the percentage is affected by the amount of unsaturated dietary fat consumed as well the isomer type present. As polyunsaturated fatty acids increase in the diet, the physiological requirements for vitamin E increase. Vitamin E is predominantly found in fatty tissue and increased dietary a-tocopherol intake leads to increased concentration in all tissues, including plasma, erythrocytes, and platelets (1).

Sickle Cell Disease

Sickle cell anemia (SCA) is a hereditary disorder confined mainly to the Black population. In sickle cell disease, normal hemoglobin (HbA) in the red cell is replaced by an abnormal hemoglobin (HbS). About 80% of the total hemoglobin in patients with sickle cell anemia is HbS. Individuals with sickle cell anemia are designated homozygous, (meaning genes from both parents will produce only HbS). Individuals with sickle cell trait are designated heterozygous, where one parent had normal hemoglobin, HbA, and the other parent had abnormal hemoglobin, HbS. Individuals with the sickle cell trait have relatively minor problems compared to patients with sickle cell anemia (7). The red blood cells of sickle cell anemia patients are distorted and undergo rupturing of the cell membrane (hemolysis) readily. Failure of the kidney and / or lungs along with bone infections usually result in the death of the individual in the mid to late twenties (8). Other clinical manifestations of the disease include chronic hemolytic anemia, recurrent vaso-occlusive painful attacks, and frequent bacterial infections. The symptoms are usually different for each patient. Some sickle cell patients have many vaso-occlusive crises and require frequent blood transfusions and hospitalizations, while other8 are hospitalized infrequently (9).

Being a hereditary disorder, sickle cell anemia is caused by a malfunctioning gene. A point mutation in DNA that codes for valine rather than glutamic acid at the sixth position of the β-globin chain occurs (9). The molecular substitution causes HbS to polymerize and form filaments, resulting in a reduction in the internal volume of the cell. The reduced volume forces the oval membrane of the cell to collapse to the shape of a sickle. This sickling process occurs in an oxygen poor environment (9). Upon subjecting the sickled red cells to oxygenation, a number of the sickled (abnormally shaped) red cells revert to normalcy. The portion of the red cells that don't revert are referred to as irreversibly sickled cells (ISC[1]s) (10). Clark and coworkers (11) suggested that the inability of ISC's to revert to a normal shape is due to their high internal viscosity, which results from cellular dehydration caused by abnormal membrane permeability. The ISC's can vary from 4.0% to 40% in individuals with sickle cell anemia (12). The hemolysis occuring in sickle cell anemia patients is directly related to the percentage of ISC's present in that individual (13).

Although the genetic defect in the DNA strand is mainly responsible for the clinical manifestations of SCA, secondary effects, such as peroxidation of the lipid membrane of the red cell, must have some accountability. S. K. Jain (14) has demonstrated that autoxidation of sickle cell hemoglobin produces various oxidation products, including

malonyldlaldehyde, which affects membrane fatty acids. In vitro studies by Jain (14) depicted the further reaction of malonyldialdehyde with the amino groups of positively charged membrane phospholipids to produce a Schiff base product. This reaction results in the cross-linking of the phospholipids in the membrane. In Figure 2, phosphatidylserine and phosphatidylethanolamine combine with malonyldialdehyde to form a Schiff adduct.

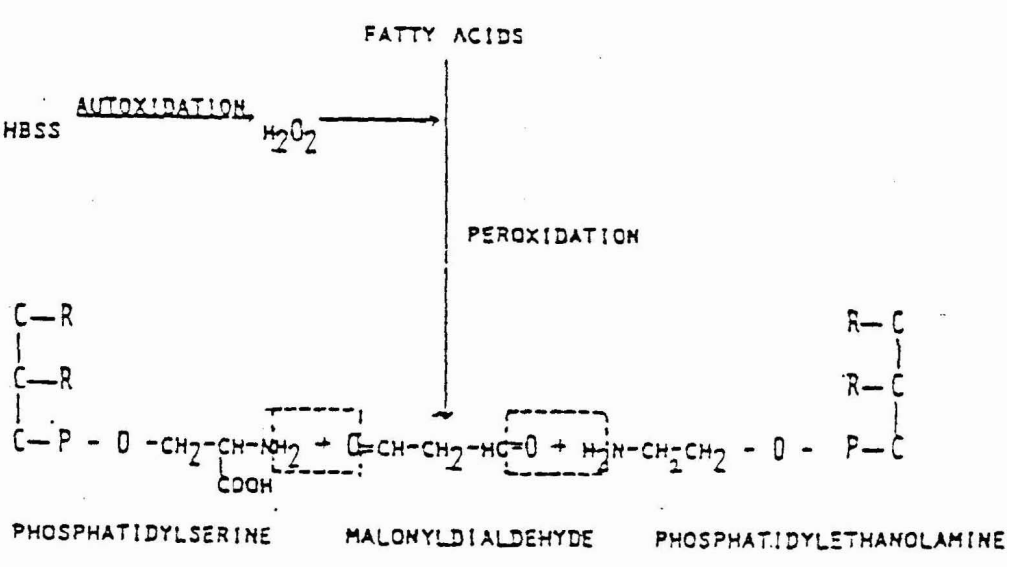


FIGURE 2. Schema showing Schiff's base adduct formation between phosphatidylserine, phosphatidylethanolamine, and malonyldialdehyde. (From Ref. 14)

Vitamin E has an important role in limiting

peroxidation in biological systems. With this fact in mind, the amount of Schiff base formed should increase when vitamin E concentrations in the blood are low, as in sickle cell anemia patients. Shohet, S. and Jain, S. (15) have demonstrated the increase of Schiff base formation in sickle cell anemia patients by employing TLC.

SCA and Vitamin E Therapy

C. L. Natta and coworkers (10) have demonstrated that sickle cell anemia patients who were given 450 IU of vitamin E per day for up to 35 weeks, showed a decrease in the percentage of circulating ISC's in their blood. The percentage of ISC's decreased from 25 ± 3% before therapy to 11 ± 1% after vitamin E was administered, and the percentage remained low as long as vitamin E therapy was continued. Plasma tocopherol levels increased from 0.7 ± 0.2 mg/g lipid before therapy, to 2.3 ± 0.3 mg/g lipid after the treatment with vitamin E. C. L. Natta and coworkers (10) were unable to predict whether prolonged administration of vitamin E would alleviate the debilitating conditions of the disease as a consequence of the reduced ISC percentage.

Chiu et al (9) did a similar study on ISC's. They started with patients with an average 12% ISC count before

vitamin E was administered and found no significant difference in the ISC count after vitamin E administration.

Chiu and coworkers (9) also studied the absorption of vitamin E in the gastrointestinal tract of sickle cell anemia patients. They found that the SCA patients did not absorb as much vitamin E into the bloodstream as the normal patients did, such that after six hours, the normal patients absorbed 3.5 times more vitamin E than the SCA patients did.

Statement of the Problem

A number of studies have been performed that determine the vitamin E content in plasma of sickle cell anemia patients. Chiu and coworkers (9) found a significant difference in plasma vitamin E content between sickle cell and normal patients by HPLC. THe vitamin E content of sickle cell plasma was low when compared to normal plasma. Natta et al (10) also found low Vitamin E content in the plasma of sickle cell anemia patients by a fluorometric procedure. Muskiet and Muskiet (16) found no significant difference in plasma vitamin E levels between sickle cell and normal patients by HPLC. However only six samples were run by the Muskiet group.

A vitamin E determination study of sickle cell anemia patients was started at YSU in an effort to duplicate and improve the previous studies mentioned. The studies

performed by the various groups have yielded contradictory information concerning the relative levels of vitamin E in plasma of sickle cell anemia patients versus normal individuals. The present study could yield valuable information which could elimate the contradiction. Also, the previous methods neglected to examine the different isomers of tocopherol in SCA patients versus normal individuals. Although not a cure, vitamin E, acting as an antioxidant, may alleviate some of the debilitating symptoms associated with SCA disease.

The method employed in this study was originated by Catignani and Bieri (17) and is titled "Simultaneous Determination of Retinal and a -Tocopherol in Serum or Plasma by Liquid Chromatography." The part of the method concerning vitamin E was used with some modifications. In this method, vitamin E was determined by reverse phase HPLC, employing a UV detector at 280 nm.

CHAPTER II

METHODS OF DETERMINATION OF VITAMIN E

Colorimetric Method: General Interferences and Pretreatment

One of the oldest methods for determining vitamin E is a colorimetric procedure (18). In this method, Vitamin E reduces ferric ions to ferrous ions. The resulting ferrous ions then react with a complexing agent to form a colored solution. Interferences from other reducing substances in the biological material being tested, resulted in erroneously high amounts of vitamin E determined by this method. Extensive sample clean up procedures were employed in an effort to diminish the interferences. Vitamin E was determined in vegetable and animal materials by this method. Other methods for determining vitamin E include the use of gas -liquid chromatography (GLC), thin-layer chromatography (TLC), and high performance liquid chromatography (HPLC).

Three major problems are encountered when vitamin E is determined in biological materials (19). First, the instability of the tocopherols must be considered during sample preparation. Vitamin E is readily oxidized, and in the presence of heat, light, alkaline pH conditions or metal

ions, the oxidation reaction is accelerated. During sample preparation, extreme care should be exercised in executing any isolation or fractionation steps. For example, in TLC determinations, the sample is developed on a thin layer plate often exposed to the air where oxidation can readily occur. Secondly, interferences resulting from the presence of lipids, particularly cholesterol, can cause problems in vitamin E assays. A variety of approaches have been devised to remove lipids from biological materials before determining vitamin E. The last problem involves the difficulty of separating vitamin E into its component isomers (a-, β-, γ-, and δ-tocopherol). Up to the present, separating β-tocopherol from -tocopherol has been extremely difficult. Only recently has normal phase HPLC afforded an accurate and simple procedure for this separation (19).

For the colorimetric and GLC procedures, extensive sample preparation techniques must be employed. The removal of lipids from the sample is necessary before the tocopherol analysis can be performed. This step is accomplished usually by employing thin-layer or column chromatography. A typical pretreatment scheme, before the removal of lipids for the colorimetric and GLC procedures, usually involves some or all of the following three steps (19).

1. Physical Treatment of the Sample:
   Samples such as oils, plasma, and some foods can forgo physical treatment that involves grinding.

However, fibrous plant and animal tissue require a homogenization step.

2. Saponification Step:

Saponification is employed to remove interfering triglycerides and to liberate tocopherol from any esters that may be present. The saponification procedure involves refluxing the sample with ethanolic potassium hydroxide for a suitable period of time. Precautions should be observed at this point to avoid oxidizing vitamin E. A nitrogen atmosphere should be employed during the saponification procedure to preclude oxidation. Antioxidants, such as pyrogallol or sodium ascorbate, can also be beneficially employed to reduce oxidation at this stage.

3. Lipid Extraction:

After saponification, the mixture is diluted with water and extracted with hexane. The saponified lipids remain in the water layer. The organic layer is separated and evaporated to dryness. The dried residue contaning vitamin E is dissolved in a suitable organic solvent and analyzed by GLC or the colorimetric method.

Gas-Liquid Chromatography (GLC)

The method of gas-liquid chromatography has been widely used to determine tocopherols in biological materials (20). Certain physical-chemical properties of these compounds make the GLC method less than ideal. Since the tocopherols have high molecular weights and low volatilities, the GLC method requires high analysis temperatures and long analysis times. The possibility of degradation of the tocopherols at the high temperatures of analysis is always present.

For the GLC determination of vitamin E, the sample preparation step is critical. Interferences from lipids in the unsaponified biological material is more than likely to occur in the GLC method. The GLC method also results in the coelution of a-tocopherol and cholesterol. For this reason, more than one step is often used to remove cholesterol. As a result, sample pretreatment in GLC is often arduous and time consuming.

The GLC method can separate a-, γ , and $-tocopherol easily. However the separation of β- and γ -tocopherol presents a challenge of immense proportions. To help achieve this goal, a more volatile tocopherol compound in the form of a derivative is made. In the GLC method of determining vitamin E, an important step is the derivatization procedure. Although tocopherols can be chromatographed without resorting

to derivatives, derivatization reduces the retention time and analysis temperature. Another benefit of derivatization is improved resolution and peak shape. Most derivatization procedures involve the preparation of trimethylsilyl (TMS) ethers (21, 22), or acetates (23, 24). A variety of other derivatives have been made. These include propionates (25), butyrates (26), trifluoroacetates (27), and pentafluoropropionates (23).

The most popular inert supports used in the GLC determination of vitamin E include silanized Gas Chrom Q, Chromosorb, and Celite. For non-derivatized tocopherol determinations, a nonpolar to medium polar stationary phase is used. These stationary phases include SE-30 (24), OV-1 (28), OV-17 (28), and SE-52 (29). Moderate to severe tailing of the tocopherol peak results when polar stationary phases such as XE-60, and OV-25 are used (28). Recently, a gas chromatographic-mass spectrometric (GC-MS) assay of vitamin E was performed using a polar stationary phase consisting of 3% Silar 10C (30).

The most widely used detector for the GLC method of determining vitamin E is the flame ionization detector. A GLC method developed by a Japanese group used a ^{63}Ni electron capture detector (ECD) for determining Vitamin E (31). The flame ionization detector and the electron capture detector are capable of determining tocopherol isomers in the mg/L range in biological materials.

For a more sensitive analysis at the picogram level, a GC-MS method can be employed (30). In this method, deuterated tocopherol acetate was employed as the internal standard and ions at M/Z 502 (molecular ion of tocopherol trimethysilyl ether) and M/Z 515 (molecular ion of tocopherol-d13-TMS ether) were simultaneously monitored using 20 eV ionization energy in the electron impact mode. The preceding GC-MS method was used to determine tocopherols in lung tissue. A quantitative GC-MS method to determine tocopherols in plasma was developed (29). The GC-MS procedure is used frequently as a qualitative tool to determine the presence of tocopherol in milk (32), vegetable oils (33), tissue (34), and pharmaceutical preparations (35).

In quantitative GLC methods, peak height or peak area is used. Calibration involves the analysis of samples to which known amounts of tocopherol are added. Standard curves can be made by plotting peak height or peak area versus the amount of tocopherol added. A suitable internal standard (IS) is added to compensate for losses during sample pretreatment and variations in injection volumes during a chromatographic run. A substitution for the peak height or peak area of the substance being determined can be made. Use of peak height or peak area ratios (tocopherol/IS) can be employed to improve accuracy. A properly chosen internal standard has a suitable partition coefficient and doesn't interfere with the tocopherol peaks on the chromatogram. A

number of suitable internal standards are 5,7-dimethyltocol (36), and a-tocopherol propionate (37).

In 1962, Wilson and coworkers (28) demonstrated that GLC separations of tocopherols occur according to the number of methyl groups present on the cyclic rings. They mare able to separate mono-, di-, and tri-methyltocols. They calculated separation factors based on the number of methyl groups on the cyclic rings and predicted the relative retention volumes for the different homologs.

Slover et al (38) were the first to prepare silyl ether derivatives, which proved to yield more symmetrical peaks than the underivatized compounds. Recently, Shen-Nan Lin and Horning (22), using capillary GLC, resolved the four tocopherols as TMS ether derivatives, employing a 32 m open tubular column coated with a polar PZ-176 liquid phase. Figure 3 demonstrates the separation of the four tocopherols which was accomplished in a 55 minute run. A similar separation was performed by Mordret and Laurent (39), using a 20 meter OV-17 capillary column. The analysis time took only 15 minutes and separated all four tocopherols and tocotrienols. Tocopherol stereoisomers can also be resolved by capillary GLC. Resolution of a-tocopherol stereoisomers has been accomplished using a 115 m glass capillary column coated with SP-2340 (40). Capillary GLC has been used to analyize crude plasma extracts without resorting to the need for saponification, TLC, or other types

of cleanup (22). However, the analysis requires 65 minutes to separate a-tocopherol from cholesterol and other lipids.

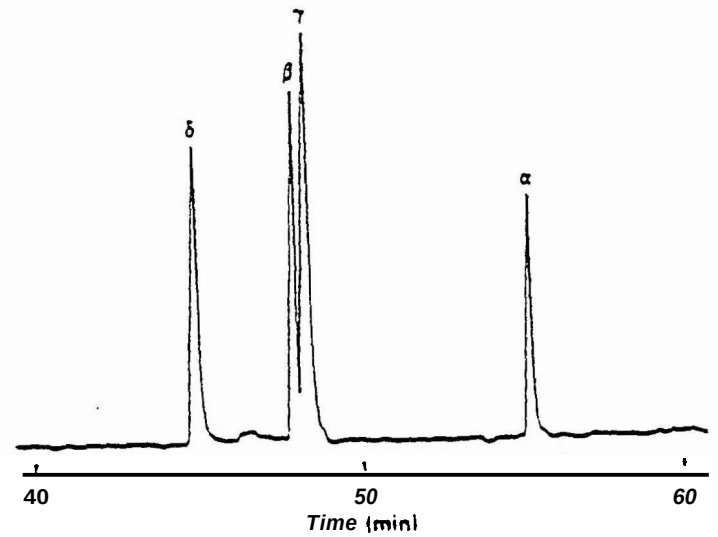


Figure 3 Separation of α-, β-, γ-, and δ-tocopherol as trimethylsilyl ethers. Operating conditions: temperature programmed from 180°C at 2°C/min; 32 *m* × 0.25 mm ID open tubular glass capillary column, coated with polar phase PZ-176; carrier *gas.* nitrogen; initial column pressure, 5 psi; split ratio, 10:1. (From Ref. 22)

Thin-Laver Chromatography (TLC)

TLC in combination with gas-liquid chromatography or colorimetry has been used to determine tocopherols and tocotrienols in blood serum and plasma (41, 42), red blood cells (43, 44), liver (45), vegetable oils (46), and pharmaceutical preparations (47). The majority of the older tocopherol assay methods for complex biological materials used TLC to separate the lipid fraction from the vitamin E fraction in the sample (19). For a typical TLC run, the

spots on the TLC plate associated with vitamin E were scraped off, eluted, and quantitated by a colorimetric or GLC procedure.

For the colorimetric procedure, the TLC plates were developed and the tocopherol spots located and scraped off. The tocopherols were eluted with ethanol, centrifuged, and the liquid mixed with the Emmerie-Engel color producing agent (19). The absorbance of the solution was then measured on a spectrophotometer.

For the GLC procedure, the tocopherol contaning eluate was evaporated to dryness and the residue derivatized. A suitable aliquot was injected into the GLC system and the peak areas were measured and quantitated.

Silica gel is the adsorbent most often used for TLC separations of vitamin E isomers. A number of other supports have been used, including Alumina, Florisil, Kieselguhr, and mixtures of zinc carbonate with Alumina or Silica gel G (19). Most TLC systems have failed to distinguish between isomeric dimethyltocopherols and dimethyltocotrienols. Pennock et al (49), developed a two dimensional technique that separated complex tocopherol and tocotrienol mixtures in plant materials. Chloroform was employed to develop the plates in the first dimension and yielded a separation of the different homologs. The second dimension was developed with a mixture of diisopropyl ether and petroleum ether, resulting in a separation of beta- and gamma-tocopherol isomers.

High Performance Liquid Chromatography (HPLC)

Classical column chromatography is used in conjunction with colorimetric and gas-liquid chromatographic methods for tocopherol determinations. However, the HPLC method can be used without the need of an additional technique and requires little or no sample pretreatment (19). The HPLC technique is characterized by high efficiency, good sensitivity, and short analysis times. The development of micro packing materials (2 to 5 microns in diameter), and the advent of high pressure pumps , have led to the improved column efficiency and resolution enjoyed by HPLC. These improvements have made HPLC the method of choice for tocopherol determinations (19).

Thompson and coworkers (52) developed the first method to determine tocopherols by a liquid chromatographic procedure. With a chromatographic column consisting of (hydroxyalkoxypropyl Sephadex) coupled to an on-line spectrofluorimetric detector, tocopherols in tissue, food, and serum were determined. The analysis time of ninety minutes, and the limited sensitivity , made this method less than ideal when compared to present day HPLC methods. However, Thompson *et al* (52) were able to separate positional isomers of the tocopherols and the tocotrienols in a single

run with good quantitative representation. Initial LC determinations of vitamin E were based on pellicular supports (53), which were later superseded by microparticulate materials offering greater column efficiency.

HPLC can determine tocopherols using polar or nonpolar stationary phases. Reverse phase chromatography. which uses a nonpolar stationary phase and a more polar mobile phase, has the advantage of good column stability, excellent reproducibility of retention times, and fast equilibration (19). Reverse phase chromatography is excellent for separating homologs, that differ from each other by the number of carbon atoms in the molecule. Reverse phase chromatography at the present time has not been able to separate the positional isomers beta-tocopherol from gamma-tocopherol. Cook et al (54) using a reverse phase C_{18} column with over 100,000 plates per meter was not able to separate beta-tocopherol from gamma-tocopherol in plasma. Most reverse phase HPLC methods for tocopherol use an octadecylsilane (C_{18}) packing material with methanol or methanol - water mixtures as the mobile phase. Small amounts of water are added to the methanol to control the retention time of the isomers.

Normal phase liquid chromatography uses a polar stationary phase and a less polar mobile phase. Normal phase liquid chromatography can separate tocopherol isomers easier than reverse phase, but suffers from column packing

deterioration at an earlier date. Normal phase chromatography also requires *less* pretreatment than reverse phase chromatography for vitamin E determinations. Van Niekerk (55) was the first person to perform a successful separation of beta- from gamma-tocopherol by a normal phase method. The method employed a silicic acfd based Corasil 11 packed column, and a mobile phase consisting of a mixture of diisopropylether-hexane (5/95) by volume. A microparticulate silica column, along with the same mobile phase, later yielded superior performance in terms of efficiency, resolution, and analysis time (56).

The first separation of all eight natural tocopherols and tocotrienols was accomplished on a Corasil II column (59). Substitution of a microparticulate packing material for the older pellicular material resulted in increased column efficiency and resolution. The vitamin E separation that took 80 minutes with the pellicular material (59) was reduced to 15 minutes with the microparticulate packing material (60). A typical separation using microparticulate packing material is illustrated in figure 4.

The detection systems most commonly used for the HPLC determination of tocopherols are UV , fluorescence, and electrochemical detection. a-Tocopherol exhibits relatively poor W absorbance, (Molar Absorptivity is 3530 $cm^2/mmol$ at 292 nm), however the absorbance is sufficient to yield

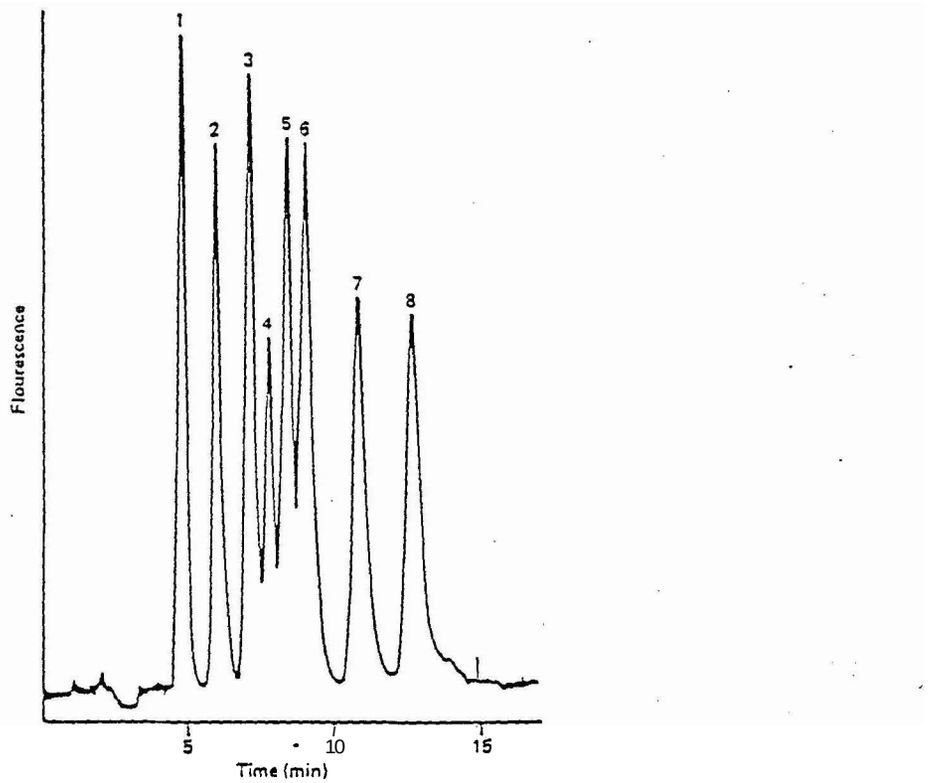


Figure 4 Segaration of tocopherols and tocotrienols. Chromatographic conditions: 25 × 0.46 cm stainless steel. column packed with 5 μm Lichrosorb Si 60; eluent, hexane-diethyl ether (95:5, v/v); flow rate, 2 ml/min; detection: fluorescence, λ_{exc} 295 nm; λ_{em} 330 nm. Peak identification: (1) a-tocopherol; (2) α-tocotrienol; (3) β-tocopherol; (4) butylhydroxyanisole (BHA); (5) y-tocopherol; (6) β-tocotrienol; (7) γ-tocotrienol; (8) δ-tocopherol. (From Ref. 60)

adequate sensitivity (61, 62). The detection limit at 292 nm for a-tocopherol was estimated to be 60 ng (63). The lowest amount of a-tocopherol that could be determined accurately in serum with this W detection system was 0.6 mg/L (63). W detectors are concentration rather than mass dependent and column size greatly affects their sensitivity (64). The greater sensitivity is achieved by using smaller diameter columns (65, 66). Some methods used fixed wavelength W detectors at 280 nm with only a slight compromise in sensitivity.

Superior performance in selectivity and sensitivity is achieved with fluorescence detection for the HPLC determination of tocopherols. Tocopherols can be made to fluoresce by exciting the molecules at the wavelengths of 205 or 295 nm (19). The excited molecules have an emission that is measured at 330 nm. Using an excitation wavelength of 295 nm, the minimum detectable amount of a-tocopherol obtained by this detection method was reported to be 9 ng (67). By using an excitation wavelength of 205 nm instead of 295 nm, Hatam and Kayden (68) reported a twenty-fold increase in sensitivity for a-tocopherol. Recently, tocopherols have been determined in plasma, platelets, vegetable oil, and liver by the fluorescence method at 205 nm (69).

Electrochemical detection of tocopherols in HPLC is employed infrequently and only when extremely high sensitivity is required. Tocopherols can be oxidized at a

glassy carbon electrode at a potential of + 0.7 V versus a Ag/AgCl reference electrode (19). Both reverse phase and normal phase liquid chromatographic systems can be employed, even in conjunction with nonaqueous solvents, providing sufficient supporting electrolyte is incorporated in the mobile phase. By using reverse phase chromatography and incorporating 0.05 M sodium perchlorate in a mobile phase mixture consisting of methanol-pyridine (99.9/0.1) by volume, tocopherol in blood was determined (70). Tocopherol levels as low as 50 pg could be detected by this method.

The calibration scheme for HPLC involves essentially the same procedure as that explained for GLC. External standard methods can be used where known amounts of tocopherol are added to a sample and peak areas or heights are calculated and plotted against concentration. Many tocopherol assays have relied on external standardization using peak height (57, 62, 66) or area measurements (60, 71). Imprecision caused by poor sample injection technique has been largely eliminated by using modern sampling valves, so that the majority of error is now the result of the extraction procedures (19). Consequently, precision can be improved by using an internal standard. The internal standard is widely employed in HPLC and compensates for losses during sample handling and provides a means to correct for fluctuations in solvent composition and flow rate.

A good internal standard should possess the quality

of structual similarity to the compound of interest (72). Tocol is the most widely used internal standard in the HPLC determination of tocopherols (58, 72). Tocol has a structure similar to tocopherol but differs by having three fewer methyl groups on the cyclic ring system. In some chromatographic methods, tocol does not elute close enough to the tocopherols, hence a-tocopherol acetate is used as an alternate internal standard, particularly in reverse phase chromatography (65, 74).

A typical analysis scheme for tocopherols by HPLC involves a double-phase solvent extraction, followed by isolation and evaporation of the organic layer. The resulting residue is dissolved in a small amount of organic solvent and injected onto the column. The tocopherol peaks are identified and the peak heights or areas are used to determine the amounts present.

CHAPTER III

SOLVENTS, MATERIALS, STANDARDS, AND APPARATUS

Solvents

The methanol, water, and hexane used were HPLC grade solvents from Mallinckrodt, Inc., Paris, Kentucky 40361. The ethanol was absolute, 200 proof, USP grade solvent from AAPER Alcohol and Chemical Co., Shelbyville, Kentucky 40065. The isopropanol was reagent grade solvent from Mallinckrodt, Inc.

Standards

Purified d-a-tocopherol, d-Y -tocopherol and d-a-tocopherol acetate were purchased from Eastman Organic Chemicals, Rochester, NY 14650. Purified d-$-tocopherol was purchased from Supelco Inc., Bellefonte, Pa. 16823. The standards were stored in the freezer upon arrival.

Materials

1. The test tubes used in the method were 6x50 mm disposable glass tubes. They were washed and reused.
2. A test tube holder with six positions for tubes was

constructed out of wood.

3. The corks were number 000 to fit the test tubes.
4. The nitrogen was supplied by a nitrogen tank with regulator set at 4.0 lb/in^2.
5. The helium gas for the HPLC system was supplied by a helium tank with regulator set at 2.5 lb/in^2.
6. The filters used to filter the mobile phase were Nylon 66 filters, 0.45 µm pore size and 47 mm in diameter from Rainin Instrument Co., Mack Road, Woburn, MA. 01901.
7. A 100 µL glass syringe from IBM instruments was used to inject a sample.

Apparatus

1. A model LC/9533 ternary gradient liquid chromatograph with work station incorporating a LC/9523 variable UV detector, a 9553 solvent degas assembly and an injection system containing a 50 µL loop was employed from IBM Instruments Inc., Danbury, Ct. 06810.
2. A reverse-phase octadecyl ($C_{,,}$), 5.0 micron particale size stainless steel column, 4.5 mm i.d. x 250 mm length was employed from IBM Instruments Inc.
3. A stainless steel 4.6 mm i.d. x 5 cm guard column packed with 10.0 micron $C_{1\,8}$ was used from Alltech Associates Inc., Applied Science Labs., 2051 Waukegan Road, Deerfield, IL. 60015.

4. A Sargent Welch model XKR strip chart recorder with S-72164-10 paper was used from Sargent Welch Co. Cleveland, OH.
5. A vortex mixer (model K-500-J) from Scientific Industries Inc., Springfield, Mass. 01103 was used to shake the sample.
6. A Sorvall GLC-1 centrffuge from Dupont Instruments, Wilmington, Del. was used to centrifuge the samples.
7. A glass capillary evaporator was made from 6 mm thick walled glass tubing drawn to a fine capillary.
8. The micro-pipets used were 25, 50, 100, and 200 µL from Cole-Parmer Instruments Co., Chicago, Illinois 60648.

HPLC Instrument System

The HPLC instrument system is depicted in figure 5. A High Performance Liquid Chromatographic system consists of five major components. They are the solvent system, pump, analytical column, detector, and recorder. The solvent system consists of a one liter glass bottle with degassing capabilities. A high pressure pump forces the prefiltered solvent through the guard column and then through an analytical column. The guard column filters out small dust particles and insolubles in the sample and mobile phase. The sample is injected into the injection port and passes through the guard column to the analytical column where separation takes place. The separated components continue on to the detector where electrical signals are formed. The signals are amplified and measured on a recorder.

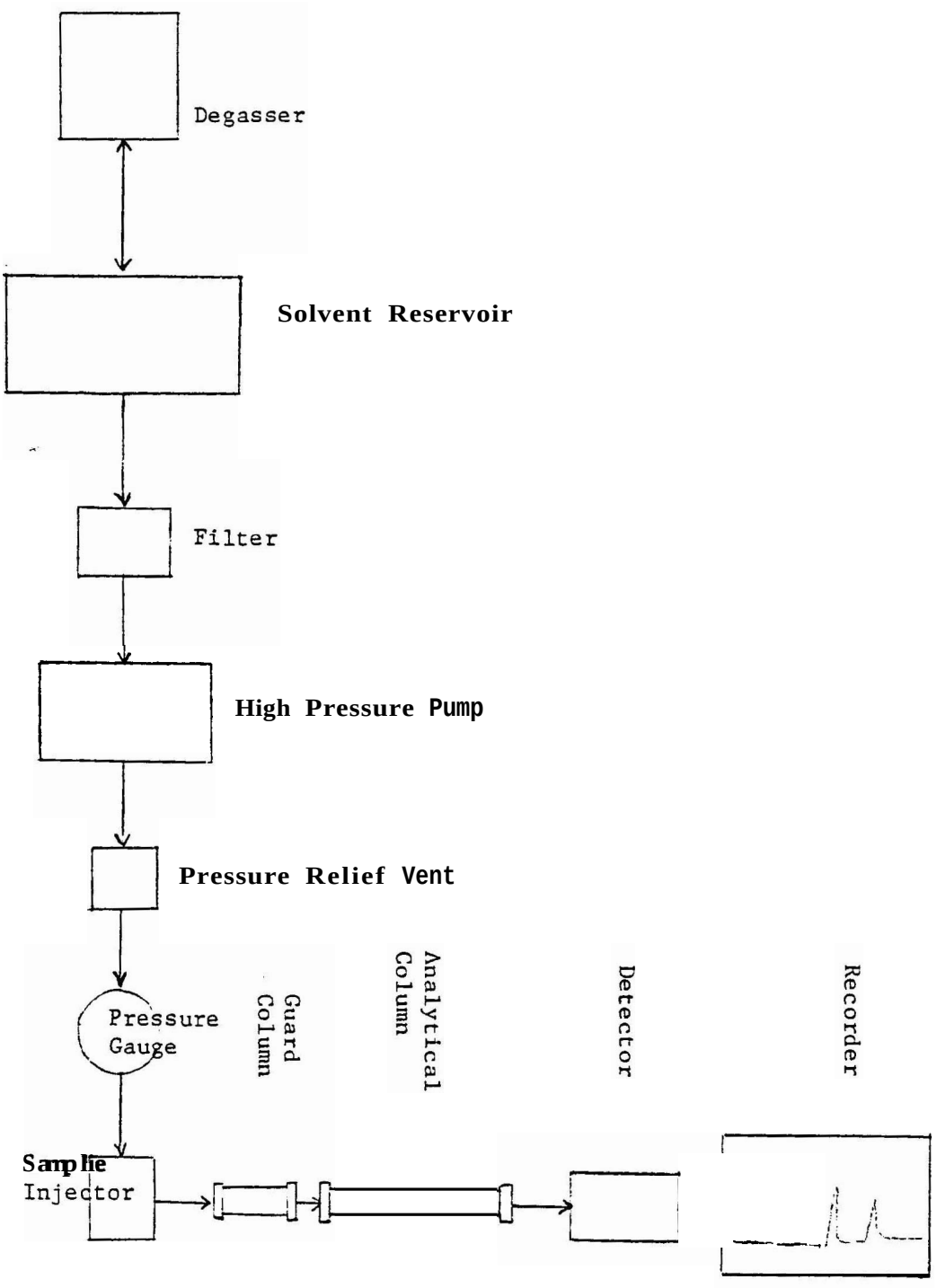


Figure 5. Block diagram showing the components of an HPLC instrument system.

CHAPTER IV

EXPERIMENTAL

Preparation of the Mobile Phase

Using a 1000 mL graduated cylinder, measure 975 mL of HPLC grade methanol. Add 25 mL of HPLC grade water to the cylinder to make one liter of 97.5 % aqueous methanol solution. The mobile phase was filtered through a Nylon-66 filter. The mobile phase was degassed for five minutes using a vacuum pump attached to the degassing system on the HPLC. The solvent container was constantly purged with helium at 2.5 lb/in^2 after degassing.

Preparation of Standards

The standard samples of d-a-tocopherol, d-'-tocopherol, and d-a-tocopherol acetate were removed from the freezer approximately one hour before being used. The samples are highly viscous materials at room temperature and are difficult to remove from the sealed vials in which they are stored. A 22.4 cm. dropper was used to remove the liquid samples from their respective vials.

Stock standard solutions of d-a-tocopherol, d-γ-tocopherol, and d-a-tocopherol acetate of 1.5 mg/mL in ethanol were prepared by weighing the liquid standard into a 10 mL volumetric flask. Working standards of d-a-tocopherol, d-γ-tocopherol, and d-a-tocopherol acetate were prepared by diluting each stock standard 100 fold with ethanol. A 50 mL volumetric flask was used and 0.5 mL of stock standard was diluted with ethanol. Stock standard solutions were stored in the freezer at -20°C and are reportedly stable for two months. (17). Working standard solutions were stored in the freezer at -20°C and are reportedly stable for one week (17).

A standard curve for d-a-tocopherol was prepared by using 50, 75, 100, and 125 µL of d-a-tocopherol working standard containing 1.5 X 10^{-2} mg/mL of d-a-tocopherol in ethanol. The standard samples were made up to a volume of 200 µL with ethanol and the sample procedure on the following page was employed. A standard curve for d-γ-tocopherol was prepared by using 50, 75, and 100 µL of d-γ-tocopherol working standard containing 1.5 X 10^{-2} mg/mL of d-)*-tocopherol in ethanol. The standard samples were made up to a volume of 200 µL with ethanol and the sample procedure on the following page was employed. Each standard sample of d-a- tocopherol and d-γ-tocopherol contained 100 µL of d-a-tocopherol acetate and 200 µL of HPLC grade water.

Sample Storage and Stability

Plasma samples were used in the procedure. Whole blood samples were collected, in most cases, after an overnight fast. Plasma samples were stored in a refrigerator at 4°C and reportedly are stable for as long as 10 days (17). Serum samples were stored in the freezer at −20°C and are reportedly stable for a period of one year (17). Plasma samples were obtained from whole blood samples by centrifuging the whole blood containing an anticoagulant at 2000 r.p.m. for 10 minutes.

Chromatographic Conditions

Samples were run at room temperature. A UV detector set at 280 nm was employed using a sensitivity setting of 0.01 AUFS. The flow rate of the mobile phase was 2.0 mL/min. A sample loop of 50 µL was employed. The chart speed of the recorder was set at 1.0 cm/min.

Procedure (17)

1. A 200 µL sample of plasma was pipetted into a 6 X 50 mm test tube. Sample size can vary from 100-200 µL, as long as an equal amount of ethanol is used to denature the

protein.

2. A 100 µL amount of a-tocopherol acetate working standard containing 1.5 x 10^{-2} mg/mL of a-tocopherol acetate in ethanol was added to the test tube.
3. A 100 µL amount of ethanol was added to the test tube. A cork was used to stopper the test tube and the tube was vortex mixed for 10 seconds.
4. A 150 µL amount of hexane was added to the test tube. The contents of the tube was vortex mixed vigorously and intermittently for 45 seconds.
5. The tube was centrifuged at 800 r.p.m. for 5 minutes.
6. A 100 µL amount of hexane containing the tocopherols (top layer) was transfered by micro-pipet to a second 6 X 50 mm test tube. The hexane was evaporated to dryness from the sample by bubbling nitrogen gas into the sample using a glass capillary tube.
7. A 50 µL amount of isopropanol was added to the residue in the test tube. A 50 µL amount of methanol was added to the test tube. A cork was placed on the test tube and the contents was shaken for 5 seconds by using the index finger.
8. A 50 µL amount of solution was injected into the liquid chromatograph.

CHAPTER V

RESULTS AND DISCUSSION

Standard Curve for d-α-Tocopherol

The standard curve for d-a-tocopherol was constructed by plotting the peak height ratio versus the weight ratio of d-a-tocopherol / d-a-tocopherol acetate. The standard curve for d-a-tocopherol is depicted in Figure 6.

The standard samples employed in constructing the curve were prepared by diluting the a-tocopherol stock standard (1.5 mg/mL) 100 fold with ethanol and taking 125, 100, 75, and 50 µL of working standard solution (1.5 $x10^{-2}$ mg/mL) for analysis. To each standard sample , 100 µL of a-tocopherol acetate working standard (1.5 $x10^{-2}$ mg/mL) and 200 µL of HPLC grade water were added. The analysis procedure previously described was employed, ultilizing a 50 µL sample injection into the chromatograph. Each standard sample was run in triplicate and the results were averaged. The peak height for a-tocopherol and a-tocopherol acetate from each standard microliter sample was measured in mm on the chromatogram. The peak height ratio of a-tocopherol / a-tocopherol acetate for each standard sample was calculated and recorded. The weights in micrograms of a-tocopherol and

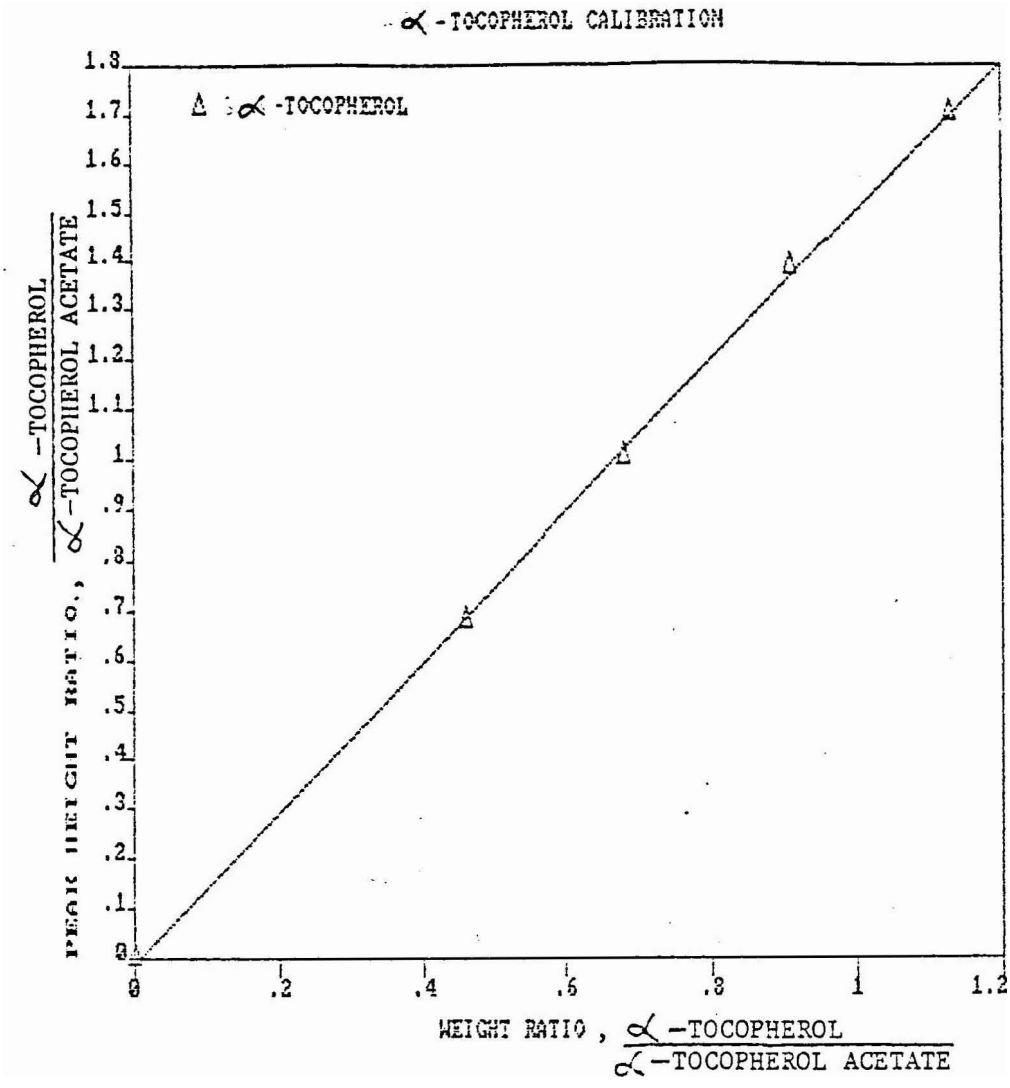


Figure 6. Standard curve for peak-height ratio vs weight ratio of ∝-tocopherol/∝-tocopherol acetate.

a-tocopherol acetate in the four standard samples were calculated from the microliters of each diluted working standard employed. The weight ratio of a-tocopherol / a-tocopherol acetate for each standard sample was calculated and recorded. The data for the calibration curve for a-tocopherol is displayed in Table 1.

TABLE 1

Data for the Calibration Curve of Alpha-tocopherol

a-tocopherol Added (µL) - -	Peak Height Ratio (a-tocopherol / a-tocopherol acetate) (mm/mm)	Weight Ratio (a-tocopherol/ a-tocopherol acetate) (µg/µg)
125	(62.0/36.5)=1.70	(1.86/1.64)=1.13
100	(49.0/35.5)=1.38	(1.49/1.64)=0.91
75	(36.0/36.0)=1.00	(1.12/1.64)=0.68
50	(24/35.5)=0.68	(0.75/1.64)=0.46

Standard Curve for d-γ-Tocopherol

The standard curve for d-γ-tocopherol was constructed by plotting the peak height ratio versus the

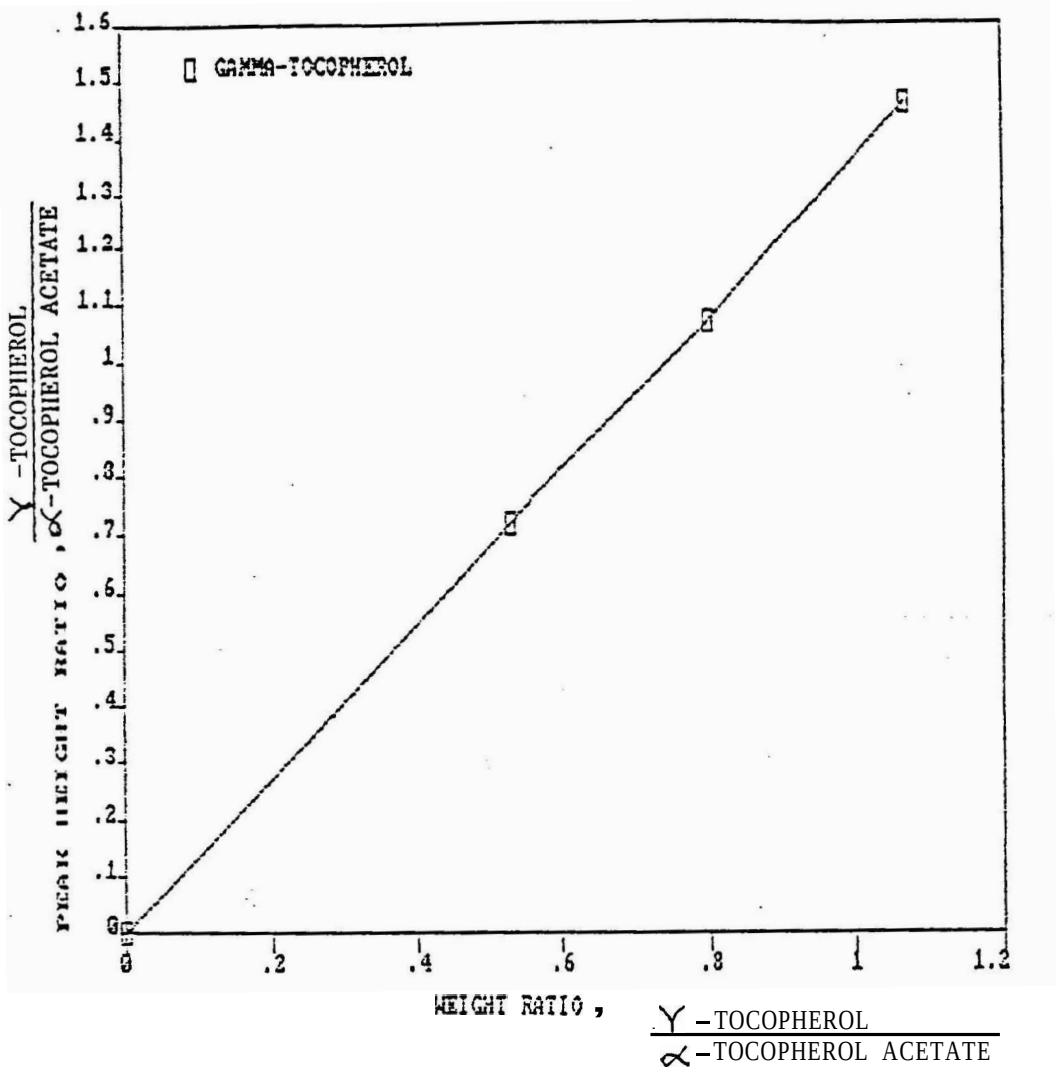


Figure 7. Standard curve for peak-height ratio vs weight ratio of γ-tocopherol /α-tocopherol acetate.

weight ratio of γ-tocopherol / a-tocopherol acetate. The standard curve for γ-tocopherol is shown in figure 7.

The standard samples employed in constructing the curve were prepared by diluting the γ-tocopherol stock standard (1.5 mg/mL) 100 fold with ethanol and taking 100, 75, and 50 µL of working standard solution (1.5 $x10^{-2}$ mg/mL) for analysis. To each standard sample, 100 µL of a-tocopherol acetate working standard (1.5 $x10^{-2}$ mg/mL) and 200 µL of distilled water were added. The analysis procedure previously described was employed, utilizing a 50 µL sample injected into the chromatograph. Each standard sample was run in triplicate and the results were averaged. The peak height for γ-tocopherol and a-tocopherol acetate for each standard microliter sample was measured in mm on the chromatogram. The peak height ratio of γ-tocopherol / a-tocopherol acetate for each standard sample was calculated and recorded. The microgram amount of 1-tocopherol and a-tocopherol acetate in the three standard samples was calculated from the microliters of each diluted working standard employed. The weight ratio of 1-tocopherol / a-tocopherol acetate was calculated and recorded for each standard sample. The data for the calibration curve for γ-tocopherol is displayed in Table 2.

Table 2

Data for the Calibration Curve of Gamma-tocopherol

γ-tocopherol Added (μL)	Peak Height Ratio (γ-tocopherol / a-tocopherol acetate) (mm/mm)	Weight Ratio (γ-tocopherol / a-tocopherol acetate) (μg/μg)
100	(56.0/38.0)=1.46	(1.75/1.64)=1.07
75	(42.0/39.0)=1.07	(1.31/1.64)=0.80
50	(28.5/39.5)=0.72	(0.875/1.64)=0.53

Calculation of the Amount of a-Tocopherol and γ-Tocopherol in a Plasma Sample

To calculate the amount of a-tocopherol in an unknown plasma sample, the following equations were employed.

$$\frac{\text{Peak height a-tocopherol}}{\text{Peak height a-tocopherol acetate}} = RT$$

$$\frac{RT \times \text{Amount of added internal standard (mg)}}{\text{Slope of standard curve} \times \text{Volume of sample (L)}} = A$$

A = a-tocopherol in mg/L

The same equation is used to calculate the amount of 1-tocopherol in a sample except the peak height of

γ-tocopherol and the slope of the γ-tocopherol standard curve are substituted in the above equations.

Retention Data

The retention times of the tocopherols investigated are listed in Table 3. Beta-tocopherol and gamma-tocopherol can not be separated by the C_{18} column used in this study. Both isomers have retention times of eight minutes and appear as a single peak on the chromatogram. Alpha-tocopherol has a retention time of 9.2 minutes and alpha-tocopherol acetate follows at 12.6 minutes.

Table 3

Retention Data of Tocopherols on C18 Column at a Flow Rate of 2 mL / min.

Isomer	Retention Time (min.)
Beta-tocopherol	8.0
Gamma-tocopherol	8.0
Alpha-tocopherol	9.2
Alpha-tocopherol acetate	12.6

Precision of the Method

A standard Fisher Diagnostics SeraChem Control Serum (Human) Level-1 sample was run six times for alpha- and gamma-tocopherol concentrations. The mean ($\bar{x}$), standard deviation (SD), and the coefficient of variation (CV), were calculated and the results displayed in Table 4.

Table 4

Precision of the Method for Alpha-tocopherol (mg/L) and Gamma-tocopherol (mg/L) by HPLC (Day to Day)

Isomer	n	$\bar{x}$	SD	CV (%)
Alpha-tocopherol	6	12.2	0.30	2.5
Gamma-tocopherol	6	1.31	o.07	5.7

Recovery of Alpha-tocopherol and Gamma-tocopherol

The percent recovery for alpha-tocopherol and gamma-tocopherol was determined on five samples. Five samples run previously for alpha-tocopherol were spiked with alpha-tocopherol and chromatographed. The amount of alpha-tocopherol added and found is noted in Table 5 along

with the percent recovery. Five samples run previously for gaama-tocopherol were spiked with gamma-tocopherol and chromatographed. The amount of gamma-tocopherol added and found is noted in Table 5 along with the percent recovery.

The percent recovery for alpha-tocopherol varied from 95.4 % to 103 % and compared favorably to the percent recoveries reported by Catignani (17). Catignani reported recoveries that ranged from 94.0 % to 116 % for alpha-tocopherol. The mean recovery for a-tocopherol for this thesis was 98.7 %.

The percent recovery for gamma-tocopherol varied from 91.8 % to 111 X. The recovery for gamma-tocopherol displayed a larger percent range when compared to alpha-tocopherol. This difference could be explained by the lower molar absorptivity of gamma-tocopherol as compared to alpha tocopherol. The mean recovery for gamma-tocopherol was 101 *f*.

Clinical Studies

Determinations of a-tocopherol and Y-tocopherol were performed by HPLC on the plasma of eleven sickle cell and eleven normal individuals. A typical chromatogram of the plasma of a normal individual is displayed in figure 8. A typical chromatogram of the plasma of a sickle cell patient is displayed in figure 9. The three relevant peaks present

TABLE 5

ANALYTICAL RECOVERIES OF ADDED α-TOCOPHEROL AND γ-TOCOPHEROL (n=3)

SAMPLE	CONCENTRATION IN PLASMA (mg/L)	CONCENTRATION ADDED (mg/L)	ADDED CONCENTRATION FOUND (mg/L)	RECOVERY PERCENT
		α-TOCOPHEROL		
20FS	5.88	7.18	7.39	103
19FN	9.33	7.18	6.96	96.9
24FS	7.71	7.34	7.39	101
24MS	4.00	7.34	7.00	95.4
24FN	8.20	7.23	7.01	97.0
		γ-TOCOPHEROL		
24FN	1.00	8.62	9.58	111
23MS	4.37	8.62	7.91	91.8
24MS	1.62	8.62	8.38	97.2
24FS	3.77	8.62	9.10	106
20FS	4.31	8.62	8.50	98.6

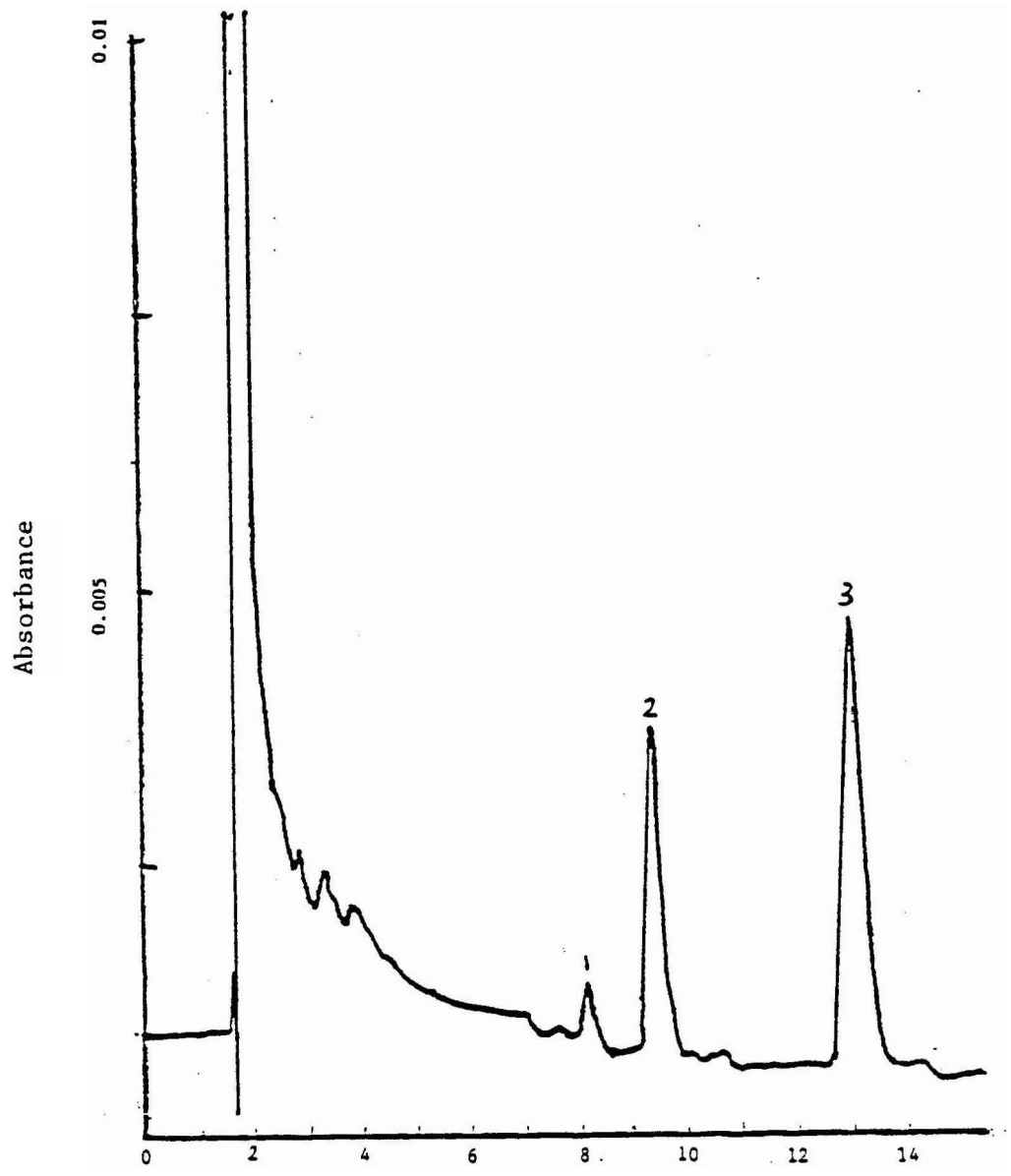


Figure 8. HPLC chromatogram of the plasma of a five year old normal male at 280 nm.
Peaks: (1) γ-tocopherol. (2) α-tocopherol.
(31.4.-tocopherol acetate.

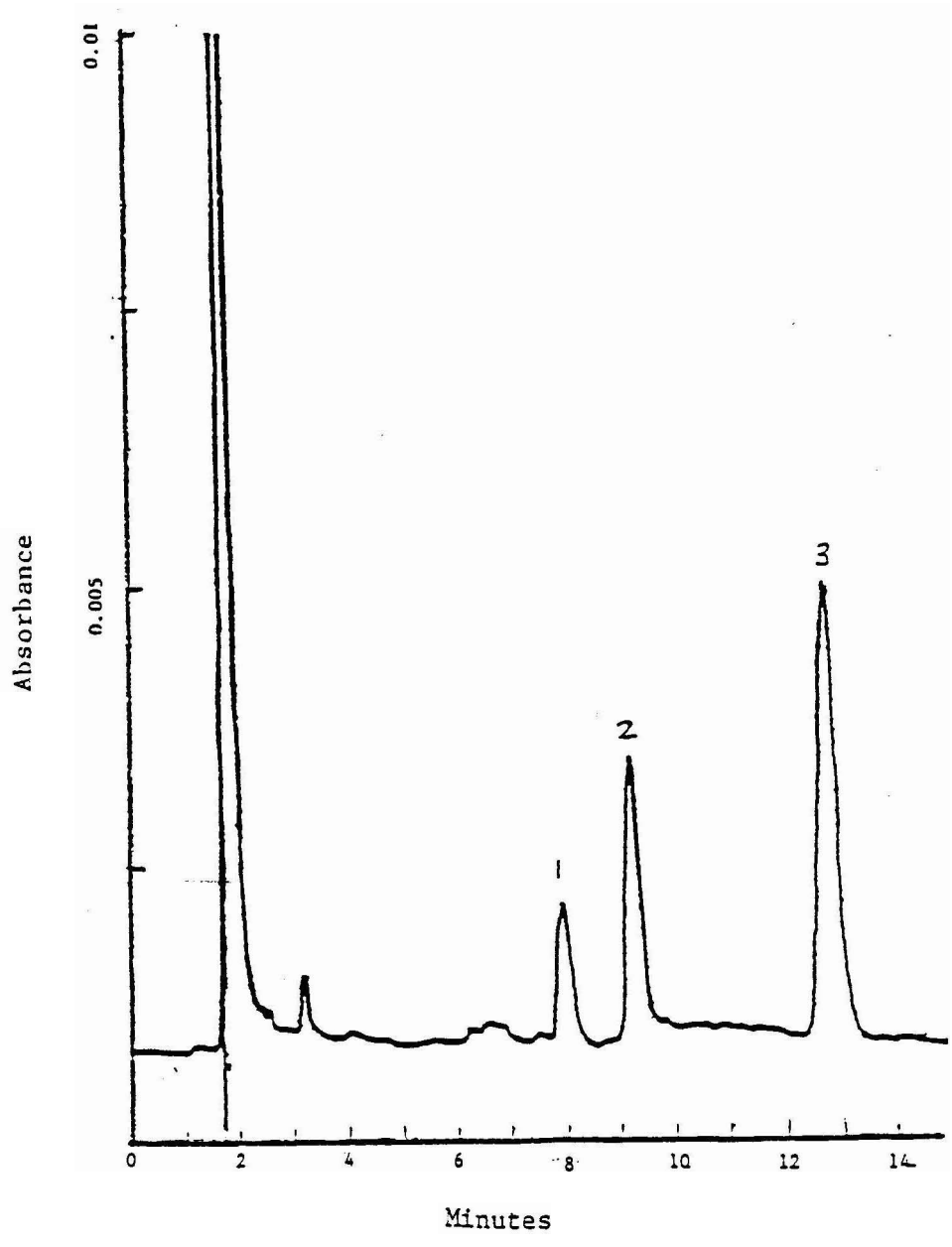


Figure 9. HPLC chromatogram of the plasma of a twenty five year old sickle cell patient at 280 nm.
Peaks: (1) γ-tocopherol. (2) α-tocopherol.
(3) α-tocopherol acetate.

in each chromatogram correspond to a-tocopherol, γ-tocopherol, and a-tocopherol acetate.

The amount of a-tocopherol and γ-tocopherol in each of the eleven plasma samples of the sickle cell patients were determined and the results found are displayed in Table 6. The results are arranged according to age, with the youngest patient first. The amount of a-tocopherol and γ-tocopherol in each of the eleven plasma samples of the normal patients were determined and the results found are displayed in Table 7. The results are arranged according to age.

According to the data in Table 6, ten of the eleven sickle cell plasma samples had a-tocopherol levels of less than five mg/L. According to the data in Table 7, ten of the eleven normal plasma samples had a-tocopherol levels higher than five mg/L. A level of a-tocopherol of five mg/L or lower is associated with a deficiency of vitamin E that can lead to an increased state of erythrocyte hemolysis by hydrogen peroxide attack on the red cell membrane (4).

According to the data in table 6, the 1-tocopherol levels in the plasma of the sickle cell patients ranged from 1.23 to 4.31 mg/L. From table 7, the γ-tocopherol levels in the plasma of the normal individuals ranged from 1.00 to 2.79 mg/L. The γ-tocopherol range for the plasma of the sickle cell patients was found to be higher than the range for the

TABLE 6

THE AMOUNT OF ∝-TOCOPHEROL ANDY-TOCOPHEROL IN PLASMA OF SICKLE CELL PATIENTS (N=11)

SAMPLE *	CONCENTRATION ∝-TOCOPHEROL (mg/L)	CONCENTRATION ϒ-TOCOPHEROL (mg/L)	TOTAL CONC. TOCOPHEROLS (mg/L)	PERCENT ∝-TOCOPHEROL IN TOTAL	PERCENT ϒ-TOCOPHEROL IN TOTAL
02FS	4.42	1.23	5.65	78.2	21.8
04MS	4.32	1.25	5.57	77.6	22.4
07MS	4.39	2.30	6.69	65.6	34.4
16FS	3.58	2.30	5.88	60.9	39.1
19MS	3.92	1.69	5.61	69.9	30.1
20FS	5.88	4.31	10.2	57.6	42.4
23MS	4.37	2.45	6.82	64.1	35.9
24FS	7.71	3.77	11.5	67.2	32.8
24MS	4.00	1.62	5.62	71.2	28.8
25FS	4.89	2.75	7.64	64.0	36.0
28FS	4.28	1.78	6.06	70.6	29.4

* NUMBER INDICATES AGE; F, INDICATES FEMALE; M, INDICATES MALE; S, INDICATES SICKLE CELL, SAMPLES RUN IN TRIPLICATE

TABLE 7

THE AMOUNT OF α-TOCOPHEROL AND γ-TOCOPHEROL IN PLASMA OF NORMAL PATIENTS (N=11)

SAMPLE *	CONCENTRATION α-TOCOPHEROL (mg/L)	CONCENTRATION γ-TOCOPHEROL (mg/L)	TOTAL CONC. TOCOPHEROLS (mg/L)	PERCENT α-TOCOPHEROL IN TOTAL	PERCENT γ-TOCOPHEROL IN TOTAL
03MN	6.94	1.50	8.44	82.2	17.8
05MN	5.27	0.96	6.23	84.6	15.4
06MN	5.98	1.69	7.67	78.0	22.0
08MN	8.59	1.07	9.66	88.9	11.1
12FN	4.79	1.78	6.57	72.9	27.1
15FN	5.36	1.58	6.94	77.2	22.8
19FN	9.33	2.27	11.6	80.4	19.6
20FN	6.58	2.10	8.68	75.8	24.2
21MN	6.00	1.34	7.34	81.7	18.3
24FN	8.20	1.00	9.20	89.1	10.9
27MN	6.88	2.79	9.67	71.1	28.9

* NUMBER INDICATES AGE; F, INDICATES FEMALE; M, INDICATES MALE; N, INDICATES NORMAL. SAMPLES RUN IN TRIPLICATE

γ-tocopherol of the normal plasma.

A statistical t test (75) was performed on the population data of the a-tocopherol and γ-tocopherol results for the sickle cell and normal plasma samples. The t test was performed to compare the means of the a-tocopherol results of the sickle cell and normal plasma samples with the intent of determining if a statistical difference exists between the means. The t test was also performed to compare the means of the γ-tocopherol results of the sickle cell and normal plasma samples with the intent of determining whether a statistical difference exists between those means. The t test was also performed on the total tocopherol results for the sickle cell and normal plasma samples.

The mean ($\bar{x}$) and standard deviation (s) for a-tocopherol for the eleven plasma samples of the sickle cell patients and the eleven plasma samples of the normal individuals were calculated and are displayed in Table 8.

Table 8

Statistical Analysis of a-Tocopherol using the t Test

Sickle Cell Plasma	Normal Plasma
$x_1 = 4.71$	$x_2 = 6.72$
$s_1 = 1.16$	$s_2 = 1.46$
$n_1 = 11$	$n_2 = 11$

The t test was performed on the data and t was found to be 3.575. For a probability of 95% (p = 0.05) and twenty degrees of freedom, t found from the t-table was 2.09 (75). From the t test, a 95% certainty of a real difference in the sample means exists between the a-tocopherol of the plasma of the sickle cell patients and the a-tocopherol of the plasma of the normal individuals.

The mean and standard deviation for γ-tocopherol for the eleven plasma samples of the sickle cell patients and the eleven plasma samples of the normal patients were calculated and are displayed in Table 9.

Table 9

Statistical Analysis of γ-Tocopherol using the t Test

Sickle Cell Plasma	Normal Plasma
$\bar{x}_1 = 2.31$	$\bar{x}_2 = 1.64$
$s_1 = 0.989$	$s_2 = 0.571$
$n_1 = 11$	$n_2 = 11$

The t test was performed on the γ-tocopherol data and t was found to be 1.95. For a probability of 95% (p = 0.05) and twenty degrees of freedom , t found from the t-table was 2.09. From the t test, the probability that a significant statistical difference exists between the means of the

γ-tocopherol in the plasma of the sickle cell patients and the γ-tocopherol in the plasma of the normal patients was inconclusive. The probability that a significant statistical difference exists for the means of the total tocopherols for the sickle cell patients and the normal individuals was found to be inconclusive from the t test.

The statistical comparison of the means for the gamma-tocopherol plasma levels between the sickle cell patients and the normal individuals was insignificant. However, from table 6 and table 7 the percent of gamma-tocopherol in the total tocopherols in the plasma of the sickle cell patients varied from 22 to 42 percent while the gamma-tocopherol percent in the total tocopherols in the plasma of the normal individuals varied from 11 to 29 percent. From this data, the percent range of gamma-tocopherol in sickle cell patients is higher than the percent range in normal individuals.

CHAPTER VI

CONCLUSIOHS

A review of the literature indicates that vitamin E in biological materials can be determined by Colorimetric, Gas Chromatographic, and HPLC methods. The HPLC method which is the least time consuming and embodies good sensitivity with few interferences evolved as the method of choice. The reverse phase HPLC method for determining a-tocopherol in plasma employed a UV detector set at 280 nm. The sensitivity of the HPLC method to determine a-tocopherol can be enhanced 20 times by employing a fluorescent detector.

Vitamin E studies on sickle cell patients has led some investigators to conclude that a difference in a-tocopherol levels in plasma between sickle cell and normal individuals does not exist (16). However, results of a-tocopherol levels from this paper and results from Chiu *et al* (9), and Natta *et al* (10) have demonstrated that a significant difference in a-tocopherol plasma levels between sickle cell and normal individuals does exist. The sickle cell patients' plasma averaged 2 mg/L less a-tocopherol than the normal individuals' plasma according to results from this study.

From this study, a statistical t test was performed

with a 95% confidence level and the t test demonstrated that a difference exists between the means of the a-tocopherol levels in the plasma of the sickle cell and normal individuals. The t test was inconclusive in demonstrating a difference between the means for $-tocopherol levels in the plasma of the sickle cell and normal individuals. The t test was also inconclusive in demonstrating a difference between the means for total tocopherols (α+β+$) in the plasma of the sickle cell and normal individuals. Since the biological activity of d-a-tocopherol, which is 1.49 International Units per milligram (IU/mg), is much higher than the biological activity of d-)/-tocopherol which is unofficially listed as 0.15 IU/mg (76), the d-a-tocopherol isomer is considered the most important in biological activity and in comparison of the means for the t test. The unofficial biological activity of the d-$-tocopherol isomer is listed as 0.75 IU/mg. However, since the amount of d-$-tocopherol in normal adult plasma is about 2% (2), the d-$-tocopherol isomer has little significance for comparison of the means.

Sickle cell anemia, being a hereditary disease, is associated with a genetic defect. Individuals suffering from genetic defects must struggle with the symptoms, since in most cases they cannot be cured. Attempts to treat individuals with sickle cell disease have focused primarily on reducing the debilitating symptoms of those affected.

Since a deficiency in a-tocopherol plasma levels in sickle cell anemia patients exists, a program involving the administration of vitamin E to sickle cell patients should be investigated. Such a program would involve a long term study of several years or more and probably would only be beneficial if the vitamin E was administered early in the life of the sickle cell patients. Such a program would involve considerable expense, time, and cooperation of a large number of people. The object of the program would be to prolong the life of persons suffering from sickle cell anemia. Given the multitude of problems such a study would entail, it is apparent why no persons to date have embraced a long term vitamin E administration program for sickle cell anemia patients. Such a program should be undertaken, since a good chance exists that administration of vitamin E to sickle cell patients could be beneficial.

A short term study of vitamin E administration has been conducted by C. L. Natta et al (10). This short term study of 35 weeks involved daily vitamin E administration to sickle cell anemia patients who consequently displayed a significant decrease in the number of irreversibly sickled cells detected in their plasma.

Another area of future study should involve the determination of vitamin E in red blood cells. Red blood cells are considered a better indicator of vitamin E

nutritional status than plasma since plasma vitamin E is affected by total lipid concentration in the blood while erythrocyte vitamin E levels are essentially unaffected (77).

In conclusion, the HPLC method developed for the determination of vitamin E in plasma is resonably fast and accurate. The plasma a-tocopherol level in sickle cell anemia patients was found to be significantly lower when compared to the plasma a-tocopherol level in normal individuals.

REFERENCES

1. Brewster, M. A. in Clinical Chemistry; Kaplan, L. A.; Pesce, A. J. Eds; C. V. Mosley: St Louis, 1984; pp 663-664.
2. McCay, P. B.; King, M. M. in Vitamin E, a Comprehensive Treatise; Machin L. J. Ed; Marcel Dekker: New York, 1980; p 289.
3. Brewster, M. A.; Naito, H. K. Nutritional Elements and Clinical Biochemistry; Plenum : New York, 1980; pp 135-167.
4. Sanberlich, H.; Dowdy, R. P.; Skala, J. Laboratory Tests for the Assessment of Nutritional Status; CRC: Boca Raton, Fla., 1974; p 23.
5. Farrell, P. M.; Bieri, J. G. Amer. J. Clin. Nutr. 1975, 28, 138.
6. Bieri, J. G.; Erarts, R. P.; Thorp, S. Amer. 3. Clin. Nutr. 1977, 30, 686.
7. Sodeman, Jr., W. A.; Sodeman, T. M. Patholoaic Physiology; W. B. Saunders: Philadelphia, 1979; p 83.
8. Latner, A. L. Clinical Biochemistry; W. B. Saunders: Philadelphia, 1975; pp 268-269.
9. Chiu, D.; Vichinsky, E.; Yee, M.; Kleman, K.; Lubin, B. *Ann.* N.Y. Acad. Sci. 1982, 323-333.
10. Natta, C. L.: Machlin, L. J.; Brin, M. Amer. J. Clin. Nutr. 1980, 33, 968.

11. Clark, M. L.; Mohandas, N.; Shohet, S. B. J. Clin. Invest. 1980, 65, 189.
12. Chien, S.; Usami, S.; Bertles, J. J. Clin. Invest. 1970, 49, 623.
13. Sergent, G. R.; Sergent, B. E.; Miller, B. F. Brit. Jour. Haematol. 1969, 17, 527.
14. Jain, S. K.; Shohet, S. B. Clin. Res. 1981, 29, 519.
15. Shohet, S. B.; Jain, S. K. *Ann.* N.Y. Acad. Sci. 1982, 229.
16. Muskiet, F. D.; Muskiet, F. A. Clinica Chimica Acta 1984, 142, 1.
17. Catignani, G. L.; Bieri, J. Clin. Chem. 1983, 29, 708.
18. Tsen, C. C. Anal. Chem. 1961, 33, 849.
19. Nelis, H. J.; De Bevere, V. O.; De Leenheer, A. P. in Modern Chromatographic Analysis of the Vitamins; De Leenheer, A.; Lambert, W.; Ruyter, M., Ed.; Marcel Dekker: New York, 1985; pp 130-182.
20. Sheppard, A. J.; Presser, A. R.; Hubbard, W. D. J. Amer. Oil Cher. Soc. 1972, 49, 619.
21. Lehmann, J. Lipids 1980, 15, 135.
22. Shen-Nan Lin; Horning, E. C. J Chromatogr. 1975, 112, 465.
23. Nair, P. P.; Machiz, J. Biochim. Biophys. Acta 1967, 144, 446.
24. Wilson, P. W.; Kodicek, E.; Booth, V. Biochem. J. 1962, 84, 524.
25. Feeter, D. K. J. Amer. Oil Chem. Soc. 1974, 51, 184.
26. Nelson, J. P.; Milun, A. J.; Fisher, H. D. J. Amer. Oil

Chem. Soc. 1970, 47, 259.

27. Oliveira, M. M.; Weglicki, W. V.; Nason, A.; Hair, P. P. Biochim. Biophys. Acta 1969, 180, 98.
28. Kovensky, B. J.; Day, R. J. Chromatogr. Sci. 1971, 9, 442.
29. Nakanishi, M.; Tsuchiya, K.; Sakaguchi, K.; Fujita, T. Chem. Abstr. 1980, 92, 34251x.
30. Thomas, D. W.; Parkhurst, R. M.; Negi, D. S.; Lunan, K. J. Chromatogr. 1981, 225, 433.
31. Morimoto, H.; Amano, T.; Toyoda, M.; Ashida, Y. Biochem. Med. 1973, 7, 169.
32. Kobayashi, H.; Kanno, C.; Yamauchi, K.; Tsugo, T. Biochim. Biophys. Acta 1975, 380, 282.
33. Govind Rao, M.K.; Perkins, E. J. Agr. Food Chem. 1972, 20, 240.
34. Nair, P. P.; Luna, Z. Arch Biochem. Biophys. 1968, 127, 413.
35. Bowman, P. B.; West, W. E. J. Pharm. Sci. 1968, 57, 470.
36. Lehmann J.; Slover, H. T. Lipids 1971, 6, 35.
37. Christie, A.; Dean, A. C.; Millburn, B. A. Analyst 1973, 98, 161.
38. Slover, H. T.; Shelley, L. M.; Burks, T. J. Oil Amer. Chem. Soc. 1967, 44, 161.
39. Mordret, F.; Laurent, A. M. Rev. Fr. Corps Gras 1978, 25, 245.
40. Slover, H. T.; Thompson, R. H. Lipids 1981, 16, 268.
41. Lovelady, H. G. J. Chromatour. 1973, 85, 81.

42. Bieri, J. G.; Prival, E. L. Proc. Soc. Exp. Biol. Med. 1965, 120, 554.

43. Bieri, J. G.; Poukka, R. K.; Prival, E. J. Lipid Res. 1970, 11, 118.

44. Kayden, H. J.; Chow, C. K.; Bjornson, L. J. Lipid Res. 1973, 14, 533.

45. Herting, D. C.; Drury, E. J. J. Chromatogr. 1967, 30, 502.

46. Slover, H. T.; Lehmann, J.; Valis, R. J. J. Amer. Oil Chem. Soc. 1969, 46, 417.

47. Crawford, R. R.; Naramore, D. C.; Esmerian, O. K. J. Pharm. Sci. 1968, 57, 1716.

48. Emmerie, A.; Engel, C. Rec. Trav. Chim. 1938, 57, 1351.

49. Pennock, J. F.; Hemming, F. W.; Kerr, J. Biochem. Biophys. Res. Commun. 1964, 17, 542.

50. Lehmann J.; Martin, H. L. Clin. Chem. 1983, 29, 1840.

51. Bollinger, H. R.; Konig, A. in Thin-laver Chromatography, Stahl, E., Ed.; Springer: New York, 1958; p 286.

52. Thompson, J. N.; Erdody, P.; Maxwell, W. B. Anal. Biochem. 1972, 50, 267.

53. Schmit, J. A.; Henry R. A.; Williams, R.; Dieckman, J. F. J. Chromatogr. Sci. 1971, 9, 645.

54. Cook, J. D.; Koch, T. R.; Knoblock, E. Clin. Chem. 1986, 32, 1193.

55. Van Niekerk, P. J. Anal. Biochem. 1973, 52, 533.

56. Matsuo, M.; Tahara, Y. Chem. Pharm. Bull. 1977, 25, 3381.

57. Abe, K.; Yuguchi, Y.; Katsui, G. J. Nutr. Sci. Vitaminol. 1975, 21, 183.

58. Hiroshima, O.; Ikenoya, S.; Ohmae, M.; Kawabe, K. Chem. Pharm. Bull. 1981, 29, 451.

59. Cavins, J. F.; Inglett, G. E. Cereal Chem. 1974, 51, 605.

60. Thompson, J. N.; Hatina, G. J. Liquid Chromatogr. 1979, 2, 327.

61. Tieback, R. K.; Schramm, M. Chromatographia 1980, 13, 403.

62. Cohen, H.; Lapointe, M. R. J. Ass. Office. Anal. Chem. 1980, 63, 1254.

63. De Leenheer, A. P.; De Bevere, V. O.; Cruyl, A. A.; Claeys, A. E. Clin. Chem. 1978, 24, 585.

64. Karger, B.; Martin, M.; Guiochon, G. Anal. Chem. 1974, 46, 1640.

65. Nilsson, B.; Johansson, B.; Jansson, L.; Holmberg, J. J. Chromatogr. Biomed. Appl. 1978, 145, 169.

66. Widicus, W. A.; Kirk. J. R. J. Assoc. Offic. Anal. Chem. 1979, 62, 637.

67. Jansson, L.; Nilsson, B.; Lindgren, R. J. Chromatogr. Biomed. Appl. 1980, 181, 242.

68. Hatam, L. J.; Kayden, H. J. J. Lipid Res. 1979, 20, 639,

69. Tangney, C. C.; McNair, H. M.; Driskell, J. J. Chromatogr. 1981, 224, 389.

70. Ikenoya, S.; Abe, K.; Tsuda, T.; Yamano, Y. Chem. Pharm. Bull. 1979, 27, 1237.

71. Vatassery, G. T.; Hagen, D. F. Anal. Biochem. 1977, 79, 129.

72. Snyder, L. R.; Kirkland, J. J. Introduction to Modern Liquid Chromatography, 2nd ed.; J. Wiley : New York, 1979; p 554.

73. De Leenheer, A. P.; De Bevere, V. O.; Claeys, A. E. Clin. Chem. 1979, 25, 425.

74. Westerberg, E.; Friberg, M.; Akesson, B. J. Liquid Chromatogr. 1981, 4, 109.

75. Freund, J. E. Statistics, Prentice-Hall : Englewood Cliffs, New Jersey, 1970; p 223.

76. Horwitt, M. K. Vitamin E Abstr. 1985, vi.

77. Vatassery, G. T.; Krezowski, A. M.; Eckfeldt J. H. Amer. J. Clin. Nutri. 1983, 37, 1020.