A high-performance liquid chromatographic method for the determination of tocopherol in plasma and cellular elements of the blood

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Abstract A rapid, sensitive, and reproducible procedure is described for the analysis of α-tocopherol in blood cells and plasma using high-performance liquid chromatography and fluorometric detection. The cardinal feature for the increased sensitivity of this high-performance liquid chromatographic procedure is that the fluorometric analysis was carried out at a short excitation wavelength (205 nm) which increased the sensitivity 20-fold over the usual excitation wavelength of 295 nm. Tocopherol levels can be measured in as little as 50 μl of plasma and 200 μl of erythrocytes. The tocopherol content of plasma, red blood cells, platelets, polymorphonuclear leukocytes, and lymphocytes of normal subjects and subjects ingesting additional quantities of vitamin E are reported. The values for the white cells are approximately 30 times higher than those of the red blood cells (polymorphonuclear leukocytes 4.47 ± 0.62 $\mu g/10^9$, lymphocytes 3.89 ± 0.85 $\mu g/10^9$, and erythrocytes 1.40 ± 0.17 $\mu g/10^{10}$ cells). The tocopherol contents of the plasma and all the cellular elements of the blood were increased by oral feeding with vitamin E.—**Hatam, L. J., and H. J. Kayden.** A high-performance liquid chromatographic method for the determination of tocopherol in plasma and cellular elements of the blood. *J. Lipid Res.* 1979. **20:** 639–645.

Supplementary key words vitamin E · α-tocopherol · erythrocytes · platelets · polymorphonuclear leukocytes · lymphocytes · lipid peroxidation · adriamycin toxicity · anemia of the premature infant

There is continued interest in the potential role of vitamin E as an important agent in human cellular metabolism. Recently, its potential protective role against the toxicity of antitumor agents has been studied in rodents, and its effect in modifying the acute respiratory-distress syndrome and development of bronchopulmonary dysplasia in the human newborn has been reported (1, 2). We have previously developed a method for the spectrophotometric analysis of tocopherol in human red blood cells (3). When this method was used to measure the tocopherol content of white blood cells, there were too few cells in blood samples of as much as 50 ml to give valid results. The results of a pilot study using a unit of blood from ambulatory donors showed that tocopherol values for the white cells were higher than the levels found in red blood cells or in platelets (4). The development of high-performance liquid chromatography (HPLC) suggested to us that a method using this technique might be more sensitive. In this report we present such a method, including the details of cell separation, the analysis of γ- and α-tocopherol after separation by HPLC, and the use of a fluorometer at a shorter excitation wave length than has previously been utilized. The method is reliable and sensitive; it requires as little as 0.1 ml of blood for plasma determinations, but at least 10 ml of blood for white blood cell analysis.

MATERIALS AND METHODS

Cell isolation

Platelets, lymphocytes, and polymorphonuclear leukocytes were isolated from 40 ml of blood collected in 5% EDTA (0.3 ml/10 ml blood) using a modification of the method of Boyum (5) to obtain a maximum yield of pure cell fractions. All procedures were carried out with plastic ware. The blood was first spun at 200 *g* for 10 min, and the platelet-rich plasma was removed. Platelets were pelleted by centrifugation at 800 *g* and washed three times with 10 ml of 0.01 M phosphate-buffered saline, pH 7.4, (PBS) containing 4 drops of 3% EDTA to prevent clumping. The washed platelet button was resuspended in PBS and an aliquot was counted in a hemocytometer after dilution in 3% procaine hydrochloride. Aliquots of the platelet suspension in PBS were extracted and analyzed as described below.

Abbreviations: HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

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After removal of the platelet-rich plasma, the remaining cell layer was diluted 1:1 with PBS and layered on a Ficoll-Hypaque (FH) gradient containing 34% Hypaque (Winthrop Laboratories, New York, NY) in 0.10 M Tris, pH 7.4, and 9% Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.01 M Tris, pH 7.4, at a ratio of 1:2.4, respectively. The diluted blood (20–25 ml) was carefully layered onto 13 ml of the FH mixture, and tubes were spun 30 min at 350 *g* in a Sorvall GLC-1 centrifuge (Dupont Instruments, Newtown, CT) using an HL-4 swinging bucket rotor. The lymphocyte layer at the interface of the FH gradient was removed, diluted with PBS, and pelleted by centrifugation at 500 *g*. In order to remove monocytes, the lymphocyte button was resuspended in PBS containing 50% autologous plasma (37°C) and applied to a 0.3-g glass wool column (15 mm × 40 mm) kept at 37°C. After 20 min, the column was drained and washed three times with PBS. The column effluents were combined, and the lymphocytes were pelleted by centrifugation. The lymphocytes were washed three times at 200 *g* with 10 ml of PBS containing 4 drops of 3% EDTA. The final suspension was counted in a hemocytometer and aliquots were removed for extraction of tocopherol as described below.

The granulocyte–RBC sediment from the bottom of the FH gradient was washed once with an equal volume of PBS, and these washed cells were diluted 1:1 with 6% Dextran in 0.9% saline (Abbott Laboratories, North Chicago, IL). This suspension was transferred to a 50-ml graduated cylinder that was placed at a 45° angle and the red cells were allowed to sediment. After 1 hr the upper phase was carefully removed, diluted with PBS, and the granulocytes were pelleted by centrifugation for 10 min at 500 *g*. The granulocytes were suspended in 0.5 ml of PBS and subjected to a hypotonic shock to remove any contaminating erythrocytes. Distilled water (6 ml) was added to these cells for 20 sec, and isotonicity was restored by the addition of 2 ml of 0.6 M NaCl. The cells were then washed three times with 10 ml of PBS containing 4 drops of 3% EDTA, using gentle centrifugation (200 *g* for 10 min) to remove red cell ghosts. The final cell suspension was counted in a hemocytometer and extracted as described below.

The purity of each cell preparation was determined by microscopic examination of slides prepared with Wright-Giemsa staining solution (J. T. Baker Chemical Co., Phillipsburg, NJ). Residual monocyte contamination of the lymphocyte preparation, as determined by latex ingestion, was less than 3%. Contamination of the platelet or granulocyte fractions with heterologous cells was less than 0.1%.

Saponification and extraction

The saponification and extraction of α-tocopherol from plasma and RBC samples were done as previously described for analysis by TLC (3). However, owing to the sensitivity of the HPLC method, the amount of sample required was reduced 10-fold in plasma (to 0.1 ml) and 4-fold in RBC (to 0.5 ml of washed cells in PBS at 50% hematocrit), and internal standardization using [^{14}C]α-tocopherol was not necessary. Routinely, 1.0 ml of platelet ($6–10 \times 10^8$ cells) or leukocyte ($2–5 \times 10^7$ cells) suspension was treated with 2.0 ml of 1% ascorbic acid in 100% ethanol in a glass-stoppered 15-ml centrifuge tube. This solution was heated at 70°C for 2 min, 0.3 ml of saturated KOH was added, and tubes were incubated 30 min in a 70°C water bath. After cooling on ice, 1.0 ml of distilled water and 4.0 ml of hexane (Burdick and Jackson Laboratories, Inc., Muskegon, MI) were added. The tubes were shaken vigorously for 2 min and phases were separated by centrifugation. An aliquot of the hexane phase (3.0 ml) was pipetted into a small conical centrifuge tube, evaporated to dryness under a stream of N_2, and redissolved in 0.1–0.3 ml of methanol. Aliquots (2–20 μl) were injected on the HPLC column. Results are expressed as the average of duplicate extractions and injections.

HPLC analysis

A Waters Associates model ALC/GPC-204 liquid chromatograph with a Model 6000 A solvent delivery system and a Model U6K injector was used (Waters Associates, Milford, MA). The detection system was a FS 970 fluorometer equipped with a 5-μl flow-thru stainless steel cuvette assembly with a quartz window and a deuterium lamp (Schoeffel Instrument Corp., Westwood, NJ). Fluorescence was recorded on an Omniscribe B-5000 strip chart recorder (Houston Instruments, Austin, TX). The analytical column was a microparticle reverse phase partition column, μ Bondapak C_{18}, 3.9 mm × 30 cm (Waters Associates); the mobile phase was methanol–water 95:5. This solvent mixture was degassed by filtration through a 0.5-μm filter (Millipore Corporation, Bedford, MA) prior to use. The flow rate of the mobile phase was 2 ml/min. The column effluent was monitored at an excitation of 205 nm with an emission filter of 340 nm. The UV spectrum of tocopherol in methanol was determined using a Cary 14 recording spectrophotometer (Applied Physics Corp., Div. of Varian, Monrovia, CA). Pure α-tocopherol was supplied by Hoffmann-La Roche, Inc., Nutley, NJ, and standards from 5 to 20 ng in methanol were injected to provide a linear concentration vs. peak height graph. β-, γ-, and δ-tocopherols

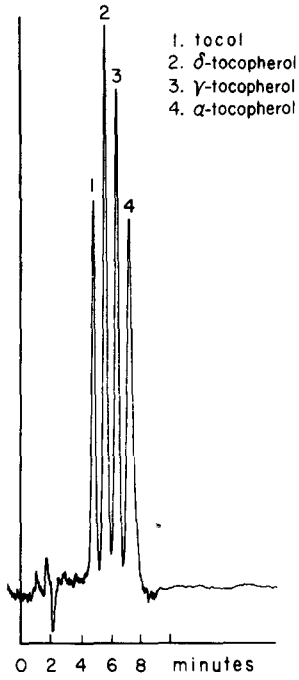

Fig. 1. Chromatogram of HPLC separation of tocol, δ-, γ-, and α-tocopherol. A 20-μl sample containing 35 ng of a mixture of different amounts of the compounds was injected. Analysis was performed on a μBondapak C18 column; the mobile phase was methanol–water 95:5; flow rate was 2 ml per min; and detection was by a fluorometer set at Ex 205 nm and Em 340 nm.

and tocol were supplied by Dr. K. Abe, Eisai Research Laboratories, Tokyo, Japan.

The minimal amount of α-tocopherol that could be detected was 1 ng. Retention time of α-tocopherol was 7.2 min. A standard curve of 5, 10, and 20 ng was determined at the beginning and end of a series of analyses. The separation of α-, β-, γ- and δ-tocopherol, and of tocol, is presented in **Fig. 1.**; β- and γ-tocopherol elute as a single peak with a retention time of 6.3 min.

RESULTS

The UV absorption spectrum of α-tocopherol in methanol showed two absorption peaks with maxima at 212 nm and 292 nm, with greater intensity at the shorter wavelength. Short excitation wavelength fluorometric detection has been used for several compounds to increase the sensitivity of detection (6). This requires a light source with sufficient energy at wavelengths below 250 nm. The fluorescence excitation spectrum of α-tocopherol in methanol–water 95:5 using the Schoeffel Model FS 970 spectrofluorometer equipped with a deuterium lamp and a cut-off emission wavelength filter of 340 nm is shown in **Fig. 2.** The difference in response between the shorter and longer excitation wavelengths was approximately 20-fold. Thus, excitation at 205 nm was used in all determinations reported in this paper in contrast to previous publications in which 295 nm was most often used for the excitation wavelength.

Representative chromatographs from a platelet extract and a polymorphonuclear leukocyte extract are shown in **Fig. 3.** The fluorescent compounds with short retentions are unidentified and may represent impurities in the solvents (hexane, ethanol) which are separated from the tocopherols by the HPLC column.

The completeness of extraction of tocopherol from plasma, cells, and tissues has been variable and, in previous methods, saponification has been necessary to obtain complete recoveries (3, 7, 8). A comparison was made between the extraction of α-tocopherol with hexane after the addition of ethanol but without saponification and extraction by hexane after saponification of the ethanol-treated sample using heat and saturated KOH, and adding known quantities of α-tocopherol. The results in **Table 1** indicate that satisfactory recoveries can be obtained using direct extraction without prior saponification. However, since the saponification step was described in the original extraction procedure, it has been used in all the analyses in this report. The recovery of α-tocopherol after the addition of known quantities to cell suspensions was greater than 90%; therefore, internal standardization was not used in the HPLC method for the determination of α-tocopherol.

The accuracy of the HPLC–fluorometric method was validated by the comparison of the analyses of five separate plasma samples by the previously published TLC–spectrophotometric method, using [^{14}C]α-

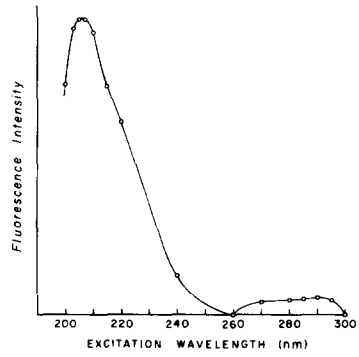

Fig. 2. Fluorescence excitation spectrum of α-tocopherol. Aliquots of 10 μl containing 10 ng of α-tocopherol were injected on a μBondapak C18 column using conditions described in Fig. 1. Fluorescence was detected by the Schoeffel FS970 fluorometer with an emission wavelength cut-off filter of 340 nm. Excitation was done stepwise by manual adjustment.

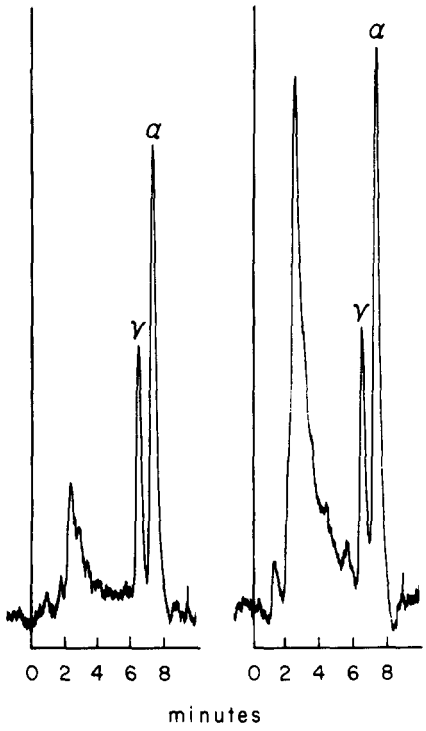

Fig. 3. Chromatogram of tocopherol analysis of a platelet extract (*A*) and a polymorphonuclear cell extract (*B*) from a normal subject. Cells were separated, extracted, and analyzed by HPLC as described in Materials and Methods. A 5-μl aliquot of the final extract in methanol was injected on the HPLC column using conditions described in Fig. 1.

tocopherol as an internal standard (3), and by the HPLC procedure. The amounts of samples analyzed by the HPLC–fluorometric method were approximately 10 to 50-fold less than in the TLC–spectrophotometric method. The correlation coefficient of the five samples by the paired *t* test was 0.99. The precision of the HPLC–fluorometric method was evaluated by the analysis of five separate extractions of a plasma sample in which saponification was not carried out. The results using saponification were 16.3 ± 0.72 μg per ml, and the results for the nonsaponified extractions were 12.7 ± 0.53 μg per ml (SD).

The α-tocopherol content of plasma and cells of subjects supplemented or not supplemented with vitamin E and of two patients with abetalipoproteinemia are presented in **Table 2.** The mean α-tocopherol values of plasma, RBC, and platelets in subjects not supplemented were 8.82 μg/ml, 1.40 μg/10^{10} cells, and 5.10 μg/10^{10} cells, respectively. These values are in agreement with data previously published by this laboratory and others (3, 4, 9–14). Polymorphonuclear leukocytes and lymphocytes of this nonsupplemented group contained 4.47 ± 0.62 μg/10^9 cells and 3.89 ± 0.85 μg/10^9 cells, respectively. These values indicate that the differences between the α-tocopherol content of the two white cell preparations are not significant. The white cell

TABLE 1. Comparison of recovery of α-tocopherol by HPLC using a hexane extraction method with and without prior saponification

Sample	Method	α-Tocopherol Added	α-Tocopherol Recovered	Saponified/ Unsaponified	% Recovery
		ng	*ng*		
Platelets	Saponified	0	414		
		100	508		99
	Not saponified	0	475	0.87	
		100	565		98
Lymphocytes	Saponified	0	102		
		100	213		105
	Not saponified	0	105	0.97	
		100	200		98
Granulocytes	Saponified	0	128		
		100	240		105
	Not saponified	0	109	1.17	
		100	218		104
RBC	Saponified	0	672		
		350	939		92
	Not saponified	0	736	0.91	
		350	1015		94
Plasma	Saponified	0	592		
		365	945		99
	Not saponified	0	492	1.20	
		365	716		84

TABLE 2. α-Tocopherol content of plasma and blood cells of human subjects

	Plasma	RBC	Platelet	Poly	Lymph
	μg/ml	$\mu g/10^{10}$	$\mu g/10^{10}$	$\mu g/10^{9}$	$\mu g/10^{9}$
Normals					
1. not supplemented					
a.	8.82 ± 3.39[a] (6)[b]	1.40 ± 0.17 (5)	5.10 ± 0.63 (6)	4.47 ± 0.62 (6)	3.89 ± 0.05 (5)
b. unit of blood (ref. 4)	10.35 ± 0.51 (15)	1.68 ± 0.12 (15)	4.58 ± 0.25 (15)	7.79 ± 1.12 (15)	6.13 ± 0.78 (15)
2. supplemented	20.41 ± 5.98 (6)	2.98 ± 2.99 (5)	10.84 ± 2.76 (5)	11.20 ± 3.57 (5)	12.53 ± 6.32 (5)
Abetalipoproteinemia patients					
1. not supplemented	0.10	0.06			
2. supplemented	0.56	0.61	2.40	1.70	1.65
Two normal subjects—repeated analyses over a 2–4 month period					
1. 19-week study	7.41 ± 0.83 (6)[c]	1.50 ± 0.22 (5)	5.21 ± 1.03 (4)	4.09 ± 0.80 (3)	3.18 ± 1.06 (4)
2. 10-week study	14.85 ± 1.75 (4)	1.50 ± 0.19 (4)	5.51 (1)	4.36 ± 0.49 (4)	4.71 ± 1.50 (4)

[a] Mean ± SD.
[b] Number of subjects.
[c] Number of analyses.

α-tocopherol values from 40 ml of blood analyzed by the HPLC–fluorometric method are similar to those previously published by this laboratory for a group of 15 normal subjects, using cells from a unit of blood (400 ml) analyzed by a spectrophotometric method for α-tocopherol after a TLC purification procedure (4).

The tocopherol content of plasma and cells of the supplemented subjects was substantially higher than that of the normal group. The mean values for plasma, RBC, and platelets were 20.41 μg/ml, 2.98 $\mu g/10^{10}$ cells, and 10.84 $\mu g/10^{10}$ cells, respectively. An increased concentration of α-tocopherol in these elements of blood after oral supplementation with vitamin E has been reported previously (14). The values for α-tocopherol in polymorphonuclear leukocytes in supplemented subjects was 11.20 ± 3.57 $\mu g/10^{10}$ cells and of lymphocytes was 12.53 ± 6.32 $\mu g/10^{10}$ cells. These values are approximately 3-fold higher than those of the normal subjects.

The effect of vitamin E supplementation on patients with abetalipoproteinemia is also presented in Table 2; the α-tocopherol content of plasma and RBC of the supplemented subject was 5–10 times that of the subject not taking supplemental vitamin E. However, the α-tocopherol content of plasma and cells of the supplemented subject was considerably less than the concentration seen in normal subjects.

The α-tocopherol concentration of plasma and cells from two normal subjects not taking supplemental vitamin E was analyzed over a 2–4 month period, and the data are presented in Table 2. The relative constancy of the values of plasma and cells provides additional evidence of the reliability of this method.

An additional peak having the same retention time as β- and γ-tocopherol was present in all samples; however, since only trace amounts of β-tocopherol have been reported to occur in plasma, RBC, and platelets (13, 15–17) of normal human subjects, this peak was designated as γ-tocopherol. A standard curve of pure γ-tocopherol was linear in the range of 1–5 ng; the fluorescent intensity at Ex 205 nm and Em 340 nm was 4 times greater than that of α-tocopherol. **Table 3** presents the content of γ-tocopherol of plasma and blood cells as percentage of total tocopherol of six normal subjects; the concentrations of γ-tocopherol in plasma, RBC, and platelets agree with previous reports (15–17). It is apparent that the percent of γ-tocopherol in polymorphonuclear leukocytes and lymphocytes (15 and 17%) was higher than in the other cellular elements of the blood.

DISCUSSION

The development of high-performance liquid chromatography has made it possible to increase the sensitivity of analytical procedures for many compounds. Several laboratories have published reports using HPLC to analyze tocopherols in animal tissues and in human plasma (8–12). The method presented in this paper was developed to reduce the volume of blood required to provide an adequate number of white blood cells for analysis of α-tocopherol. The

TABLE 3. γ-Tocopherol concentration expressed as percent of total tocopherol in plasma and blood cells of normal subjects

	Plasma	RBC	Platelet	Poly	Lymph
L.H.	8.9	16.7	14.3	22.2	23.8
M.T.	10.5	12.5	12.5	14.3	
H.K.	6.2	7.7	6.7	12.5	13.3
A.L.	6.7	5.9	7.7	10.7	10.6
R.G.	10.9		13.1	17.2	20.8
A.M.	11.6	16.7	13.9	17.2	18.2
	9.13 ± 2.26[a]	9.7 ± 6.8	11.4 ± 3.3	15.7 ± 4.1	17.3 ± 5.4

[a] Mean ± SD.

extraction step in the analysis retains the important feature of our previously described method, namely the addition of an unusually large amount of antioxidant to protect against the loss of tocopherol. In the original method, we used either pyrogallol or ascorbic acid as the antioxidant, but in subsequent years and in the present study we have found ascorbic acid to be more reliable and suitable. This extraction system with saponification has been used by another laboratory (7) for the analysis of tocopherol in tissues by a fluorometric method, eliminating the TLC purification step, which usually results in considerable loss of tocopherol.

The difficulties in obtaining adequate quantitation of low levels of tocopherol in cells and tissues have been decreased by the use of an internal standard. In our previously reported work, we utilized [^{14}C]tocopherol as the internal standard, but the specific activity of either ^{14}C- or ^{3}H-labeled tocopherol is so low as to make them unsuitable as internal standards in the HPLC–fluorometric method. Other laboratories have used tocol as the internal standard in analyzing α-tocopherol using HPLC; we attempted to use tocol for this purpose but found it unstable in our procedures involving saponification with as much as 30% loss during analysis, although it was quantitatively recovered when saponification was not used. On the other hand, the recovery of tocopherol quantitated by the addition of known amounts of pure tocopherol to samples before the extraction step was excellent. The data reported here were obtained using the saponification step in the analysis of the different components of blood. It is doubtful that it is a necessary procedure in the analysis of tocopherol levels in white cells and in platelets, but it may be useful in plasma, in red blood cells, and in adipose tissue.

An improvement in the sensitivity of the detection system for α-tocopherol using fluorometric analysis was obtained by shortening the wavelength of excitation from 295 to 205 nm, which resulted in a 20-fold greater response. At Ex 205 nm and Em 340 nm, the detection of tocol is 2.5 times greater than for α-tocopherol per ng, and the γ-tocopherol detection is 4.0 times greater than for α-tocopherol.

The results for α-tocopherol content of plasma, red blood cells, and platelets of the normal subjects are in agreement with previous values. The α-tocopherol contents of polymorphonuclear leukocytes and of lymphocytes were not significantly different from each other, but the value per cell was 10-fold greater than per platelet, and 35-fold greater than for the erythrocyte. The significance of the increased content of α-tocopherol in the white blood cells remains to be clarified; part of the increase may be related to the larger size of the white blood cell and the essential role for α-tocopherol as a constituent of the cell membrane.

The tocopherol contents of plasma and blood cells of subjects taking supplemental vitamin E were measured in hyperlipidemic individuals. Although we and others have reported that plasma values of tocopherol are correlated with total lipid values in hyperlipidemic subjects who are not taking supplemental vitamin E (18–21), there was no significant difference in the α-tocopherol content of their platelets, red blood cells, or white cells from those found in normal subjects (data not shown). By contrast, subjects who take supplemental vitamin E achieve unusually high levels in their erythrocytes, platelets, and white cells.

The precise time course for these changes has not been shown for white cells, although it has been studied in platelets (12). If the tissues of patients who are supplemented with oral vitamin E accumulate tocopherol in a manner similar to white cells and platelets, the potential protective role of α-tocopherol in cells can be achieved by oral supplementation. We would speculate that the availability of the ingested supplemental vitamin E to blood cells and tissues occurs during the metabolism of chylomicrons, which is the major route of absorption of tocopherol from the intestinal tract; and that the high concentration of tocopherol in the chylomicron and

smaller secondary particles facilitates the transfer of tocopherol to cells and tissues.

In hyperlipidemic subjects who are not being supplemented with vitamin E, there is an increased amount of tocopherol in very low density lipoprotein (VLDL) and in low density lipoprotein (LDL), which are the major tocopherol-containing lipoproteins in hyperlipidemic humans; but exchange between the increased amount of tocopherol in the lipoprotein and the tissues may be limited by tocopherol being distributed to a considerable extent in the core of the lipoproteins, and only a small proportion of the tocopherol is distributed to the surface of the lipoprotein where it is available for transfer to the tissues (18, 19).

This HPLC–fluorometric method for the measurement of α-tocopherol may have particular value in the management of premature infants and neonates with either anemia or respiratory-distress syndrome, in whom repeated measurements of plasma tocopherol content may be useful in evaluating tocopherol therapy. We plan to use this method to follow levels of tocopherol in the white cells of patients receiving the anti-tumor agents that presumably cause lipid peroxidation with resultant toxicity (22). For such patients supplemental vitamin E may have an important protective role, and quantitating cell and plasma levels of tocopherol would be important.

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