The measurement of nanograms of tocopherol from needle aspiration biopsies of adipose tissue: normal and abetalipoproteinemic subjects

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Abstract A method for the analysis of tocopherol in human adipose tissue using high performance liquid chromatography and fluorescence spectrometry is described; results are expressed relative to total triglyceride content measured by the reaction of the methylated fatty acids with hydroxylamine and ferric chloride. The tocopherol contents of adipose tissue obtained at surgery and by the needle aspiration biopsy technic of ambulatory human subjects (who did not take supplemental vitamin E) were found to be virtually identical. The tocopherol content of adipose tissue by the needle aspiration technic was 262 ± 33 ng tocopherol/mg triglyceride; this value was increased twofold or more in persons ingesting additional vitamin E. Patients with abetalipoproteinemia (ABL) who absorb tocopherol poorly and have extremely low levels of plasma and red blood cell tocopherol also had a low concentration of adipose tissue tocopherol. However, some ABL patients on massive supplementation with vitamin E (approximately 10 g daily) did achieve normal concentrations of adipose tissue tocopherol.—**Kayden, H. J., L. J. Hatam, and M. G. Traber.** The measurement of nanograms of tocopherol from needle aspiration biopsies of adipose tissue: normal and abetalipoproteinemic subjects. *J. Lipid Res.* 1983. **24:** 652–656.

Supplementary key words cholestatic liver disease • normotriglyceridemic abetalipoproteinemia • supplemental vitamin E • fluorescence detection with HPLC • adipose tissue cholesterol and fatty acid

There is continued interest in the role of vitamin E in cellular metabolism, and a corresponding interest in methods for assessing levels of tocopherol in the body. Plasma levels have been widely used, especially when expressed along with total serum lipid values, as a guide to the state of tocopherol concentration. We have previously described methods for measuring tocopherol in plasma and the formed elements of the blood (erythrocytes, granulocytes, platelets, and lymphocytes), using a high performance liquid chromatographic (HPLC) system with fluorescence detection (1).

In patients with abetalipoproteinemia (ABL), plasma and erythrocyte levels of tocopherol are very low due both to an inability of these patients to form chylomicrons, thus absorption of lipid-soluble vitamins is impaired, and to the absence of circulating lipoproteins containing apoprotein B, which transport the bulk of tocopherol in humans (2). We documented the deficiency of tocopherol in patients with ABL almost two decades ago (3), and the beneficial and prophylactic effect of vitamin E supplementation has been recorded in many publications. It is still not certain by what route the oral vitamin E is absorbed in these patients, whether by synthesis of high density lipoprotein (HDL) by the intestine and incorporation of tocopherol into this lipoprotein, or by the absorption of tocopherol into the portal stream, from the intestine, attached to circulating HDL or to albumin. We have recommended that supplemental vitamin E be taken in divided doses, but the appopriate dosage is uncertain. One of the goals of the present study was to see if an apparent dose–response curve could be identified, using adipose tissue as a guide since both plasma and erythrocytes are always low in these patients.

We therefore developed a method for the measurement of tocopherol in ambulatory human subjects using the needle aspiration biopsy technic of adipose tissue to obtain the sample. The sensitivity of this method is such that as little as 1 ng of tocopherol could be measured reliably. The accuracy of the method is demonstrated by the comparison of values obtained from samples of adipose tissue from patients undergoing elective surgery and in ambulatory human subjects. The ultrasensitivity of the method is amply demonstrated in the measurement of tocopherol in needle biopsies of the adipose tissue of patients with ABL.

MATERIALS AND METHODS

Informed consent was obtained from 18 subjects, including 10 patients with ABL. Adipose tissue was obtained either during elective surgery or by the needle

Abbreviations: ABL, abetalipoproteinemia; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography.

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aspiration biopsy procedure (described below). The surgical specimens were obtained from the abdominal wall or from the omentum of 4 subjects. Needle aspiration biopsies of adipose tissue from the gluteal area were performed on 14 subjects, including 10 ABL patients. The biopsy procedure, originally described by Hirsch et al. (4), was carried out as follows. The skin was cleansed with alcohol, infiltrated with 1% novocaine, and additional novocaine was injected into the subcutaneous tissue. A 50-ml glass syringe fitted with a Luer Lok and a 1 1/2-inch 18-gauge needle was inserted into the gluteal area away from the sciatic nerve. One to 2 ml of sterile saline solution was injected using the 50-ml syringe into the subcutaneous/adipose tissue. Suction was then vigorously applied by drawing the plunger of the syringe; the saline was aspirated into the syringe along with droplets of adipose tissue, and often with shreds of tissue in the fluid or in the needle. The aspirate usually contained between 0.7 and 16 mg of adipose tissue and was placed into a screw cap tube. The syringe and needle were then washed with a total of about 1 ml of 0.01 M phosphate buffered saline, pH 7.4 (PBS). The adipose tissue suspension and the PBS washes were pooled, diluted with twice the volume of 1% ascorbic acid in 100% ethanol, and placed in a 70°C water bath for 2 min. Then saturated KOH was added (one-tenth the volume of the total suspension) and the tubes were incubated in the water bath for an additional 30 min. After cooling to room temperature, 1 ml of distilled water and 4 ml of hexane (Burdick and Jackson Laboratories, Inc., Muskegon, MI) were added. The tocopherol and cholesterol were extracted into the hexane by shaking the tubes vigorously for 2 min. After centrifugation, the hexane layer was removed and the aqueous phase was reserved for analysis of its fatty acid content.

To measure tocopherol, an aliquot of the hexane was evaporated to dryness under a stream of N_2(g), then an appropriate volume of methanol was added. The tocopherol content was determined as previously described (1) using a Waters Associates Model ALC/GPC-204 liquid chromatograph with a Model 6000A solvent delivery system, model U6K injector, data module, and system controller (Waters Associates, Milford MA). Detection of tocopherol fluorescence was with a fluorometer (FS970 fluorometer, Schoeffel Instrument Corp., Westwood, NJ) set to an excitation wavelength of 205 nm and with an emission filter of 340 nm.

The cholesterol content of the methanol solution (used for tocopherol measurement above) was determined by gas–liquid chromatography (GLC) (Hewlet Packard model 7620A, with a flame ionization detector) using 3% OV-17 on Gas ChromQ with an isothermal setting of 256°C. Stigmasterol was used as the internal standard; quantitation was by integration of the cholesterol and stigmasterol curves.

For measurement of the total fatty acid of the adipose tissue (biopsy or surgical specimen), the remaining aqueous phase of the saponified sample was acidified with 12 N HCL and the free fatty acids were extracted by vigorous shaking with heptane (Burdick and Jackson). Standards of triolein (Analabs) were also saponified, extracted, and included throughout the following procedure. After centrifugation an aliquot of the heptane layer was placed in a screw-capped test tube and evaporated to dryness under N_2(g). The methyl esters of the fatty acids were formed by incubating the samples with 1 ml of 5% sulfuric acid in methanol overnight in a 65°C oven. After cooling, the fatty acid methyl esters were extracted into petroleum ether. An aliquot of petroleum ether containing between 0.5 and 2 μeq of fatty acid was evaporated under a stream of N_2(g). Three ml of alcohol–ether 3:1 was added to each of the dried samples and 0.5 ml of 2 M hydroxlamine and 0.5 ml of 3.5 N NaOH were added; following vigorous mixing the samples were allowed to stand 20 min at room temperature. Then 0.6 ml of 4 N HCl and 0.5 ml of 0.37 M $FeCl_3$ in 0.1 N HCl were added. The absorbance of the product of this color reaction was measured using a Gilford Spectrophotometer at 525 nm (5). The triglyceride content was calculated from the fatty acid content with the assumption that fatty acids from phospholipids or cholesteryl ester were of minimal quantity.

The adipose tissue tocopherol is expressed as ng tocopherol/mg triglyceride. The sensitivity of this method was such that as little as 1 ng of tocopherol could be measured. Data of cholesterol content per mg triglyceride is also given where available. Measurement of the tocopherol content of plasma was carried out as previously published (1).

RESULTS

Analysis of abdominal adipose tissue obtained during surgery in four subjects is shown in **Table 1.** Subjects A and B had been taking supplemental vitamin E for more than a year; Subject A 800 mg/day; Subject B 1200 mg/day from 1975 to 1979, then 400 mg/day until January 1980, some 20 months prior to surgery when she discontinued taking the supplement. Their values averaged 905 and 619 ng tocopherol/mg triglyceride, respectively. Subjects C and D had adipose tissue values of 333 and 247 ng tocopherol/mg triglyceride, respectively. The plasma value for A, 22.4 μg/ml, was an elevated value, but B had a normal value of 10.5 μg/ml. Although Subject B had discontinued supplementing her diet with vitamin E approximately

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TABLE 1. Tocopherol, triglyceride, and cholesterol content of abdominal adipose tissue samples obtained from four subjects during surgery

Subject	Site	Adipose Tissue: Tocopherol/TG Mean Value	Adipose Tissue: Tocopherol/TG Individual Value	Adipose Tissue: Cholesterol/TG	Plasma Tocopherol
		ng/mg		*μg/mg*	*μg/ml*
A[a]	Upper[b]	964	812 1117		22.4
	Middle	798	851 744		
	Lower	954	966 943		
Mean ± SD			906 ± 132		
B[a]	Upper	652	642 662	1.49	10.5
	Middle	617	631 628 592	1.34	
	Lower	590	643 545 581	1.22	
Mean ± SD			616 ± 39	1.35 ± 0.14	
C	Upper	307	300 315	1.76	15.2
	Middle	359	352 359 366	1.65	
	Lower	334	342 338 323	2.06	
Mean ± SD			337 ± 23	1.82 ± 0.21	
D	Upper	207	200 231 190	2.39	14.8
	Middle	232	198 233 265	2.93	
	Lower	303	313 290 306	1.91	
Mean ± SD			247 ± 47	2.41 ± 0.51	

[a] Subjects A and B had been taking supplemental vitamin E for more than 1 year.

[b] Sites of incisions on the abdominal wall from which specimens were taken.

20 months prior to surgery, there was a delayed fall in the adipose tissue level of tocopherol in comparison with the prompt fall in the plasma value. This response agrees with results from studies carried out in the guinea pig by Machlin et al. (6).

Table 2 presents the data on needle aspiration biopsies of adipose tissue of ambulatory patients; E, F, and H had familial hypercholesterolemia and subject H was receiving supplemental vitamin E for several months prior to sampling of adipose tissue. The mean of the biopsy values was 262 ± 33 ng tocopherol/mg triglyceride, and compares favorably with the average of the surgical specimens from subjects C and D, which was 290. As further confirmation of the validity of the needle aspiration biopsy method, the value of tocopherol in the adipose tissue aspirate of subject B (Table 1) was 503 ng/mg triglyceride, which compares with the surgical sample value of 616 ± 39 ng/mg triglyceride.

Needle aspiration biopsies of adipose tissue were carried out in ten patients with ABL, including the patient (AF) with normotriglyceridemic ABL reported by Malloy et al. (7) (**Table 3**). Many of the ABL patients had been receiving supplemental vitamin E in their diet; the approximate level is noted in the table. The plasma levels of tocopherol remained uniformly low in ABL despite dietary supplementation with vitamin E. Adipose tissue levels ranged from undetectable levels in a 5-year-old boy (RR), who presumably had no supplemental vitamin E, to the normal value in a 29-year-old man who had been taking 9 g of vitamin E for several years; his value was 242 ng tocopherol/mg triglyceride. In the other patients no obvious dose relation between oral intake and level of tocopherol in adipose tissue was apparent.

The patient AF, whose clinical and metabolic abnormalities were described in detail by Malloy et al. (7), had normotriglyceridemia and ABL with absent B_{100} apolipoprotein. Chylomicron formation occurred in this patient and tocopherol absorption could be demonstrated following an oral dose. Plasma tocopherol levels were distributed in the fasting state as follows: d < 1.063 g/ml, 2.43 μg/ml (57% of total); d 1.063–1.21 g/ml, 1.56 (37%); d > 1.21 g/ml, 0.25 (6%). Adipose tissue level was a high value of 565 ng tocopherol/mg triglyceride, despite the modest level of supplementation of 400 mg/day of vitamin E.

TABLE 2. Tocopherol content of adipose tissue samples obtained by needle aspiration biopsy from four subjects

Subject	Average Adipose Tissue Content: Tocopherol	Triglyceride	Tocopherol/TG	Plasma Tocopherol
	ng/biopsy	*mg/biopsy*	*ng/mg*	*μg/ml*
E	3159	10.5	301	18.0
F	1557	6.7	232	13.1
G	934	3.9	239	11.3
H	3813	13.7	278	24.7
Mean ± SD			262 ± 33	

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TABLE 3. Tocopherol and triglyceride content of adipose tissue samples obtained by needle aspiration biopsy from ten patients with abetalipoproteinemia

Subject	Sex	Age	Vitamin E[a]	Mean of Duplicate Samples of Adipose Tissue			Plasma Tocopherol
				Tocopherol	Triglyceride	Tocopherol/TG	
		yr	*mg*	*ng/biopsy*	*mg/biopsy*	*ng/mg*	*μg/ml*
AMV	F	25	1200	170	5.52	31	0.58
MS	M	28	800?	80	0.68	118	0.16
KL	F	2.5	1200	24	0.62	39	1.60
LF	F	23	3200	759	5.38	141	2.50
RH	M	29	9000	482	1.99	242	0.81
RR	M	5	none	<1	7.50		0.03
KM	M	3	400	12	0.58	22	0.11
BL	F	29	800	56	0.37	151	0.66
PC	F	19	14,000	480	7.30	66	1.10
AF[b]	F	10	400	2481	4.71	526	5.85

[a] Amount of supplemental vitamin E taken daily by each patient.
[b] Patient AF is the normotriglyceridemic ABL with absent B100 component; triplicate samples were analyzed for this patient.

The single patient with cholestatic liver disease studied had very low levels of plasma tocopherol (1.1 μg/ml) and virtually no tocopherol in adipose tissue samples (≪1 ng tocopherol/mg triglyceride).

DISCUSSION

The data presented in this report establish that sufficient adipose tissue can be obtained by needle aspiration biopsy to permit the measurement of tocopherol level, total fatty acid content, and cholesterol content of the sample. The extraction procedure follows saponification of the total sample, with separation of tocopherol and cholesterol into an organic solvent phase and fatty acids into the aqueous phase. The tocopherol is quantified by HPLC with fluorescence spectrometry, cholesterol by GLC, and fatty acids, following methylation, by reaction with hydroxylamine and ferric chloride. The method was developed using large samples of adipose tissue from surgical specimens, and then modified for the small quantities of tissue obtained by needle biopsy.

The needle aspiration biopsy technic was chosen for its simplicity; it is virtually pain-free and does not require a second visit by the patient to remove sutures. The aspirated tissue is promptly protected against oxidation of the tocopherol by the addition of 1% ascorbic acid to the sample. In order to compare samples from different patients and from the same patients on repeated sampling, we have selected the measurement of total fatty acids as the parameter for comparison; data are presented on a per mg triglyceride basis.

There is continued interest in the role of vitamin E in human cellular metabolism. In patients with ABL, long term administration by mouth of supplemental vitamin E appears to protect against much of the neuromuscular abnormalities and may even cause regression of these abnormalities when they occur (8). We have documented that tocopherol levels in the plasma and erythrocytes of these patients are extremely low, but vitamin E is absorbed, particularly when supplemental doses are given in sufficient amounts to correct abnormal peroxide hemolysis and autohemolysis of the red blood cells.

There appears to be no simple dose to tissue level relationship in this group of ABL patients. It is clear that failure to take any tocopherol supplement will give very low tissue tocopherol levels (and clinical signs of tocopherol deficiency) and the one subject, RH, who injested 9 g/day for more than a year had a normal adipose tocopherol level. But other patients, despite high oral intake (PC on 14 and 12 g/day), had continued low levels of tocopherol in tissue. We are currently raising the doses in other patients and plan to re-measure their adipose tocopherol levels after months of treatment to record any changes.

Of interest is the patient AF, reported in detail by Malloy et al. (7), whose genetic abnormality of absent B_{100} apolipoprotein component results in abetalipoproteinemia, but in normal chylomicron formation and triglyceride levels. On the supplement of 400 mg/day, a modest dose for ABL patients, her adipose tissue level was high, despite plasma values in the fasting state of 5.85 μg/ml. The importance of chylomicron formation in tocopherol absorption is again documented; it is possible that distribution into the tissues occurs during chylomicron catabolism. In the ABL patient, abnormalities in HDL structure and composition may also contribute to the limitation in the transport of tocopherol by this lipoprotein.

Published data on the content of tocopherol in hu-

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man adipose tissue usually relates tocopherol content to the total weight of the adipose tissue obtained whether at post mortem or by surgical biopsy. One report by Dju, Mason, and Filer (9), on the tocopherol content in human adipose tissue of seven adolescents and adults who died by sudden accidents, has percent of lipid as well as tocopherol per unit weight. Calculation of the values for tocopherol in these subjects averages 270 ng tocopherol/mg triglyceride, assuming that percent lipid was all triglyceride. Rates of storage and depletion of tocopherol in human adipose tissue were measured by McMasters et al. (10) in normal subjects by administering 1 g of vitamin E daily for 2 weeks and comparing plasma and adipose tissue levels of tocopherol before, at the end of 2 weeks of daily supplementation, and after an additional period of 2 weeks without supplementation. Although changes in plasma levels rose and fell, the rapidity of changes in adipose tissue content was surprising (from an average of 38 ± 18 μg tocopherol/g tissue to 80 ± 27 μg at the end of 2 weeks of supplementation, to 50 ± 17 μg at the end of the additional 2 weeks). In the guinea pig (6) and in the rat (11) the adipose tissue tocopherol had been considered to be virtually unavailable to the circulating blood during periods of vitamin E deprivation. Subject B in Table 1 had a high level of 619 ng tocopherol/mg triglyceride in her surgical specimens and she had been taking supplemental vitamin E (1200 mg/day) from 1975 to 1979. During 1979 she reduced her intake to 400 mg daily and in January 1980, some 20 months prior to surgery, she discontinued the supplement, which we believe was the reason for the normal value of her plasma tocopherol of 10.5 μg/ml, but the adipose tissue level of tocopherol remained elevated.

The importance of tocopherol in preventing and ameliorating the neuromuscular disease associated with cholestatic liver disease has been recently reported (12, 13). These patients have low plasma levels and have difficulty in absorbing tocopherol, but respond briskly to parenteral therapy. We have examined the plasma and adipose tissue of one patient and could not detect any measurable tocopherol in the adipose tissue (despite a good-sized sample); the plasma tocopherol concentration was also very low, 1.1 μg/ml. Further studies on tissue levels and the difficulties in absorption of tocopherol in these patients are being carried out.

The authors would like to thank Ms. Claudia Farb for technical assistance. The following physicians provided samples of adipose tissue from ABL patients: Aubie Angel, Mitchell Brin, Fredrick Daum, Roger Illingworth, John Kane, and Mary Malloy. This study was supported in part by grant HL 06481 from the National Heart, Blood, and Lung Institute and from a grant by Hoffmann La-Roche, Inc., Nutley, New Jersey 07110.

Manuscript received 15 August 1982 and in revised form 15 December 1982.

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