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CONCURRENT DETERMINATION OF α-TOCOPHEROL AND FREE FATTY ACIDS IN HUMAN PLASMA BY GLASS OPEN TUBULAR CAPILLARY COLUMN GAS CHROMATOGRAPHY

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SUMMARY

A new gas chromatographic procedure was developed for the concurrent analysis of mixtures of long-chain acids (as methyl esters), cholesterol (as the trimethylsilyl ether) and the tocopherols (as trimethylsilyl ethers). This method was used in a study of free fatty acids and α-tocopherol in the plasma of normal subjects and stroke patients. The chromatographic separation was based upon the use of thermostable glass open tubular capillary columns prepared with Silanox (procedure of Lin, Pfaffenberger and Horning) and a new polar liquid phase (Schwartz–Mathews polyphenyl ether sulfone phase). The German–Horning injection system was used in a modified gas chromatograph.

INTRODUCTION

Compositional studies of long-chain acids in biological samples and in food fats and oils were one of the early analytical applications of gas chromatography (GC). While the principles of quantitative analytical work based on GC separations of methyl esters of long-chain acids have not changed[1], the widely used procedures of packed column analysis with polyester liquid phases are not highly useful in applications involving acids of relatively long chain length. The principal difficulties arise from the temperature limitations imposed by high bleed rates above about 225–250°, and the relatively low resolution provided by the ordinary packed column. The procedure described here is based on two recent advances. These are the synthesis of polar polyphenyl ether sulfone phases with very high thermal stability (to 320–350°) by Mathews *et al.*[2] and the development at the same time of methods for preparing high resolution glass open tubular capillary columns with polar liquid phases[3,4]. The columns used in this investigation provided excellent separations of long-chain acids as methyl esters, up to methyl octacosanoate, and also of the tocopherols and cholesterol as trimethylsilyl ethers. These properties permitted the development of an analytical procedure for the concurrent determination of α-tocopherol and free fatty acids (FFA) of human plasma. This procedure was used in a comparative study of plasma FFA and α-tocopherol in stroke patients and in normal subjects.

EXPERIMENTAL

Gas chromatography

An F & M Model 400 gas chromatograph was modified to include the injection system of German and Horning[5], and to accept glass open tubular capillary columns. The conditions of operation were: injector temperature, 250°; detector temperature, 280°; temperature programming, 2°/min; carrier gas, nitrogen; initial column pressure, 5 p.s.i.; split ratio, 10:1. The picoammeter was a Keithley Model 417, in place of the original electrometer–amplifier.

The glass column used in this study was 32 m × 0.25 mm I.D.; it was drawn to provide a coil with a diameter of 12 cm. The coating of phase PZ-176 (ref. 2) was applied as described earlier[4]. During a period of approximately six months of operation, no deterioration or loss of efficiency was observed for the column.

Mass spectrometry

An LKB Model 9000 gas chromatograph–mass spectrometer was used to confirm the identity of the compounds shown in Figs. 3, 4 and 6. The column was a 4 m × 3.4 mm I.D. glass coil packed with 3% PZ-176 on 80–100 mesh acid-washed and silanized Gas-Chrom P. The conditions of operation were as usual for long-chain methyl esters and sterols; temperature programming at 2°/min was used. The mass spectra of plasma components were identical to those observed for authentic samples. A detailed study of electron impact and chemical ionization mass spectra of the tocopherols will be published separately.

Analytical samples

Blood samples were obtained during studies of patients which required, for other purposes, the insertion of a catheter in the jugular vein. Samples from normal subjects were taken from an antecubital vein with a disposable syringe. All samples were centrifuged within 15–20 min after collection. Lipids were extracted essentially according to the procedure of Dole and Meinertz[6]. One ml of plasma, in a 15-ml tube with a PTFE-lined screw cap, containing a known quantity of heptadecanoic acid (usually 30–50 μg) in pyridine (30–50 μl) as an internal standard, was treated with 5 ml of a mixture of isopropanol–isooctane–1 *N* sulfuric acid (40:10:1). After vigorous mixing (Vortex mixer), 2 ml of glass-distilled water and 5 ml of isooctane were added. The tube was shaken, centrifuged, and the supernatant organic solution was separated. The solvent was removed with the aid of a nitrogen stream.

Derivatization was carried out in two steps. The acidic components were converted to methyl esters by short treatment with diazomethane; this was accomplished by dissolving the lipid sample in 0.5 ml of methanol and adding 2 ml of a solution of diazomethane in ether (prepared from Diazald, Aldrich, Milwaukee, Wisc., U.S.A.). After 10–15 min, the solvents and excess reagent were removed with the aid of a nitrogen stream. To the residue there were added 50 μl of pyridine, 50 μl of bis(trimethylsilyl)acetamide and 25 μl of trimethylchlorosilane. The mixture was heated at 90° for 1 h. The resulting solution was used directly for GC analysis.

Analytical calculations

Area comparisons for peaks (Figs. 3 and 4 show typical charts) were made by

hand measurements with a magnifying scale. The approximation used was based on height (to the outside of the pen line) and width at half-height (to pen line centers, by using inside and outside of the pen line of a peak). The analytical values for FFA composition and for FFA concentration were based on direct comparisons of peak areas.

Standard mixture NHI-D[1] was employed to establish the precision and accuracy of the GC procedure. The reproducibility of the Dole–Meinertz extraction procedure was examined through repetitive analyses of extracts from aliquot samples of a plasma sample. Standard deviations were calculated according to the usual method.

The response factor for α-tocopherol with respect to heptadecanoic acid was found to be 0.90.

The mass–instrumental response relationship for α-tocopherol analyses was determined by the analysis of aliquot samples of plasma (not containing detectable α-tocopherol) to which increasing amounts of α-tocopherol had been added.

Some charts showed small peaks near 30 and 50 min retention time; these compounds have not been identified, but they are associated with septa from some of the tubes used in the analytical procedure. Myristic acid was omitted from the calculations. Tocopherols other than α-tocopherol were not observed in plasma in this study.

Subjects

The normal subjects were young (20–40 years) volunteers with no history of atherosclerotic vascular disease. A description of the patients, along with analytical values of other kinds, will be included in a separate publication. All were in the immediate post-stroke period. No attempt was made to correlate clinical evaluations of patient status with the values shown in Fig. 8; these data are considered to represent a risk factor analysis rather than data serving for prognostic purposes.

RESULTS AND DISCUSSION

Compositional studies of mixtures of long-chain acids from biological sources are best carried out by GC. Procedures developed during 1960–1965, many of which are still in use, employed polyester liquid phases and packed columns for methyl ester separations. These methods, while useful for the analysis of most seed oils, have disadvantages in other applications. The relatively high bleed rate of polyester phases at elevated temperatures makes it difficult to analyze mixtures containing acids of very long chain length, and a high bleed rate also interferes with mass spectrometric (MS) identification procedures when gas chromatograph–mass spectrometer–computer analytical systems are used. The relatively low resolution that can be achieved with ordinary packed columns is satisfactory if the samples under study do not contain geometric or bond isomers of unsaturated acids; if these are present, as is often the case, it is not possible to arrive at a complete analysis on a molecular basis. "Oleic acid" values represent a combination of *trans* and positional isomers as well as oleic acid, unless the sample is free of these isomers. The recent introduction of polysiloxane phases containing cyanopropyl groups has provided additional polar phases, but these are still not sufficiently thermostable for many purposes.

The procedure described here was originally developed for the detection and estimation of long-chain acids of the type occurring in brain phospholipids. It was based upon the use of new, thermostable (to 320–350°) polyphenyl ether sulfone phases synthesized by Mathews *et al.*[2], and upon a newly developed procedure for the preparation of polar-phase high-resolution glass open tubular capillary columns for GC[4]. These columns, with phase PZ-176 (a designation of one of the Schwartz–Mathews phases), provided excellent separations of all of the common long-chain acids (methyl ester derivatives) up to octacosanoic acid. An initially unrelated study of separation methods useful for work with the tocopherols was combined with the long-chain acid study when it was found that PZ-176 capillary columns could be used to separate all of the tocopherols (α-, β-, γ- and δ-) from each other and from cholesterol (as trimethylsilyl ether derivatives). A new procedure was developed which permitted the concurrent determination of FFA of human plasma, and of α-tocopherol in plasma, without additional steps involving saponification or thin-layer chromatographic separation. This procedure was used in a study of stroke patients and normal subjects.

Since the literature dealing with these determinations has heretofore had no overlap in methodology, the two analytical problems are discussed separately.

FFA in human plasma

Free (non-esterified) long-chain acids are always present in human plasma. They are derived from adipose tissue stores, and their concentration in plasma is influenced by many different types of physiological stimuli. Measurements of concentration have generally been made in order to relate the values to different conditions of lipid and carbohydrate metabolism, or to varieties of stress. The normal distribution of values is wide, and there is evidence that rapid fluctuations occur. In work of this kind, it is customary not to estimate individual acids, but to rely on a class determination. Compositional studies have generally been made in order to relate FFA to adipose tissue triglyceride composition.

The most widely used method of extraction is that of Dole and Meinertz[6]. The plasma sample is acidified with dilute sulfuric acid and a lipid fraction is extracted with heptane–isopropanol. The extract contains plasma triglycerides, cholesterol, cholesterol esters and α-tocopherol as well as FFA, but these neutral lipids do not interfere with the colorimetric procedures that are generally used. A comparison of published methods was made in 1973 by Falholt *et al.*[7]; values obtained by a number of methods, developed since the original procedure of Dole[8] was published in 1956, were compared. A chloroform–heptane–methanol solvent mixture, used with an aqueous phosphate buffer solution, was recommended for the extraction step. An automated procedure, based on the Dole extraction, was recently described by Kashket[9]. A semi-automated fluorimetric method, also employing the Dole extraction, was developed by Carruthers and Young[10].

Our initial interest was in compositional data, and not in total FFA values. By adding an internal reference compound (heptadecanoic acid in this case) at the extraction step, however, it is possible to obtain both compositional and total FFA values. The FFA concentration, in mM, is obtained by addition of individual concentration values.

The Dole–Meinertz extraction procedure was used in this work, with the sub-

stitution of isooctane for heptane. The extracted long-chain acids were converted to methyl esters by reaction with diazomethane, and the entire sample was then subjected to silylating conditions. The principal plasma lipid fractions present in the sample, after these reactions, are triglycerides, cholesterol esters, cholesterol as the trimethylsilyl ether, FFA as methyl esters and α-tocopherol as the trimethylsilyl ether. Triglycerides and cholesterol esters are not eluted under the conditions employed here; long-chain methyl esters are eluted first, and these are followed by α-tocopherol and cholesterol (trimethylsilyl ethers). The relative concentrations of each long-chain acid and of α-tocopherol were calculated through comparisons of peak areas, with the use of heptadecanoic acid as an internal reference compound.

The linearity of response of the hydrogen flame detector with respect to sample mass, and the general applicability of GC methodology in long-chain acid analyses, have been established in many earlier studies. This procedure, however, is based upon the use of a new phase, and upon new capillary column technology, and it was necessary to validate both the qualitative and quantitative aspects of the work.

Table I contains methylene unit (MU) values[11] for many of the commonly occurring unbranched long-chain acids (as methyl esters), observed with phase PZ-176 in a temperature-programmed separation. The term "methylene unit" is used as a specific indication that the values were determined by temperature programming[11], rather than by the use of isothermal conditions as is conventional for retention index comparisons[12]. The phase is sufficiently polar so that monoenoic esters are eluted after the corresponding saturated esters. The nature of the separation is illustrated in Fig. 1. Baseline separation is not difficult when open tubular capillary columns are used, but it would be difficult to achieve similar separations with this phase with the use of an ordinary packed column. Fig. 2 shows a separation of the principal acids (methyl esters) that would be encountered in compositional studies of plasma FFA, cholesterol esters, triglycerides or phospholipids. This should be compared with the separation shown in Fig. 3. The principal acids present as FFA are, in the usual order of

TABLE I

METHYLENE UNIT (MU) VALUES[11] FOR METHYL ESTERS OF LONG-CHAIN ACIDS

Observed with a glass open tubular capillary column with phase PZ-176; temperature programmed at 2°/min from 160°. Me = methyl.

Compound	*MU*	*Compound*	*MU*
Me palmitate	21.62	Me arachidate	25.68
Me palmitoleate	21.86	Me 11-eicosenoate	25.81
Me heptadecanoate	22.63	Me 11,14-eicosadienoate	26.28
Me stearate	23.63	Me 11,14,17-eicosatrienoate	26.56
Me oleate	23.83	Me arachidonate	27.65
Me elaidate	23.88	Me behenate	27.74
Me petroselenate	23.80	Me erucate	27.92
Me vaccenate	23.93	Me brassidate	27.89
Me linoleate	24.10	Me lignocerate	29.60
Me linolelaidate	24.20	Me nervonate	30.00
Me linolenate	24.78	Me hexacosanoate	31.64
Me γ-linolenate	24.43	Me octacosanoate	33.70

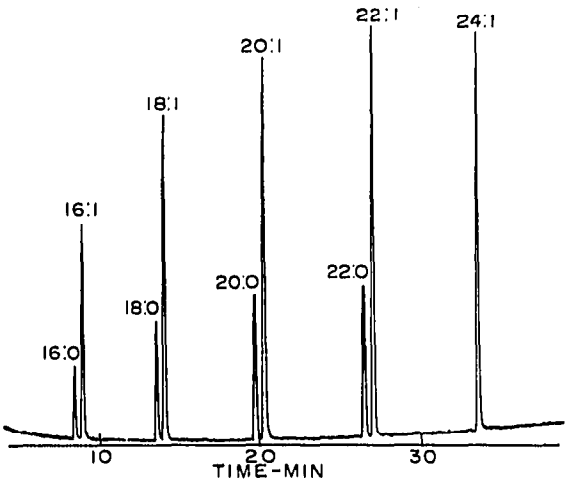

Fig. 1. Separation of methyl esters of monoenoic and saturated long-chain acids. Operating conditions: temperature programmed from 180° at 2°/min; 32 m × 0.25 mm I.D. open tubular glass capillary column with polar phase PZ-176.

abundance, oleic, palmitic, linoleic, stearic and palmitoleic acid. Myristic acid was omitted in these analyses; it is normally present in small quantity (see Figs. 3 and 4). Heptadecanoic and nonadecanoic acids are normally present as trace components, but the amount of heptadecanoic acid is so low that this compound may be used as an internal reference. Longer-chain acids were not observed as part of the FFA fraction for adults in this study. We have not had an opportunity to analyze plasma of patients or normal subjects whose diet included substantial amounts of arachidic or

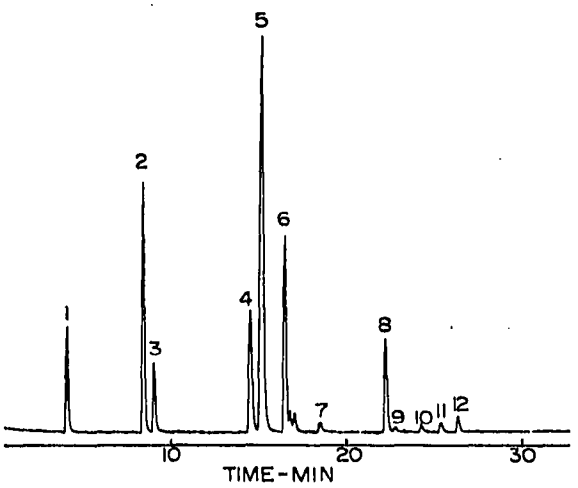

Fig. 2. Separation of methyl esters of long-chain acids which may occur in human plasma lipids. The acids are: 1 = myristic acid; 2 = palmitic acid; 3 = palmitoleic acid; 4 = stearic acid; 5 = oleic acid; 6 = linoleic acid; 7 = linolenic acid; 8 = arachidic acid; 9 = 11-eicosenoic acid; 10 = 11,14-eicosadienoic acid; 11 = 11,14,17-eicosatrienoic acid; 12 = arachidonic acid (5,8,11,14-eicosatetraenoic acid). The conditions were the same as for Fig. 1.

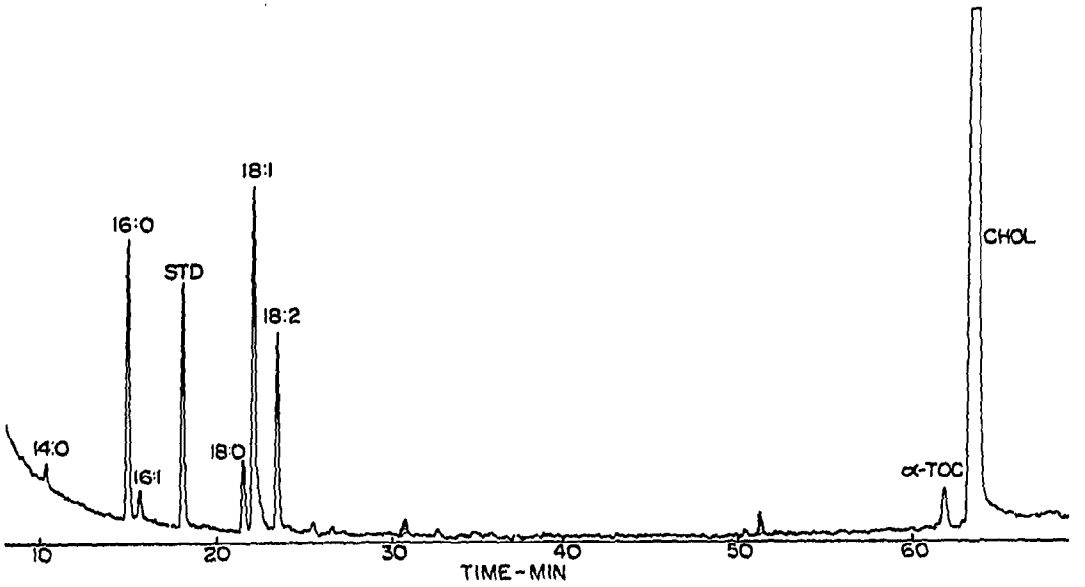

Fig. 3. Profile of FFA methyl esters, α-tocopherol trimethylsilyl ether and cholesterol trimethylsilyl ether from plasma of a normal human adult. The conditions were the same as for Fig. 1, except that the initial temperature was 165°. 14:0, Methyl myristate; 16:0, methyl palmitate; 16:1, methyl palmitoleate; STD, methyl heptadecanoate; 18:0, methyl stearate; 18:1, methyl oleate, with evidence of isomers; 18:2, methyl linoleate; α-TOC, α-tocopherol trimethylsilyl ether; CHOL, cholesterol trimethylsilyl ether.

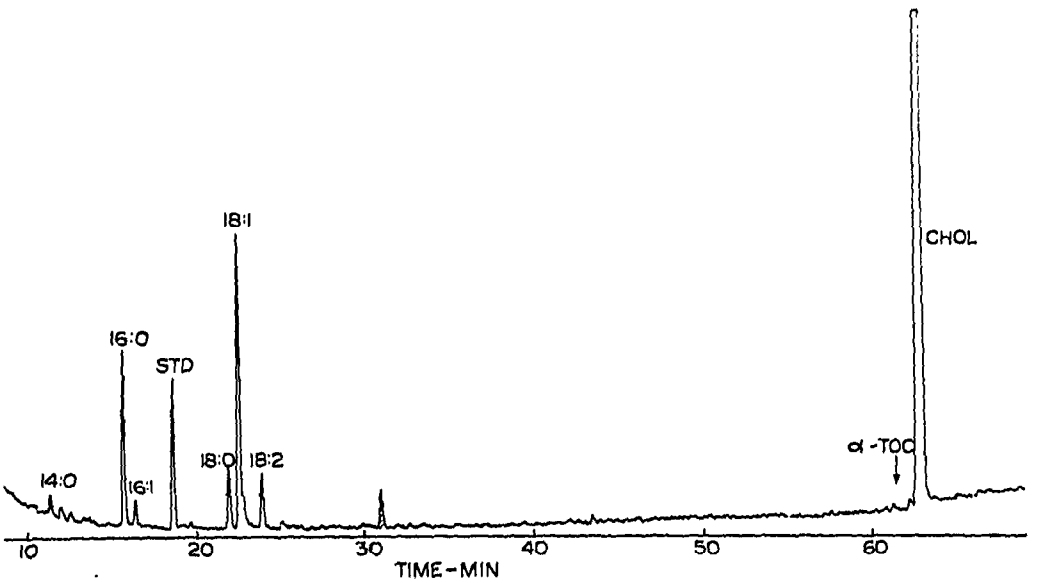

Fig. 4. Profile of FFA methyl esters, α-tocopherol and cholesterol trimethylsilyl ethers from plasma of a stroke patient. The conditions and compounds were the same as for Fig. 3.

TABLE II

ANALYSES OF STANDARD MIXTURE NHI-D[1]

Analyses were carried out over a period of several months while the same column was being used for analyses of biological samples. Figures in parentheses show the deviation of the found values from the true composition.

Experiment	*Composition (%)*				
	14:0	*16:0*	*16:1*	*18:0*	*18:1*
1	10.8	24.1	6.9	12.8	45.3
	(−1.0)	(+0.5)	(0.0)	(−0.3)	(+0.7)
2	11.5	23.4	7.4	12.7	45.0
	(−0.3)	(−0.2)	(+0.5)	(−0.4)	(+0.4)
3	11.8	23.2	8.2	10.8	45.9
	(0.0)	(−0.4)	(+1.3)	(−2.3)	(+1.3)
4	12.7	25.6	6.7	11.8	43.3
	(+0.9)	(+2.0)	(−0.2)	(−1.3)	(−1.3)
5	11.8	23.9	6.2	13.4	46.3
	(0.0)	(+0.3)	(−0.7)	(+0.3)	(+1.7)
6	9.5	23.5	6.3	14.2	46.5
	(−2.3)	(−0.1)	(−0.6)	(+1.1)	(+1.9)
Average	11.4	24.0	7.0	12.6	45.4
S.D.*	1.1	0.9	0.8	1.2	1.2
Composition	11.8	23.6	6.9	12.1	44.6
Accuracy S.D.**	1.2	1.0	0.8	1.4	1.5

* Standard deviation.
** Standard deviation with respect to known composition.

erucic acid. It is generally agreed that the composition of a plasma FFA fraction reflects the triglyceride composition in adipose tissue, subject to the "last in–first out" relationship[13]. The presence of at least two oleic acid isomers is evident in Fig. 3.

Standard mixture NHI-D[1] was used to monitor the FFA analyses. Table II contains the results of six analyses, carried out over a period of several months while the same column was being used for analyses of biological samples. There was no evidence of deterioration of the column, loss of column efficiency, or systematic drift of analytical values. The precision and accuracy of the determinations for myristic, palmitic, palmitoleic, stearic and oleic acids are well within the usual range.

The Dole–Meinertz extraction procedure is recognized as being satisfactory for FFA extraction from plasma, but the analytical sample contains many substances (neutral lipids and traces of phospholipids) that might lead to variable results in a GC determination of the acids. Tables III and IV contain the results of analyses carried out to test the reproducibility of the extraction procedure and the analytical method. Four samples from a single plasma collection were extracted separately, and 2–4 GC analyses were carried out for each of the four analytical samples. The variation is about that which would be expected from the GC analyses alone.

The total FFA values observed in this work for normal subjects were 0.11–0.57 m*M*; these are in the ranges reported in the studies summarized by Falholt *et al.*[7]. The values for stroke patients are included as part of the clinical studies carried out with these patients, and will be reported separately. A direct comparison with a titration method was not made; according to Ko and Royer[14] GC analytical values are

TABLE III

REPRODUCIBILITY OF THE DOLE EXTRACTION PROCEDURE AND THE GC ANALYTICAL METHOD FOR FFA COMPOSITION

All analyses were carried out with aliquot samples of the same plasma sample.

Extraction	*Composition (%)*				
	16:0	*16:1*	*18:0*	*18:1*	*18:2*
1	40.4	3.4	13.7	33.0	9.5
	40.3	3.8	14.5	31.8	9.6
	38.9	3.7	13.1	34.7	9.6
Average	39.9	3.7	13.8	33.1	9.6
2	40.3	3.5	12.7	34.3	9.2
	37.2	4.5	12.6	36.2	9.6
Average	38.7	4.0	12.7	35.3	9.4
3	40.4	4.0	13.6	32.4	9.6
	39.3	3.7	14.6	33.9	8.5
	39.1	3.9	14.1	34.1	8.9
	39.9	3.4	14.4	34.1	8.1
Average	39.5	3.8	14.2	33.6	8.8
4	40.8	4.3	13.5	31.3	10.2
	38.2	3.6	13.2	34.1	10.8
	40.5	3.6	13.5	32.5	10.0
Average	39.8	3.8	13.4	32.7	10.3
Average*	39.5	3.9	13.5	33.7	9.5
S.D.**	0.5	0.1	0.7	1.1	0.6

* Average of all values.
** Standard deviation.

lower than titration values (perhaps because of small amounts of phospholipids in the extract).

The advantages of high-efficiency columns in studies of isomer separations have been described by Ackman[15] and by Ackman and Hooper[16]. An examination of Table I and Fig. 3 indicates that the conditions employed in this work will not provide separations of all oleic acid isomers, and it is possible that some linoleate isomers will not be separated. The peak shape for methyl oleate shown in Fig. 3 demonstrates the well known fact that human "oleic acid" is not a single compound for most adults.

Fig. 4 shows a profile for a stroke patient; this should be compared with Fig. 3. The compositional change in the long-chain acid part of the profile is evident. The linoleic acid peak is smaller and the oleic acid peak is larger for the stroke patient, when compared with a normal subject. The linoleic acid:oleic acid ratio was calculated for each individual participating in the study. Compositional data are given in Tables V and VI.

Determination of α-tocopherol in plasma

A recent review by Sheppard *et al.*[17] summarizes GC methods used up to 1972 for the determination of the tocopherols in plant and animal tissues, and in foods. A review by Bunnell[18] summarizes all methods used up to 1971, including colorimetric procedures. The chief analytical problems have been those arising from the generally

TABLE IV

REPRODUCIBILITY OF THE DOLE EXTRACTION PROCEDURE AND THE GC ANALYTICAL METHOD FOR FFA CONCENTRATION

All analyses were carried out with aliquot samples of the same plasma sample. Concentrations were obtained by employing heptadecanoic acid as internal standard.

Extraction	*Concentration in plasma (mM)*				
	16:0	*16:1*	*18:0*	*18:1*	*18:2*
1	0.15	0.01	0.05	0.13	0.04
	0.15	0.01	0.05	0.12	0.04
	0.15	0.01	0.05	0.13	0.04
Average	0.15	0.01	0.05	0.13	0.04
2	0.15	0.01	0.05	0.13	0.03
	0.15	0.02	0.05	0.14	0.04
Average	0.15	0.02	0.05	0.14	0.04
3	0.15	0.02	0.05	0.12	0.04
	0.16	0.02	0.06	0.14	0.04
	0.15	0.02	0.05	0.13	0.03
	0.15	0.01	0.06	0.13	0.03
Average	0.15	0.02	0.06	0.13	0.04
4	0.17	0.02	0.06	0.13	0.04
	0.16	0.02	0.06	0.14	0.05
	0.17	0.02	0.06	0.14	0.04
Average	0.17	0.02	0.06	0.14	0.04
Average*	0.16	0.02	0.06	0.14	0.04
S.D.**	0.01	0.01	0.01	0.01	0.00

* Average of all values.
** Standard deviation.

TABLE V

PLASMA FFA COMPOSITION AND α-TOCOPHEROL CONCENTRATION FOR NORMAL ADULT HUMANS

Subject	*Composition (%)*					*Ratio 18:2/18:1*	*α-Tocopherol (mg/100 ml)*
	16:0	*16:1*	*18:0*	*18:1*	*18:2*		
1	28.6	3.5	18.4	33.9	15.6	0.46	1.22
2	27.4	1.8	15.7	33.0	21.9	0.66	0.70
3	24.3	3.1	11.3	44.6	16.7	0.37	0.51
4	30.8	2.7	14.6	38.0	14.4	0.38	0.44
5	28.9	2.2	16.0	35.8	17.0	0.48	0.50
6	24.9	2.7	10.9	40.9	20.5	0.50	0.58
7	28.3	3.5	18.9	35.4	13.9	0.39	0.71
8	26.6	1.9	16.1	36.8	18.5	0.50	1.07
9	34.7	1.1	14.7	30.6	18.9	0.62	0.95
10	23.1	3.0	12.3	40.3	21.4	0.53	0.75
11	27.1	1.7	12.9	39.6	18.6	0.47	0.62
12	30.9	1.6	20.1	34.6	12.7	0.37	1.12
13	25.1	2.6	8.8	41.2	22.3	0.54	1.20
14	27.6	1.8	18.4	34.4	17.8	0.52	0.51

TABLE VI

PLASMA FFA COMPOSITION AND α-TOCOPHEROL CONCENTRATION FOR PATIENTS

Subject	*Composition (%)*					*Ratio 18:2/18:1*	*α-Tocopherol (mg/100 ml)*
	16:0	*16:1*	*18:0*	*18:1*	*18:2*		
1	32.3	5.4	9.8	47.2	5.2	0.11	0.41
2	38.9	4.2	10.7	40.0	6.2	0.15	0.36
3	27.6	1.9	11.6	45.1	13.9	0.31	0.55
4	27.6	2.0	15.2	41.9	13.3	0.32	0.21
5	24.3	4.2	7.4	51.0	13.2	0.26	0.31
6	29.3	1.4	10.7	45.8	12.8	0.28	0.23
7	27.4	2.0	12.4	42.8	15.4	0.36	0.74
8	26.7	3.5	10.4	48.6	10.7	0.22	0.16
9	28.9	4.2	6.4	51.4	9.0	0.18	0.15
10	26.8	2.7	10.2	49.2	11.1	0.23	0.37
11	30.8	5.9	8.0	45.1	10.2	0.23	—*
12	32.8	2.5	9.2	51.5	4.0	0.08	0.21
13	25.8	3.9	9.9	52.0	8.3	0.16	0.23
14	32.7	4.1	7.4	47.8	8.0	0.17	0.44
15	35.7	4.1	8.6	43.8	7.8	0.18	—*
16	34.0	2.1	10.2	43.8	9.9	0.23	—*
17	29.2	4.6	9.0	43.7	13.4	0.31	0.22
18	31.6	4.7	7.6	41.0	13.5	0.33	0.23

* Not measurable.

low concentration of the tocopherols in lipid fractions, from the relatively high temperature needed for the elution of the tocopherols during GC analysis, and from an inability to separate β- and γ-tocopherols in analyses of foods and α-tocopherol from cholesterol in plasma analyses. The method described here is based upon the use of recently developed open tubular capillary column technology[3-5,19,20] which provides columns possessing both high theoretical plate efficiency and high capacity. Fig. 5 shows a separation of the four known tocopherols (α-, β-, γ- and δ-) as trimethylsilyl ethers with a PZ-176 (ref. 2) open tubular capillary column. The positional isomers, β- and γ-tocopherol, are separated; this has not been accomplished previously by GC techniques. The separation of α-tocopherol and cholesterol, as trimethylsilyl ethers, is shown in Fig. 6. This has not been accomplished previously by GC techniques.

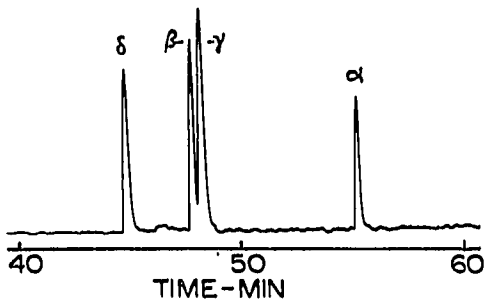

Fig. 5. Separation of the four known tocopherols (α-, β-, γ- and δ-) as trimethylsilyl ethers. The conditions were the same as for Fig. 1 with longer programming.

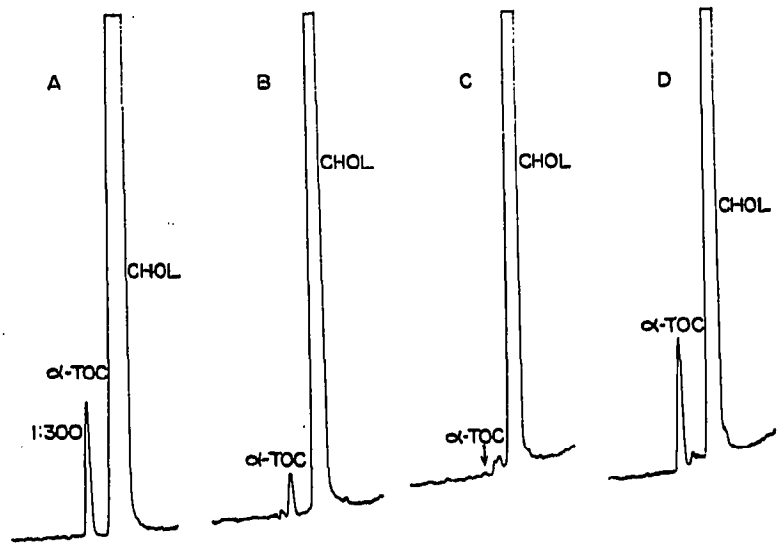

Fig. 6. Separation of α-tocopherol and cholesterol as trimethylsilyl ethers. A, reference compounds in 1:300 ratio. B, analysis for a normal human subject. C, analysis for a stroke patient. D, analysis of the same plasma used for C, but with addition of α-tocopherol.

Through use of open tubular columns it is no longer necessary to use thin-layer chromatographic procedures recommended for tocopherol separations[21–23] or a digitonide precipitation procedure for the separation of α-tocopherol and cholesterol[24].

The lipid fraction of plasma obtained by use of the Dole–Meinertz extraction procedure contains triglycerides and cholesterol esters, as well as free cholesterol, FFA and α-tocopherol. In the past, saponification procedures have generally been recommended as the first step in analytical methods for the tocopherols; the unsaponifiable fraction has usually been regarded as the starting point for separation procedures, and the chief problem is that of separating sterols from the tocopherols. The method used in this work is based upon the vaporizer–forecolumn design of German and Horning[5], which has a high sample capacity, and upon column technology which also permits use of relatively large samples. Fig. 6A shows a separation of α-tocopherol and cholesterol in a mass ratio of 1:300. Fig. 3 shows a separation of these compounds for a plasma sample. The presence of other neutral lipids (retained in the forecolumn) does not affect the separation.

Fig. 4 shows the result obtained when a plasma sample from a stroke patient was analyzed. The α-tocopherol peak is missing. This result, which was obtained in the early stages of our work, has considerable biological interest. The unusual nature of this result made it necessary to examine both the extraction and instrumental analysis steps in greater detail. Fig. 6 shows the effect of adding α-tocopherol to a plasma sample previously found to have no detectable concentration of this compound. Fig. 6B shows an analytical chart for a normal plasma sample, for comparison. Fig. 6C shows an analytical chart for a stroke patient, and Fig. 6D shows a chart obtained after addition of α-tocopherol to the plasma sample used for the analysis shown in Fig. 6C. There are apparently no special properties attached to plasma from stroke

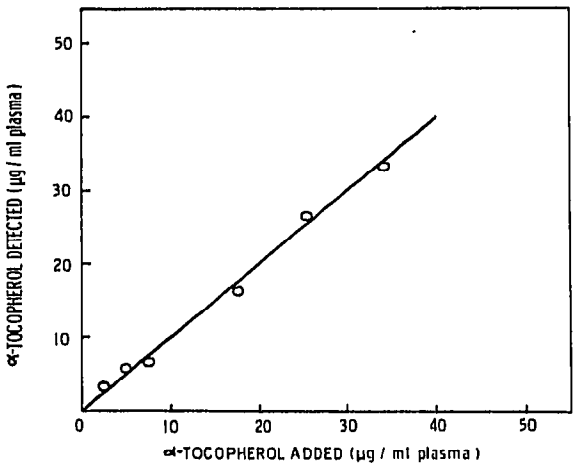

Fig. 7. Results of recovery experiments carried out to define the mass–instrumental response relationship for α-tocopherol. Increasing amounts of α-tocopherol were added to aliquot samples of the same plasma, and the analysis was carried out as usual.

patients which would make it impossible to obtain reliable α-tocopherol concentration data. Recovery experiments were also carried out. Fig. 7 contains the results. A plasma sample which contained no detectable α-tocopherol was used as a blank. Increasing amounts of α-tocopherol were added to aliquot samples of plasma, and the analyses were carried out as usual. The results (Fig. 7) indicate that the mass:instrumental response ratio is linear, and that the extraction step is essentially quantitative, for concentrations up to about 3 mg/dl of α-tocopherol.

Many studies of plasma α-tocopherol concentration have been carried out; Harris *et al.*[25] summarized the results obtained up to 1961. Bieri *et al.*[26] obtained similar results in a later study. The values for normal subjects found in this study are in the range found in earlier work. The distribution of values is usually between about 0.3 and 1.8 mg/dl, with a mean of about 1 mg/dl. It is known that circulating α-tocopherol is associated with the β-lipoprotein fraction, and variations in concentration are due both to variations in size of the exchangeable pool of α-tocopherol and to differences in β-lipoprotein plasma concentration. The ingestion of α-tocopherol results in higher plasma concentration values, if intestinal absorption mechanisms are not impaired[27]. There is insufficient evidence to define an optimal range, and the kinetics of α-tocopherol metabolism have not been adequately defined.

Since the values found for normal subjects are in accord with previous observations, and since there is no difficulty in analyzing plasma from stroke patients after the addition of α-tocopherol, the results for stroke patients are considered to be analytically valid. Low plasma concentrations have been observed previously in human studies, as indicated in a later section, but not for stroke patients.

Risk factor analyses

The current approach to preventive medicine with respect to atherosclerotic vascular disease and its complications is to define risk factors. For stroke, two widely accepted risk factors are hypertension and elevated serum cholesterol concen-

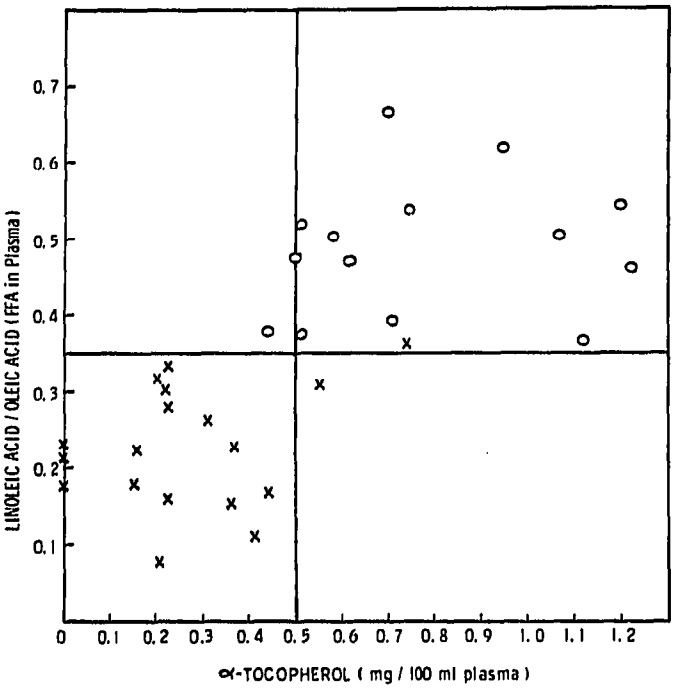

Fig. 8. Relationship between plasma α-tocopherol concentration and FFA linoleic acid: oleic acid ratio found for stroke patients (×) and for normal human subjects (○).

tration. Fig. 8 shows two additional parameters that warrant consideration as risk factors. One is plasma α-tocopherol concentration, and the other is expressed as the FFA linoleic acid:oleic acid ratio. The values clustered in the upper-right quadrant are those for healthy, young (20–40 year) adults with no history of atherosclerotic vascular disease. The values in the lower-left quadrant are for stroke patients. The division of α-tocopherol values at 0.5 mg/dl is based on the accepted view that values below this concentration are undesirable (because of the demonstrable increase in peroxide-induced hemolysis of erythrocytes that results when the concentration of α-tocopherol falls below 0.5–0.6 mg/dl).

Proponents of the antioxidant hypothesis of α-tocopherol action may view these results as virtual confirmation of the hypothesis, implying that a low concentration (less than 0.5 mg/dl) of circulating α-tocopherol results in peroxidative destruction of linoleic acid. These data, however, can be used only to define associative, and not causative, relationships. The risk factor approach is based on associative relationships, and in the absence of direct causative information emphasis should be placed on evaluating this analytical method as a risk factor analysis. The chromatographic procedures appear to be valid, and biological studies will be required to evaluate the significance of the results shown in Fig. 8. This will probably require the development of additional new analytical methods, as discussed in the next section.

ANALYTICAL REQUIREMENTS FOR FURTHER WORK

This paper is concerned with analytical methodology, rather than with

biological relationships. The analytical results, however, suggest that an increased effort should be made to define the biological function of α-tocopherol. This will almost certainly depend upon new analytical methods. It is therefore appropriate to discuss the problem in this context.

α-Tocopherol

The classical approach to defining function for a vitamin-like substance is to characterize physiologic and/or biochemical changes associated with the deficiency state. In this instance, animal studies have led to conflicting views. A variety of pathologic circumstances may result when an α-tocopherol-deficient state is established. These include anemia, encephalomalacia, exudative diathesis, muscular dystrophy and reproductive failure, but not all of these occur together and in some studies no major effects have been found. The complexity of the problem is illustrated by the fact that carp (*Cyprinus carpio*) develop muscular dystrophy when fed an α-tocopherol-free diet, but rainbow trout (*Salmo irideus*) do not[28], and that antioxidants with no known specificity of action will in some instances protect animals against deficiency effects. Human studies have been disease-oriented. Low plasma concentrations of α-tocopherol have been observed in association with a-β-lipoproteinemia[29], with disorders having as a common element a severe impairment of lipid absorption[27,29,30], and with cystic fibrosis[29,31,32]. The nature of the association in humans indicates that these disorders cause low plasma concentrations, rather than the reverse.

The view that α-tocopherol has no specific function other than that of a lipid-soluble antioxidant has many adherents. Whether or not this is true, it is important to recognize that measurements of α-tocopherol concentration alone will not resolve this issue. The carotenes, retinol, retinoic acid, retinyl palmitate, the ubiquinones and long-chain poly-unsaturated acids react readily with singlet oxygen and other reactive oxygen species. These shared properties suggest that it might well prove to be difficult to devise an experiment to define the function of α-tocopherol in a biological system unless analytical methods for estimating some or all of these compounds are used at the same time. The sparing action of α-tocopherol on liver retinyl palmitate is an example of the complex balance of oxygen-sensitive compounds existing in biological systems[33].

It has proved difficult to carry out definitive experiments through whole animal depletion studies. The circulating α-tocopherol can be reduced, but residual α-tocopherol permits cell function to continue. A deficiency effect would be expected to show first in newly generated cells. This is the situation observed for humans. It has been reported that cells of the intestinal villi are abnormal in a deficiency state[34], and the sensitivity of erythrocytes to peroxide-induced hemolysis rises sharply when the plasma concentration of α-tocopherol falls to 0.5–0.6 mg/dl[27]. These effects may be due to a functionally significant deficiency of α-tocopherol in new cells, but it is also possible that the altered properties of these cells are due partly to faulty assembly processes, or to altered long-chain acid membrane composition. If the observed effects are predominantly late expressions of events set in motion early in the life of the affected cells, late analyses may not be fully informative.

The analytical requirements for further study of this problem are not simple. It is a virtual certainty that it will be necessary to measure concentrations of a number

of other substances, as well as that of α-tocopherol, under a variety of circumstances. If multicomponent analyses of single cells, or of small cell populations, are required, it will be necessary to develop procedures with very high sensitivity in detection. If larger samples can be used, it will still be necessary to develop new analytical procedures. For example, procedures are not now available for the estimation of retinoic acid and the ubiquinones in small samples of biological origin.

The analytical results for normal subjects found in this study are not different from those reported in other studies, as indicated earlier. Plasma analyses for patients with one or more indications of atherosclerotic vascular disease, for individuals with a history of transient ischemic attacks, and for long-term stroke survivors would be of interest. The relatively low cost of GC analyses is appealing in this connection, but the development of reference procedures based upon gas chromatograph–mass spectrometer–computer techniques should be regarded as a requirement. GC methods with mass detection alone lack the specificity contributed by MS.

Unsaturated long-chain acids

The total number of naturally occurring long-chain acids is very large, but plasma FFA fractions are among the simplest in composition. These acids are released by adrenergic action from adipose tissue. There is considerable evidence indicating that the composition of adipose tissue triglyceride stores is diet-related, but it is highly unlikely that the differences observed in this study for FFA $C_{18:2}/C_{18:1}$ ratios were diet-determined. The question posed by these results is whether differences in FFA composition, or in the composition of other blood lipid fractions, are caused by an α-tocopherol-deficient state, and whether the current analytical methods are adequate for further studies.

Evidence pointing to a plasma linoleic acid–α-tocopherol relationship was recently reported by Rosenlund *et al.*[35]. Children with cystic fibrosis have low plasma concentrations of α-tocopherol; the average value for 20 patients in the study of Underwood *et al.*[31] was 0.21 mg/dl. Compositional analyses for plasma cholesteryl esters, phospholipids and triglycerides for the Philadelphia patients[35] showed marked differences from normal subjects; the linoleic acid content was depressed, particularly in the cholesteryl ester and phospholipid fractions, and the oleic acid content was increased. In the study of Underwood *et al.*[31] of long-chain acids in tissues of cystic fibrosis patients, it was found that the content of linoleic acid was decreased, with an increase in oleic acid, for liver, heart, kidney and skeletal muscle, when compared with normal subjects. In our study, only plasma FFA data were obtained, but the same effect was observed.

The fish feeding studies of Watanabe *et al.*[28] are of interest. The proportion of linoleic acid observed in carp visceral lipids was 31.8% for a control group and 7.1% for an α-tocopherol-deficient group. The corresponding values for carp muscle lipids were 15.5 and 0%. These are very large differences. Carp developed muscular dystrophy; rainbow trout, in similar feeding experiments, did not[28].

All of these observations, together with the data obtained in this study, suggest that the pool of available linoleic acid is reduced in size, and that the rate of metabolic transformation of ingested linoleic acid is faster in a deficiency state than in a normal state. Comparisons of rates and routes of metabolism of linoleic acid under different circumstances can not be arrived at solely by composition studies.

Radioactive tracer methods can be used, but there are advantages in the use of stable isotope-labeled compounds in human studies. A decreased halflife of exchangeable linoleic acid might be due to an accelerated conversion to and increased consumption of arachidonic acid, rather than to increased oxidative degradation of linoleic acid. The observations could also be explained by destruction of linoleic acid in the gut. These and other possibilities require tracer methods of analysis, rather than compositional analyses alone, for their study.

The triggering event in thrombus formation in stroke is not known, but it may be the release from platelets of an endoperoxide derived from arachidonic acid. The requirement of a compound of this kind for platelet aggregation is suggested by the studies of Hamberg and coworkers[36–38], Willis[39], and Smith *et al.*[40]. The extent of peroxidation occurring in the body is believed to be inversely related to α-tocopherol availability, but direct methods for estimating the products of specific peroxidation reactions, and of non-specific peroxidation products, are not available. Substitutes that have been suggested are the measurement of long-chain conjugated diene acids in the blood[41] and measurement of ethane generation[42]. Measurement of malondialdehyde production[43] from liver mitochondria has been used in *in vitro* studies of peroxidation. The sensitivity of erythrocytes to peroxidative damage when the plasma α-tocopherol concentration is reduced is well established; platelets may also act differently from normal either because of a lowered α-tocopherol concentration or because of membrane structural changes.

LITERATURE REFERENCES

Approximately fifty years of investigative work has not brought agreement on the biological function of α-tocopherol, and the vastness of the literature makes it difficult to discuss without omissions even such a limited topic as the chemical analytical aspects. Current views of α-tocopherol function in man are summarized in a review by Olson[44]. A recent conference report[45] includes examples of most lines of investigation, together with reviews of current hypotheses of biological action. As far as is known, the associative relationship shown in Fig. 8 for stroke patients has not been defined previously.

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