Determination of α-tocopherol in erythrocytes by gas–liquid chromatography

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ABSTRACT A method is described for the determination of submicrogram amounts of α-tocopherol in 0.5 ml of packed erythrocytes. The α-tocopherol in a lipid extract is oxidized to α-tocopherylquinone which is separated by thin-layer chromatography, eluted, and quantitated by gas–liquid chromatography. Calculation is based on the recovery of added α-tocopherol-^{3}H.

Erythrocytes from stock rats had an average α-tocopherol concentration of 344 μg/100 ml of packed cells, while for human cells the average was 235 μg/100 ml. The ratio of red cell to plasma α-tocopherol was 0.482 for rat blood, and 0.244 for human blood.

SUPPLEMENTARY KEY WORDS erythrocyte α-tocopherol · red cell vitamin E · blood α-tocopherol · vitamin E determination

MUCH OF THE CURRENT interest in the nutrition and metabolism of vitamin E has centered on its role in stabilizing the erythrocyte both in vivo (1–3) as well as in vitro (4–6). Despite considerable research over 20 yr on the effect of vitamin E nutrition on the red blood cell, there is a paucity of information on its vitamin E content. Bratzler, Loosli, Krukovsky, and Maynard (7) indirectly determined the tocopherol content of swine erythrocytes. Sternberg and Pascoe-Dawson (8), and Krishnamurthy and Bieri (9) measured red cell radioactivity after administering α-tocopherol-^{14}C to rats. Kaludin (10, 11) analyzed both rat and rabbit erythrocytes for α-tocopherol. To our knowledge, no information is available on human red cells. Prior to undertaking studies of the relationship between plasma and red cell α-tocopherol, it was considered important to develop a reliable method for the determination of the α-tocopherol content of erythrocytes.

Preliminary experiments using a method (12) which gave 85–90% recovery of α-tocopherol from a variety of animal tissues were found to yield erratic results and only 30–60% recovery of α-tocopherol-^{14}C added to erythrocytes. This procedure entailed the thin-layer chromatograph separation of α-tocopherol from an unsaponfiable extract of red cells and then its colorimetric determination. The loss was due primarily to the oxidation of the α-tocopherol to more polar compounds, primarily α-tocopherylquinone. Attempts to reduce the oxidation by a variety of methods, e.g. addition of antioxidants or conversion of the hemoglobin to methemoglobin or carboxyhemoglobin, did not improve the recovery. Attempts to separate α-tocopherol-^{14}C by TLC from a total lipid extract of labeled red cells indicated that considerable oxidation of the tocopherol occurred during extraction.

These results showed that the analysis of α-tocopherol in erythrocytes was more difficult than for serum (or other tissues). In view of the persistent oxidation of α-tocopherol during extraction from red cells despite various precautions, it was decided to oxidize the α-tocopherol to α-tocopherylquinone and to determine this latter compound. The procedure which was developed utilizes GLC and an isotope-dilution technique. It is in many respects analogous to current methods of plasma steroid analysis (13, 14).

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

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EXPERIMENTAL PROCEDURE

Materials

All solvents were reagent grade and were redistilled (except chloroform) using a 60 cm Vigreaux column. Each solvent was checked for purity by evaporating 1.0 ml and injecting the residue, dissolved in carbon disulfide, into the gas chromatograph at the highest sensitivity to be used.

Glass tubes were cleaned by soaking them overnight in chromic acid. They were then washed with detergent and rinsed with tap and distilled water. Prior to use, tubes were rinsed with 1 ml of hexane. Particular care was taken not to touch fingers to the ground glass portion of glass stoppers since cholesterol contamination interfered with the gas chromatographic separation.

Thin-layer plates (10 × 20 cm) coated with Silica Gel H (0.25 mm thick) were purchased from Analtech, Inc. (Wilmington, Del.). These were cleaned by predevelopment in the solvent system (see below) for 2 hr. They were then dried at 100°C for 1 hr and stored in a desiccator.

Tritiated 5-methyl-*d*-α-tocopherol was synthesized by the chloromethylation procedure of Green, McHale, Marcinkiewicz, Mamalis, and Watt (15) as modified by Dr. A. S. Csallany.[1] *d*-α-Tocopherol (Eastman Chemical Products Inc., Rochester, N.Y.) was reacted with tritiated paraformaldehyde (Nuclear-Chicago Corp., Des Plaines, Ill.), and the reaction mixture was reduced with zinc dust. The product was purified by two one-dimensional thin-layer chromatographic separations on Silica Gel G. For the first separation benzene–ethanol 99:1 was used, and the α-tocopherol area was eluted with methanol. An equal volume of water was added to the methanol solution, and the tocopherol was extracted into hexane. The hexane solution was concentrated, and a second chromatographic separation was carried out using benzene–ethyl acetate 3:1. The α-tocopherol area was eluted as described above. The specific activity of tocopherol was 2.28×10^8 dpm/mg.[2]

Counting was done with a Packard Model 3003 scintillation counter using Liquifluor (New England Nuclear Corp., Boston, Mass.) as scintillation fluid.

GLC was performed with a Barber Colman Model 15 instrument using a hydrogen flame detector. Glass U columns 180 cm × 3 mm I.D. were packed with 3% QF-1 on 80–100 mesh silanized Supelcoport (Supelco, Inc., Bellefonte, Pa.). Ends of the column were plugged with silanized glass wool, and packing was added by using a weak vacuum and by light tapping of the glass tube with a pencil. The top of the column was 2 cm from the end of the syringe needle when fully inserted. The column was conditioned overnight at 230°C. Operating temperatures were: column, 230°C; injection heater, 250°C; detector bath, 275°C. Flow rates in ml/min were: argon carrier, 50–70; air, 275–330; hydrogen, 11–13. Attenuation was 9×10^{-12} amp for most analyses. Samples were injected in 2–5 μl of carbon disulfide using a 10 μl double-action syringe (Glass Engineering Co., Inc., Houston, Texas). A flush volume of 1.5 μl of solvent was taken into the syringe before the sample.

An aliquot of a stock solution of *d*-α-tocopherylquinone (Eastman Chemical Products, Inc.) in benzene (1 mg/ml) was diluted with carbon disulfide to give a working standard of 0.025 μg/μl. This solution was prepared fresh every 5 days.

The response of the detector as determined by peak area (height × width at half height) was linear in the range 0.025–0.25 μg of α-tocopherylquinone. Prior to injection of samples each day, an overload (0.2 μg) of tocopherylquinone was injected twice in order to saturate the active sites on the column. After every one or two samples, an amount of standard α-tocopherylquinone approximating the sample size, was injected since the instrument response after 3–4 hr of operation was usually greater than earlier in the day.

A typical gas–liquid chromatogram from normal human red cells is shown in Fig. 1. Under the conditions described above, α-tocopherylquinone came off the column in 7–8 min. Cholesterol, the only significant contaminant, appeared about 2 min before α-tocopherylquinone.

Erythrocytes

Venous blood (heparinized) was centrifuged for 10 min in an International Model PR-1 refrigerated centrifuge at 5°C and 2400 rpm (1000 *g*). The plasma and buffy layer were removed by aspiration, and the red cells were washed three times using 2 volumes of 0.89% sodium chloride for each wash. Before samples of the washed cells were taken for analyses, they were mixed to distribute light and heavy fractions. All blood used in these experiments had hematocrit values of 40–50%.

Stepwise Procedure for Analysis of Erychrocytes

Stage 1: Extraction. Pipet into a 15 × 150 mm glass-stoppered test tube 50 μl of α-tocopherol-^{3}H in benzene (15,000–20,000 cpm). Add 0.5 ml of water and 0.5 ml of washed red cells. Mix briefly on a tube vibrator, and let stand for 5 min. Slowly add 3 ml of methanol (previously cooled in an acetone-dry ice bath), while mixing on the vibrator, and then extract three times using 3, 2, and 2 ml of hexane. The tubes are shaken vigorously by hand for 1 min and centrifuged between extractions. Transfer the extracts to a 5 ml glass-stoppered test tube

[1] Personal communication.

[2] DL-α-Tocopherol (5-methyl-tritium) is available from Amersham Searle Corp., Des Plaines, Ill.

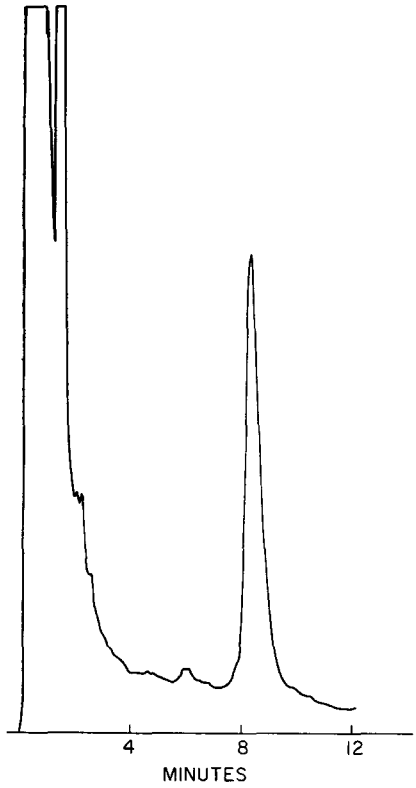

FIG. 1. GLC tracing of α-tocopherylquinone (0.07 μg) from 0.5 ml of human red cells after TLC separation. The sample was dissolved in 50 μl of CS_2, and 5 μl was injected. Column was 3% QF-1, 228°C; argon, 50 ml/min; air, 330 ml/min; hydrogen, 11 ml/min; attenuation was 9×10^{-12} amp.

evaporate the hexane under a stream of nitrogen and with the tube in a water bath at 60°C.

Stage 2: Oxidation of α-Tocopherol to α-Tocopherylquinone. To the tubes add 0.1 ml of chloroform to dissolve the lipid. Add 0.5 ml of ethanol and 0.25 ml of 5% ferric chloride in 1% hydrochloric acid. Mix the contents and let the tubes stand 5 min at room temperature. Add 0.5 ml of 1% bipyridine in ethanol and then mix, stopper the tubes, and place them in a 50°C water bath for 10 min. Cool the tubes, add 1 ml of water, and extract the contents three times with 2, 1.5, and 1 ml of benzene. After centrifuging, transfer the benzene layer to a 13 ml glass-stoppered centrifuge tube and wash twice with 3 ml of 0.9% sodium chloride by inverting gently to avoid emulsion formation. Centrifuge the tubes briefly and remove the water layer by aspiration. Repeat the washing two times with 3 ml of water. Add a small amount (50–60 mg) of anhydrous granular sodium sulfate to dry the benzene, and after 5 min centrifuge briefly. Decant the benzene into a 5 ml conical centrifuge tube, rinse the sodium sulfate with 0.5 ml of benzene and decant, being careful not to transfer particles of sodium sulfate. Evaporate the benzene, and rinse the tube walls down twice with 0.5 ml benzene to concentrate the lipid in the tip. Evaporate the benzene prior to TLC.

Stage 3: Thin-Layer Chromatography. The lipid is dissolved in 25 μl of benzene and applied to a TLC plate by means of a microsyringe with a repeating dispenser (Hamilton Company Inc., Whittier, Calif.) as a streak 5 cm long beginning 1 cm from the left edge of the plate. Two 20-μl rinses of the tube are also applied. On the same origin line, but 2 cm from the end of the lipid streak, 10 μg of α-tocopherylquinone is spotted. The plates are developed for 40–45 min in benzene–ethyl acetate 2:1 in jars lined with filter paper. After evaporating the solvent for 8 min in an oven at 100°C, the left two-thirds of the plate are covered with a plain glass plate and the right one-third is sprayed with 10% phosphomolybdic acid in ethanol. An area of silica gel opposite the standard α-tocopherylquinone spot (R_f, 0.5–0.6; Fig. 2) and no wider than 1.4–1.7 cm is scraped into a 13 ml centrifuge tube. If too wide an area is taken, contamination with cholesterol (R_f, 0.40–0.45) will occur.

The silica gel is eluted three times with methanol (2, 1.5, and 1 ml) by mixing vigorously for 30 sec on a tube vibrator. Centrifuge the tubes and carefully draw off the methanol and transfer it to a 13 ml glass-stoppered centrifuge tube. Add 2.0 ml of water and extract three times with hexane (3, 2, and 1 ml). Transfer the hexane extracts to a 5 ml conical centrifuge tube, and evaporate the solution to dryness under nitrogen. Rinse the walls with 0.25 ml hexane to concentrate the lipid in the tip, and again evaporate the solvent. Place the tube in an ice bath.

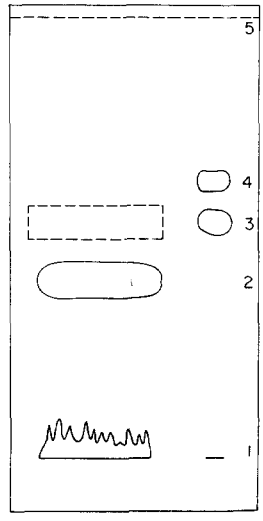

FIG. 2. Diagram of thin-layer chromatographic separation on Silica Gel G of the oxidized total lipid extract from 0.5 ml of red cells. Standard spots on right side of plate were visualized by spraying. The location of cholesterol is drawn in to show its proximity to tocopherylquinone. No. 1 is the origin; 2, cholesterol; 3, α-tocopherylquinone; 4, α-tocopherol; 5, solvent front. Solvent was benzene–ethyl acetate 2:1.

Stage 4: Gas–Liquid Chromatography. Dissolve the lipid in 50–150 μl of carbon disulfide, depending on the α-tocopherol content of the red cells, and inject 2–5 μl on to the column. Immediately thereafter, transfer three 5-μl aliquots to counting vials. A final 5 μl aliquot is taken into the syringe in the event a duplicate injection is required. This procedure should take only 3–4 min so that evaporation of the carbon disulfide does not become a significant source of error.

Stage 5: Calculation.

$$\left[\left(\frac{A}{B} \times C \times \frac{D}{E} \times \frac{100}{F}\right) - G\right] \times \frac{100}{H}$$

= μg α-tocopherol per 100 ml of packed red cells.

Where:

A = Peak area of unknown.
B = Peak area of standard.
C = μg of tocopherylquinone in standard peak.
D = Volume of carbon disulfide used to dissolve the sample.
E = Volume of the sample injected.
F = Percent recovery of α-tocopherol–^{3}H.
G = Amount of tocopherylquinone contributed by the α-tocopherol–^{3}H.
H = Volume of erythrocytes taken for analysis.

Two samples required a full day for analysis, but eight samples were easily completed in a two-day period.

RESULTS AND DISCUSSION

Extraction of the red cell lipids with hexane was selected in preference to methods using chloroform because hexane removes less cholesterol and also requires less time for subsequent evaporation. The efficiency of the methanol–hexane extraction for α-tocopherol, was compared with a procedure for red cells (16) in which isopropanol–chloroform is used. Washed cells from each of three vitamin E deficient rats which had been fed various amounts of α-tocopherol–^{14}C for 4 days were extracted with each of the two solvent mixtures. Yields of radioactivity from the cells extracted with methanol–hexane (480, 930, and 1380 cpm/ml cells) were essentially the same as those obtained with isopropanol–chloroform (480, 920, and 1300 cpm/ml cells).

Experiments to determine the recovery of α-tocopherol through the entire procedure were carried out by adding unlabeled *dl*-α-tocopherol to erythrocytes from vitamin E deficient rats. The amounts of α-tocopherol added were in the low to normal range found in human red cells (ca. 100–200 μl/100 ml). As shown in Table 1, the mean recovery was 98.9%.

To determine the reproducibility of the method, eight aliquots of a sample of washed red cells from a normal adult male were analyzed simultaneously. The mean value and standard deviation was 183.5 ± 9.3 μg/100 ml cells.

TABLE 1 RECOVERY OF α-TOCOPHEROL ADDED TO ERYTHROCYTES OF VITAMIN E DEPLETED RATS*

Added†	Blank Correction‡	Found	Recovery
μg	μg	μg	%
0.414	0.000	0.408	98.6
0.491	0.006	0.539	109.7
0.510	0.008	0.475	93.1
0.982	0.009	0.984	100.2
1.020	0.008	0.946	92.7
		Mean ± SEM	98.9 ± 3.1

* Male rats depleted of vitamin E for 4–6 months.
† The indicated amount of *dl*-α-tocopherol was added to 0.5 ml of washed red cells which were then carried through the entire procedure. All determinations were carried out in duplicate.
‡ This "background" amount of α-tocopherol was found in 0.5 ml of red cells analyzed at the same time as the recovery samples.

In the initial development of this procedure, a stationary phase of 1% SE-30 was used for the GLC determination of α-tocopherylquinone. Although this packing gave a fair separation of the quinone from cholesterol when the latter was present in only trace amounts (17), it was found that occasionally sufficient cholesterol was present in samples so that distortion of the α-tocopherylquinone peak made its quantitative estimation invalid. An OV-1 packing gave similar results, but 3% QF-1 was found to give an adequate separation of cholesterol from α-tocopherylquinone so that the occasional appearance of cholesterol in a sample offered no problem.

α-Tocopherylquinone peaks on the QF-1 column were generally very symmetrical as shown in Fig. 1. Occasionally, however, a human red cell sample gave a peak with a slight distortion of the leading edge of the base. This interfering material could not be attributed to a contaminant in the reagents but may possibly have been β-tocopherylquinone. However, β-tocopherylquinone when mixed with α-tocopherylquinone gave a distinct but unseparated peak.

No attempt was made to determine the presence of other tocopherols in erythrocytes. In the TLC separation, β- and γ-tocopherylquinones, if present, would not have separated distinctly from α-tocopherylquinone. R_f values for the tocopherylquinones were: α-, 0.54; β- and γ-, 0.50; δ-, 0.44. The GLC retention times relative to α-tocopherylquinone were: β-, 0.91; γ-, 1.09; δ-, 1.00. The QF-1 stationary phase thus is not suitable for a clear separation of the various tocopherylquinones. In any case, β- and γ-tocopherol constitute no more than 10–15% of the total tocopherol found in normal human plasma (18).

Consideration was given to analyzing α-tocopherylquinone as the silyl derivative by GLC. α-Tocopherylquinone has on the side chain a tertiary hydroxyl group which was found to react quantitatively with *bis*-(trimethylsilyl) acetamide. This derivative on a QF-1 column had a retention time which was 0.67 that of α-tocopherylquinone and gave a slightly greater detector response. It was felt that the extra step of silylation did not improve the over-all procedure sufficiently to justify its use.

An internal standard of cholesteryl acetate for quantitating the GLC response was tested in the early phases of this work with the OV-1 packing. No significant advantage of the internal standard was apparent when compared with the use of standard α-tocopherylquinone after each sample, as described. With the QF-1 packing, no suitable compound with a retention time close to that of α-tocopherylquinone could be found.

Oxidation of α-tocopherol to α-tocopherylquinone with ferric chloride was adapted from the procedure of Nair, Sarlos, and Machiz (19). When 1.0–1.6 μg of α-tocopherol-^{3}H was oxidized, and the extract separated by TLC, 80–90% of the counts were recovered in the α-tocopherylquinone area of the plate. When oxidation of this same amount of α-tocopherol was carried out in the presence of the total lipid extracted from 0.5 ml of erythrocytes from vitamin E deficient rats, then the recovery of radioactivity as α-tocopherylquinone was only 65%.

Recoveries of radioactivity from the complete procedure as described ranged from 43–64% with erythrocytes. With plasma, recoveries were always higher and ranged from 60–85%.

In Table 2 are the results of analyses of both erythrocytes and plasma from rats with various dietary intakes of α-tocopherol. All of the plasma values, with the exception of two over 1000 μg/100 ml, can be considered to be in the normal range (400–1000 μg/100 ml). Red cell α-tocopherol content ranged from 209 μg/100 ml for rats fed a relatively low intake to 619 μg/100 ml for rats with a high intake of α-tocopherol. Of particular interest is the ratio erythrocyte concentration:plasma concentration, which ranged from 0.330 to 0.482. It thus appears that in the rat the erythrocyte α-tocopherol concentration is slightly less than half that in the plasma.

In the only report of a direct analysis of α-tocopherol in rat erythrocytes, Kaludin (10) separated the α-tocopherol by paper chromatography and made a colorimetric determination. In rats 15–17 days old which had been fed a natural diet, the red cell:serum ratio was 0.284 while in older rats it was 1.50. Sternberg and Pascoe-Dawson (8) reported that the erythrocyte:plasma ratio of α-tocopherol-^{14}C ranged from 0.25–0.35 in 150 g rats at an unspecified time after a single oral dose. In earlier work in our laboratory (9) with α-tocopherol-^{14}C, plasma radioactivity was only slightly higher than that in the erythrocyte. In these studies labeled α-tocopherol with relatively low radioactivity was used, and counting was by means of a Geiger tube.

Examination of the values in Table 2 leads to the suggestion that the rat erythrocyte has a limited capacity for taking up α-tocopherol. As the plasma concentrations became quite high, over 1800 μg/100 ml, the red cell:plasma ratio was the lowest.

Analyses of blood from normal laboratory personnel are shown in Table 3. Plasma was analyzed by the same method as for erythrocytes. The plasma concentrations of α-tocopherol found were in the range reported earlier from this laboratory for a large number of normal adults (20). The concentration in human plasma is slightly

TABLE 2 α-TOCOPHEROL CONTENT OF ERYTHROCYTES AND PLASMA OF MALE RATS WITH VARIOUS DIETARY INTAKES OF α-TOCOPHEROL

Diet	Dietary α-Tocopherol	No. of Rats	Red Cells	Plasma*	Red Cells: Plasma
	mg/kg of diet		*μg/100 ml*	*μg/100 ml*	
Stock†	66	4	344 ± 45	714 ± 63	0.482
Purified‡	27–41	10	209 ± 8	526 ± 22	0.397
	200	2	618 ± 7	1873 ± 23	0.330

* Determined colorimetrically after separation from a total lipid extract by TLC. Values are means ±SEM.

† Purina Lab Chow. α-Tocopherol content approximate as furnished by manufacturer.

‡ Rats were depleted of vitamin E for 4–5 wk and were then given the indicated levels of *dl*-α-tocopheryl acetate (27 or 41 mg/kg of diet) for 8–10 days. The two rats given 200 mg/kg of diet received this amount for 2 months. All rats were fasted for 24 hr before they were killed.

TABLE 3 α-TOCOPHEROL CONTENT OF NORMAL HUMAN ERYTHROCYTES AND PLASMA*

Sex	Red Cells	Plasma	Red Cells: Plasma
	μg/100 ml	*μg/100 ml*	
M	223	534	0.418
F	249	820	0.304
M	323	1115	0.290
F	232	855	0.271
F	217	816	0.266
M	184	800	0.230
F	271	1239	0.219
M	328	1516	0.216
M	169	782	0.216
F	216	1605	0.135
M	175	1420	0.123
Mean ± SEM	235 ± 16.3	1045 ± 107	0.244 ± 0.02

* Blood samples from fasting subjects were analyzed in triplicate. For the analysis of plasma, 0.5 ml was mixed with 1.0 ml methanol, followed by extraction with hexane as for erythrocytes. The remainder of the procedure was the same as for erythrocytes.

higher than that in rats with moderate α-toco pherolin-takes, but the content of α-tocopherol in human erythrocytes is lower. This difference is most apparent from the mean erythrocyte : plasma ratio of 0.244 for human blood compared with a ratio of 0.330–0.482 for rat blood (Table 2). The method has been applied successfully to red cells and plasma from rats and humans with α-tocopherol concentrations as low as one-fifth the normal amount. In current studies we are exploring the relationship between plasma and erythrocyte α-tocopherol in a variety of patients with abnormal concentrations of blood lipids.

Manuscript received 21 August 1969 and in revised form 10 November 1969; accepted 8 December 1969.

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