A MICROMETHOD FOR ASSAY OF TOTAL TOCOPHEROLS IN BLOOD SERUM*

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A simple, accurate micromethod for vitamin E in blood would be a desirable addition to assay procedures now available. There appears to be no vitamin E method which requires less than 2 to 5 ml. of serum or plasma, and this amount is frequently unobtainable from small children or experimental animals. Economy of space and time is another advantage of using a micromethod.

The micromethod for measuring vitamin E in blood to be described here appears to fit these requirements. A pair of analysts can do 80 assays in a working day (and carotene is also measured in the method). The volume of blood serum or oxalated plasma which is required (0.06 ml.) is easily obtained by finger-tip puncture. The method employs techniques previously described, whereby the Beckman spectrophotometer is adapted to measurement of optical density of very small volumes and micro pipettes of the Lang-Levy type are used (1, 2).

For determining vitamin E in blood the direct measurement of its absorption peak at about 295 mμ is attractive. However, owing to the very low extinction value of tocopherol and to the presence of many interfering substances which absorb in this region, this method is not practical. Of the oxidimetric color reactions for vitamin E, only two seem to be sensitive enough for use in a blood method. One is oxidation (*e.g.* with silver nitrate) to give tocopherylquinone with an absorption peak at about 265 mμ; it does not seem useful for microassay because of carotene interference and because of the rigid control required to obtain precise results. The other is the Emmerie and Engel (3) color reaction with ferric chloride and α,α'-dipyridyl to give a red color. Since the latter reaction is precise and easy to perform, it has been used.

The method is based on the macroprocedure of Quaife and Harris (4). However, the hydrogenation step, which obviates interference due to carotene, has been omitted since it was not found feasible to hydrogenate

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small samples without loss of tocopherol, presumably because the catalyst sensitizes it to oxidation. (In the hydrogenation apparatus used in the blood macromethod (5), this loss is prevented by use of a closed system, from which the air is rapidly swept out by a stream of hydrogen.) Instead, the light absorption due to the carotenoids is measured at 460 mμ and a correction made for the contribution of carotene to the Emmerie-Engel reaction color at 520 mμ.

Procedure

Reagents and Apparatus—

1. Absolute ethyl alcohol, purified as follows: Distil the alcohol from a flask containing pellets of potassium hydroxide and crystals of potassium permanganate. Discard the first portion.

2. Xylene, C.P.

3. α,α′-Dipyridyl in *n*-propyl alcohol, 0.120 gm. per 100 ml.

4. Ferric chloride hexahydrate in absolute ethyl alcohol, 0.120 gm. per 100 ml. Keep this solution in a dark brown or red glass bottle, fitted with a glass or cork stopper.

5. Pyrex test-tubes, 6 × 50 mm., fitted with cork (*not rubber*) stoppers.[1]

6. Lang-Levy constriction pipettes (2), 60, 40, and 13 c.mm. It is desirable to have the upper constriction quite small to facilitate pipetting organic solvents with low surface tension. (They can be purchased from the Microchemical Specialties Company, Berkeley 3, California.)

7. A Beckman spectrophotometer fitted with a micro attachment and 2 mm. quartz cuvettes (1). (The micro attachment and cells are obtainable from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28.)

8. Test-tube racks about 5 × 5× 1½ inches to hold 100 tubes. These may be made from wire screen (two pieces of ½ inch mesh) or sheet metal.

9. A high speed hand drill, fitted as described by Bessey *et al.* (6).

Method

60 c.mm. of serum or plasma and 60 c.mm. of absolute ethyl alcohol[2] are put into a small (50 × 6 mm.) test-tube. The contents of the tube are mixed, immediately after the alcohol is added, by touching the side of

[1] Rubber stoppers are avoided because they contain substances readily extracted by organic solvents, which give a reaction with the Emmerie-Engel reagents. Corks are preferable, but contact with the solution must still be avoided. If small pieces of cork fall in the extraction mixture, false high Emmerie-Engel values will result. Corks covered with aluminum foil are satisfactory.

[2] All quantities of solution referred to in the method should be accurately measured. The exact amounts taken will depend on the volumes of the micro pipettes, and will not usually correspond exactly to the amounts stated.

the tube near the bottom to the whirling nail in the motor drill which has been mounted in a clamp with the nail up. 60 c.mm. of xylene are next added to each tube, and the tubes are corked to prevent loss by evaporation. Each tube is uncorked and mixed by touching to the motor drill long enough to allow violent agitation of the contents for at least 30 seconds, and then recorked. The tubes are centrifuged 10 minutes at 3000 R.P.M. To 40 c.mm. of the xylene supernatant layer, which has been transferred to another 50 × 6 mm. test-tube, are added 40 c.mm. of the α,α'-dipyridyl reagent. Each tube is touched to the motor drill to mix the contents and is then recorked. 60 c.mm. of the mixture are pipetted into a Beckman cuvette and the absorption read at 460 mμ with the use of distilled water as a reference blank. To the cuvette are then added 13 c.mm. of the ferric chloride reagent. The cuvette is rapidly rocked sideways through a 90° arc for 30 seconds to insure thorough mixing of the contents, and the absorption is measured at 520 mμ at $1\frac{1}{2}$ minutes after the addition of the ferric chloride. Similar readings are made on blank solutions which have been prepared by applying all of the procedure described above for serum or plasma to an equal volume of distilled water. At least two blanks should be included for each Beckman cuvette. As with other micromethods, three Beckman cuvettes, each containing an individual sample, are used along with the reference cuvette which has distilled water.

It is imperative to clean the Beckman cuvettes prior to each color reaction by filling with acetone and sucking dry twice. Use of ether is avoided, as it frequently contains peroxides, which destroy tocopherol, and also impurities, which give a color with the Emmerie-Engel reagents.[3]

Calculation

Calibration data are based on pure, natural, d,α-tocopherol and on crystalline (90 per cent β-, 10 per cent α-) carotene. The tocopherol calibration curve is made from results obtained on solutions in absolute ethyl alcohol, ranging in concentration from 0.500 to 2.000 mg. per 100 ml. 60 c.mm. aliquots of these are added to equal volumes of distilled water and treated as described for blood. The carotene is dissolved in xylene (in a concentration range of 50 to 500 γ per 100 ml.), diluted 1:1 with the dipyridyl in propyl alcohol, and readings made at 460 mμ and also at 520 mμ after ferric chloride addition.

Calibration data should, of course, be determined by each individual, since slight deviations in technique frequently cause considerable varia-

[3] Pipettes and other glassware are cleaned in conventional fashion, *e.g.* with chromic acid. Then they are repeatedly rinsed with distilled water to remove any trace of acid or base which might affect the color reaction. Then they are rinsed with acetone and dried.

tions in results in a given analytical method. This is especially true for the color reaction employed here, since it gives increasing color with time. Once established, the calibration factor has remained surprisingly constant over a long period.

Under the conditions described and with the given volumes of solution, 57.4 c.mm. of xylene-dipyridyl reagent and 12.2 c.mm. of ferric chloride reagent, it was found that carotene gives a reading at 520 mμ in the Emmerie-Engel reaction, which is 29 per cent of that of carotene itself at 460 mμ. Therefore, readings on blood extracts at 520 mμ are corrected to this extent.

If in the standardization the same pipettes are used for standard α-tocopherol solution as for serum or plasma, the pipette volumes cancel out. When all values of standard and unknown are corrected for reagent blanks, the calculation for vitamin E (total free tocopherols) in plasma or serum becomes

$$\text{Mg. \% vitamin E} = \frac{(D_{520}-0.29D_{460})}{D_{520}\text{ of standard}} \times \text{mg. \% vitamin E in standard}$$

With use of the volumes given above, a value of 6.10 was determined for the ratio of mg. per cent of vitamin E of standard to D_{520} of the standard.

Carotene itself can, of course, be determined from the 460 mμ readings. (The formula determined in this laboratory is micrograms per cent of carotene = $856 \times D_{460}$.)

DISCUSSION

On a macro scale the Emmerie-Engel reaction is preferably carried out with ethyl alcohol as the solvent, since rapid and complete color development occurs; also, possible color repression, which sometimes occurs when there is excess fat in the reaction mixture (7), is minimized. But it is too volatile for use in the micromethod. Acetic acid-petroleum ether mixtures as solvents have been avoided because they have the disadvantages mentioned above.

After some preliminary trial with various combinations of polar and hydrocarbon solvents, a 1:1 mixture of *n*-propyl alcohol and xylene was selected. Xylene is a hydrocarbon solvent which is sufficiently nonvolatile for use in extracting vitamin E from serum in the micromethod. It must be diluted with at least an equal volume of polar solvent in order to give rapid and complete color development of the Emmerie-Engel reaction for tocopherol in a blood extract. The polar solvent chosen, *n*-propyl alcohol, is a compromise between the greater polarity of ethyl alcohol and the lesser volatility of the higher alcohols. (It has a boiling point 16° higher than that of isopropyl alcohol.)

Aliquots of a solution of carotene in a 1:1 mixture of *n*-propyl alcohol and xylene, when contained in micro Beckman cuvettes in minimum cell volumes, gave stable readings at 460 mμ for at least 30 minutes. The time required to read them in the actual blood procedure should not exceed 5 minutes.

Although the fact that a timed reaction is used on the Beckman instrument might appear to be a handicap, in practice little difficulty is encountered in obtaining readings, both on pure tocopherol and carotene and on blood samples, which show good reproducibility. This applies to readings made on successive samples in the three micro cuvettes (plus the reference solution, distilled water) which are contained in the Beckman cuvette holder.

A large number of assays (*e.g.* 50 to 100) can be run simultaneously. Blanks must be run with each batch, but once the method is well standardized, standard solutions of vitamin E need not be run. The samples can be allowed to stand overnight in the refrigerator, after extraction and centrifugation, or after dilution of the xylene supernatant with the dipyridyl solution.

Tocopherol which had been added to the original serum extraction mixture at a level of 0.995 mg. per cent was recovered quantitatively (107 per cent) in the micromethod. (An absolute ethyl alcohol solution of pure α-tocopherol replaced ethyl alcohol in the extraction step.) This would be expected, since there are no steps in the procedure which are conducive to tocopherol loss, *e.g.* saponification.

Precision of the method appears as good as that of other micromethods. A few coefficients of variation are listed in Table I; they range from 2.7 to 5.7 per cent.

Results of assay by the micromethod were compared with those by the macromethod for a series of blood samples from presumably normal subjects, which include men and pregnant and non-pregnant women. They are listed in Table I. Good agreement is shown between them. The mean value for eighteen subjects was 1.11 mg. per cent by macroassay, and 1.07 mg. per cent by microassay.

Since the micromethod for vitamin E gives values identical with those given by the macromethod, within experimental error, the range of "normal" values would be the same: 0.8 to 1.2 mg. per cent for venous blood plasma or serum.

The micromethod was used to assay blood sera of rats which had been kept on a vitamin E-deficient diet for a year. The samples were obtained by cutting off the tip of the tail. The zero tocopherol levels found in the rat blood provide further evidence that extraneous materials other than tocopherol (and carotene) are not measured by the Emmerie-Engel reaction in the micromethod.

TABLE I

Comparison of Total Tocopherol Content of Human Blood Serum As Determined by Macro- and Microassay Methods

Sample No.	Macro values, assayed in duplicate	Micro values		Coefficient of variation
		Concentration	No of determinations	
	mg. per cent	*mg. per cent*		*per cent*
1	1.75	1.75 ± 0.06*	9	3.6
2	1.05	1.045 ± 0.028*	6	2.7
3	0.56	0.54 ± 0.03*	6	5.7
4	1.95	1.90	3	
5	0.73	0.71	3	
6	0.92	0.75	3	
7	0.76	0.76	3	
8	0.83	0.74	3	
9	0.97	0.92	3	
10	0.78	0.71	3	
11	1.33	1.26	3	
12	1.15	1.09	3	
13	1.39	1.21	3	
14	1.57	1.44	3	
15	1.05	1.03	3	
16	1.13	1.24	3	
17	0.91	0.87	3	
18	1.15	1.30	3	
Mean.....	1.11	1.07		

* Standard deviation.

TABLE II

Stability of Vitamin E in Blood Sera Stored in Deep Freeze at −22°

Sample No.	Tocopherols*		Loss
	Original	After 8 wks.	
	mg. per cent	*mg. per cent*	
1	1.90	1.73	9
2	0.54	0.54	0
Mean.....			4.5

* Mean value of triplicate assay.

A preliminary check of the stability of vitamin E in blood sera on storage in the deep freeze has been made. The results (Table II) suggest that they are stable, within experimental error of the microassay, for at least 8 weeks when stored at −22°.

SUMMARY

A micromethod is described for determining total tocopherols in 0.06 ml. of blood serum or plasma, which consists of (1) precipitation of protein with ethyl alcohol and extraction of tocopherols into xylene; (2) dilution of an aliquot of xylene extract with α,α'-dipyridyl in *n*-propyl alcohol; (3) measurement of light absorption due to carotenoids at 460 mμ; and (4) addition of ferric chloride to the Beckman cuvette and measurement of absorption at 520 mμ.

The requirements of an analytical method with regard to precision, accuracy, and quantitative recovery of added tocopherol are satisfactorily met. Results agree well with those of the Quaife-Harris macroprocedure.

Blood sera stored at $-22°$ appear to be stable for at least 8 weeks with respect to tocopherol content.

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