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Drug Standards

Assay of Vitamin E in Pharmaceutical Products

By ROBERT W. LEHMAN

Control assays for vitamin E may appear to be hopelessly complex to the pharmaceutical analyst. "The National Formulary" describes a different assay method for each of the six forms or sources of vitamin E listed. Alkaline saponification is used for some materials and acid hydrolysis for others. Ceric sulfate titration is used for some; reaction with ferric chloride and dipyridyl for others. The derivation of the factors used in the calculations is not readily apparent. The job of the analyst is further complicated because the N.F. methods do not automatically apply to these same forms or sources when they are used in finished pharmaceutical products. This review is intended to help the analyst who must modify the N.F. assay methods for vitamin E in order to apply them to finished pharmaceutical products not covered by the N.F. monographs. This review mentions some reasons for the differences between methods and suggests modifications that can be used in assaying vitamin E in pharmaceutical products.

ALPHA TOCOPHEROL can be easily oxidized by ferric chloride or by ceric sulfate to the "yellow quinone" as shown in Fig. 1. The N.F. assay methods depend on either measuring the amount of ferrous ions produced (they form a red complex with α,α'-dipyridyl) or titrating until ceric ions are left in solution to oxidize an indicator (diphenylamine).

α-Tocopheryl esters such as the acetate and acid succinate shown in Fig. 2 cannot be oxidized until the ester group has been removed by hydrolysis (either with potassium hydroxide or sulfuric acid).

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Acid Hydrolysis versus Alkaline Saponification

Table I shows the differences in assay procedure that are specified for the six forms or sources of vitamin E that are now listed in "The National Formulary" (1). The two high purity acetates are hydrolyzed with acid, while the *d*-α-tocopheryl acid succinate requires saponification because it is not completely hydrolyzed by the N.F. acid treatment. Either method could be used for the acetates; each has the following advantages and disadvantages. Acid hydrolysis requires 3 hours of reaction time, but very little attention from the analyst. When acid hydrolysis is followed by ceric sulfate titration, the reaction mixture can be diluted directly, with no work-up. On the other hand, alkaline saponification requires only 20 minutes of reaction time. While saponification requires a lengthy work-up involving solvent extraction, washing, evaporation, and change of solvent, it would still be the procedure of choice when it is to be followed by the ferric

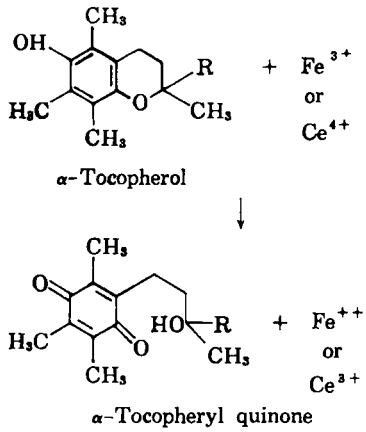

Fig. 1.—Oxidation of α-tocopherol.

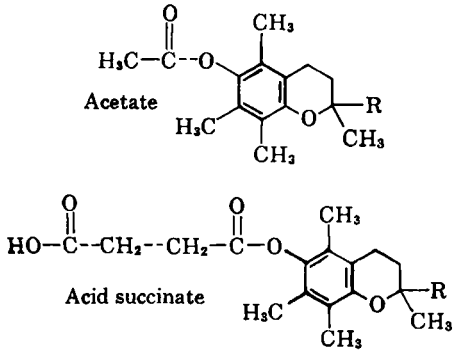

Fig. 2.—Esters of α-tocopherol.

chloride–dipyridyl procedure. Acid hydrolysis would have no advantage here, because it, too, would have to be worked up by the same procedure.

Ceric Sulfate versus Dipyridyl

Because it is more precise, ceric sulfate titration would usually be chosen over ferric chloride–dipyridyl colorimetry wherever it can be applied. Table II shows the relative precision of the N.F. assay methods which was obtained in a collaborative study of five samples among nine laboratories (2). Both the intra and the interlaboratory variation of the ceric sulfate titration are significantly less than those of the dipyridyl procedure.

Unfortunately the ceric sulfate titration can be applied only to tocopherol samples of high concentration. It fails on mixed tocopherols concentrate because of fading end points, which are a result of a solubility problem. Tocopherol is soluble in ethanol, but not in water; ceric sulfate is soluble in aqueous sulfuric acid, but not in ethanol. As the aqueous ceric sulfate solution is run into the ethanol solution of tocopherol, one reactant or the other tends to be thrown out of solution. When excess oil phase is present, this causes fading end points. Sharp end points exist only within very narrow solubility limits.

The ferric chloride–dipyridyl reaction can be applied to extremely dilute tocopherol solutions. In almost all pharmaceutical dosage forms, it would present fewer problems than the ceric sulfate procedure.

Derivation of Assay Factors

In the two N.F. monographs that specify the dipyridyl assay method, the derivation of the "factors," 28.2 and 32.0, is not presented. To obtain proper factors needed for accurate application to "pure" tocopherols or esters in pharmaceutical products, the analyst needs the following information.

The formulas follow the pattern

$$A \times \text{factor}/C \times L = \text{mg. per Gm.}$$

where A is absorbance, C is concentration of sample, and L is length of the cell.

This "factor" in the mixed tocopherols dipyridyl assay calculation is derived as

$$\text{Factor} = \frac{25 \times 1000}{2 \times 443.3} = 28.2$$

The 25, 1000, and 2 come from the dilutions made for the spectrophotometry, and 443.3 is the extinction coefficient (E, 1%, 1 cm.) calculated for the particular mixture of α-, β-, γ-, and δ-tocopherols present in the average lot of mixed tocopherols concentrate. These four tocopherols have extinction coefficients in the dipyridyl assay of 414, 437, 455, and 561, and they are present in the average proportions 52.0, 9.3, 28.0, and 10.7%, respectively. Thus, the average E, 1%, 1 cm., is (414 × 0.520) + (437 × 0.093) + (455 × 0.280) + (561 × 0.107) = 443.3.

For *d*-α-tocopheryl acetate concentrate, the proportions are 68, 22, 5, and 5%, respectively, and the calculated extinction coefficient is 428.5. However, these calculations are in terms of milligrams of unesterified tocopherols, while the assay result is to be expressed in milligrams of tocopheryl acetate. Thus, the ratio of molecular weights must also be introduced, and the factor is

$$\text{Factor} = \frac{25 \times 1000 \times 472.76}{2 \times 428.5 \times 430.72} = 32.0$$

Multicomponent pharmaceutical products might be assayed for vitamin E by the dipyridyl procedure rather than by the ceric sulfate titration, even when a pure form of vitamin E is used in its preparation. In such cases, the extinction coefficient, 414, for α-tocopherol should be used, and a molecular weight adjustment should be made to yield milligrams of the particular ester present. Thus, the proper factor for *d*- or *dl*-α-tocopheryl acetate is

Table I.—Assay Methods Specified by "The National Formulary"

N.F. Product	N.F. XI (1) Page	Purity	Hydrolysis	Analytical Method	Nitroso
dl-α-Tocopherol	376	High	(None)	Ceric SO_4	No
d-α-Tocopheryl acetate	377–378	High	Acid	Ceric SO_4	No
dl-α-Tocopheryl acetate	379–380	High	Acid	Ceric SO_4	No
d-α-Tocopheryl acid succinate	380	High	Saponification	Ceric SO_4	No
Mixed tocopherols concentrate	376–377	Low	(None)	Dipyridyl	Yes
d-α-Tocopheryl acetate concentrate	378–379	Low	Saponification	Dipyridyl	Yes

TABLE II.—PRECISION OF ASSAY METHODS

	Coefficient of Variation, % Intralab	Interlab
Ceric sulfate	0.45	0.70
Dipyridyl	1.52	2.80
Nitroso	5.45	17.50

$$\text{Factor} = \frac{25 \times 1000 \times 472.76}{2 \times 414 \times 430.72} = 33.1$$

and the one for *d*-α-tocopheryl acid succinate is

$$\text{Factor} = \frac{25 \times 1000 \times 530.80}{2 \times 414 \times 430.72} = 37.2$$

Colorimetry

A method using a spectrophotometer rather than one using a colorimeter was proposed to The National Formulary because the spectrophotometric method can be set up quickly in any laboratory without a need for reference standards. But since the spectrophotometer is a null-point instrument, and not direct reading, it is difficult to use where absorbance is changing rapidly with time. Therefore, the time interval of 10 minutes was recommended to The National Formulary.

Figure 3 shows how the rate of reaction and the degree of color development may vary with the tocopherol homolog. The problem caused by the different responses of the β, γ, and δ forms can be avoided by using a colorimeter instead of a spectrophotometer and by using a reaction time of 50 seconds instead of 10 minutes.

By using a direct reading colorimeter and a time interval of 2 minutes (see Fig. 3), it is less necessary to calibrate against all four tocopherols or to know the relative abundance of the four tocopherols in each sample for assay.

By changing the concentration of the reagents and the order of addition, these four reaction curves can be made to cross each other at a time interval of about 50 seconds. Quaife (4) developed a specific procedure for determining tocopherols in biological samples that could be very useful in a pharmaceutical control laboratory. The reaction rates are shown in Fig. 4.

Conditions needed to duplicate the relationships in Fig. 4 are fairly critical; directions for conducting the assay are as follows.

50-Second Assay for Total Tocopherol.—*Apparatus.*—An Evelyn colorimeter (Rubicon Instruments Division, Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.) or similar instrument having a constant, low intensity light source, means for filtering this source to a narrow wavelength region, and a direct reading, instantaneously responding photocell and galvanometer. For this determination it should be equipped with a 515 or a 520-mμ filter.

Reagents.—Ethanol (absolute ethyl alcohol); α,α′-dipyridyl solution. (dissolve 0.25 Gm. in 50 ml. of ethanol, protect from light); and ferric chloride solution (dissolve 0.10 Gm. of $FeCl_3 \cdot 6H_2O$ in 50 ml. of ethanol, protect from light) are utilized.

Procedure.—Place in the colorimeter tube 8 ml. of ethanol and 1 ml. of α,α′-dipyridyl solution. With the tube in the colorimeter, adjust the light intensity so that the galvanometer reads 100% transmittance. Add 1 ml. of ferric chloride solution. Shake well for 5 seconds, and record the per cent transmittance (G) exactly 50 seconds after adding the ferric chloride. Convert this to "L_B" (2 − log G).

Place in the colorimeter tube 8 ml. of ethanol containing sufficient sample to supply about 60 mcg. of tocopherol. Add 1 ml. of α,α′-dipyridyl solution and, with the tube in the colorimeter, adjust the light intensity so that the galvanometer reads 100% transmittance. Add 1 ml. of ferric chloride solution. Shake well for 5 seconds and record the per cent transmittance (G) exactly 50 seconds after adding the ferric chloride. Convert this to "L_S" (2 − log G). Subtract L_B, determined for the blank reagents, from L_S, determined for the sample and record the difference as "L_D."

Prepare a calibration curve, using solution of pure α-tocopherol in place of the sample. On linear graph paper, plot L_D *versus* milligrams of α-tocopherol. Draw the best fitting smooth line through the points and through zero. Do not necessarily attempt to draw a straight line.

From the calibration curve, find milligrams of tocopherol corresponding to L_D found for the sample. From the concentration of sample in the colorimeter tube, calculate milligrams of total tocopherol per gram of sample.

Nitroso Assay.—For the two "sources" of vitamin E in "The National Formulary," mixed tocopherols concentrate and *d*-α-tocopheryl acetate concentrate, a nitroso reaction is specified to determine the tocopherols "other than alpha-." The relatively poor precision of the nitroso assay procedure is shown in Table II. The supplier of these concentrates needs to use this assay routinely; but because of the assay uncertainty, the industrial analyst might use an average figure for the ratio of alpha to total tocopherol, relying on the dipyridyl assay for control purposes.

The ratio of alpha to total tocopherol will not

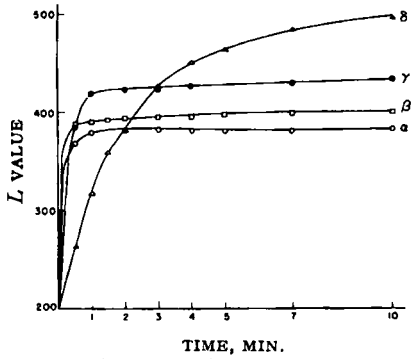

Fig. 3.—Reaction rates of tocopherols in Stern and Baxter (3) procedure.

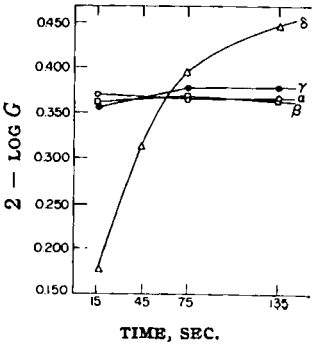

Fig. 4.—Reaction rates of tocopherols in Quaife (4) procedure.

change, except under conditions of severe destruction, and these would always be avoided in pharmaceutical practice. Once it has been established for a given lot of concentrate, that ratio can be applied to the total tocopherol content of finished products made from the known lot.

The N.F. monographs do not specify that the nitroso assay be used on the high purity "forms" of vitamin E. When it is applied to them it shows an absorption corresponding to 1 to 2% of *apparent* non-α-tocopherol. However, this occurs even with the synthetically prepared forms and with highly purified experimental preparations; thus it seems probable that the slight color observed is caused by a side-reaction between α-tocopherol and the reagent.

Detection of d-Isomers

Until recently, there was no accurate way to distinguish between the *d* epimer (from natural sources) and the *dl*-racemic mixture. It is important to develop methods for distinguishing because the *d* form is credited with 36% more "potency" than the *dl*. While the *d* epimer does exhibit optical rotation, the α_D is only a fraction of a degree, and impractical to use in control work.

Of course, the pharmaceutical control chemist will not usually need this type of test for work on his company's product. But he may wish to check on the raw materials. And law enforcement chemists may wish to examine products off the shelf.

A control procedure can probably be worked out, based on the method published in 1962 by Nelan and Robeson (5). They oxidized a sample of 400 mg. of tocopherol by shaking for 3 minutes in 50 ml. of ether with 10 ml. of 10% potassium ferricyanide in 0.2 *N* aqueous sodium hydroxide. After washing and drying over sodium sulfate, the solvent was removed, the sample taken up in iso-octane, and the optical rotation determined on a 5% solution using a Rudolf polarimeter (model 70). The crude oxidation product gave an $[\alpha]_D^{25}$ of +27.5.

By using only 3 ml. of 5% solution in a 20-cm. tube, a rotation of at least 0.25° (which can be reliably distinguished from zero) can be obtained with a sample of only 15 or 20 mg. of α-tocopherol. This can be applied directly to high purity forms of vitamin E. Finished dosage forms might require a clean-up by chromatography or other method modifications if other optically active ingredients are present.

SUMMARY

This review discusses limitations in acid hydrolysis and ceric sulfate procedures that are specified in some of "The National Formulary" monographs for forms and sources of vitamin E. It suggests that vitamin E in pharmaceutical products not covered by the N.F. monographs be assayed by saponification followed by the dipyridyl determination. It shows how the calculation factors in the dipyridyl assay can be modified to apply to any of the vitamin E sources. A more simple, colorimetric method is described for the dipyridyl assay. The nitroso assay and detection of *d* isomers are discussed briefly.

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