Simplified Assay of Vitamins A and E in Mixtures

By WILLIAM T. FISHER, NANCY M. EDWARDS, and ROBERT W. LEHMAN

Assay for vitamins A and E in mixtures has required separate saponifications; the extraction for vitamin A had to be carried out from the alkaline mixture, while the extraction for vitamin E had to be from an acidic one. Furthermore, vitamin A interferes with the ferric chloride-dipyridyl determination of vitamin E, and vitamin E interferes with the spectrophotometric determination of vitamin A. Ascorbic acid in the alkaline medium was found to permit a common saponification and extraction for both vitamins A and E. The extraction of the unsaponifiable fraction can be shortened by using a single extraction with a large volume of ether instead of three or four extractions with smaller volumes. Hydrogenation of portions of the unsaponifiable fraction was shown to eliminate the mutual interference. These three improvements have been incorporated into a proposed procedure which is valid for the determination of vitamin A and vitamin E in the presence of each other.

THE ASSAY for vitamins A and E in mixtures using official procedures is lengthy and may yield inaccurate results. Separate saponifications are necessary, and several ethyl ether extractions are required to isolate the unsaponifiable fraction. In addition, vitamin E interferes in the spectrophotometric determination of vitamin A, often lowering the assay result, and vitamin A interferes in the colorimetric assay of vitamin E, often raising the assay result.

EXPERIMENTAL

Three simplifications in the assay of mixtures of vitamins A and E were considered in the course of this work. These are: (*a*) a common saponification, (*b*) a single extraction, and (*c*) elimination of the mutual interference so that accurate assay values can be obtained.

Common Saponification.—Adaptations of "The National Formulary" assays (1) for vitamin E in pharmaceutical products would usually require alkaline saponification, acidification of the saponification mixture, and extraction of the unsaponifiable fraction with three portions of ethyl ether. The U.S.P. assay (2) for vitamin A requires alkaline saponification and extraction of the unsaponifiable fraction with four portions of ethyl ether. A common saponification is not possible for both vitamins unless modifications are made in one of the official procedures. Unesterified tocopherol is very unstable to air in an alkaline medium. Therefore, the N.F. procedure calls for acidification of the saponification mixture with hydrochloric acid. Vitamin A, on the other hand, is unstable in the presence of strong acids, dehydrating to form anhydrovitamin A (3).

To prevent destruction of vitamin A by the acid added to the saponification mixture to preserve vitamin E, ascorbic acid was substituted for the hydrochloric acid. After several levels were tried, an amount of 4.5 Gm. was found satisfactory. Smaller quantities did not afford complete protection to the vitamin E; larger quantities might have caused anhydrovitamin A formation. It was necessary to use ascorbic acid U.S.P. having a minimum purity of 99%. Inadequate protection of vitamin E was obtained using a less pure preparation. If a pharmaceutical product has a high pH, more ascorbic acid may be required. This could be determined by titration of the sample.

Simplified Extraction.—Another simplification considered in this work was the reduction in number of extractions. Various proposals for a single extraction of the unsaponifiable portion of oil-soluble vitamins have appeared in the literature. These utilize a variety of solvents, including benzene (4), chloroform (5), and ethyl ether (6, 7).

To reduce the number of extractions required in working up the saponification solution, two of the

TABLE I.—EVALUATION OF SIMPLIFIED EXTRACTION PROCEDURES FOR ASSAYING COMMERCIAL VITAMIN A PALMITATE

Extraction Procedure	Vitamin A Content, u./Gm.	Morton-Stubbs Factor
U.S.P.	1,565,440	1.005
U.S.P.	1,562,710	1.014
Av.	1,564,080	1.010
Mulder	1,528,050	0.955
Mulder	1,556,260	0.974
Av.	1,542,160	0.965
Napoli, *et al.*	1,543,750	1.000
Napoli, *et al.*	1,531,230	0.988
Av.	1,537,490	0.994

TABLE II.—EVALUATION OF SIMPLIFIED EXTRACTION PROCEDURES FOR ASSAYING *d*-α-TOCOPHERYL ACID SUCCINATE N.F.

Extraction Procedure	Vitamin E Content, u./Gm.
N.F.	1190
N.F.	1220
Av.	1205
Mulder	1180
Mulder	1240
Av.	1210
Napoli, *et al.*	1200
Napoli, *et al.*	1230
Av.	1215

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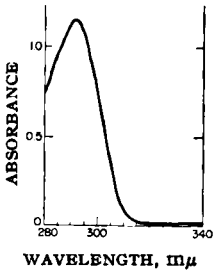

Fig. 1.—Ultraviolet absorption spectrum of *d*-α-tocopherol.

methods (4, 7) were compared with the official methods (1, 2). Results of these assays are shown in Tables I and II. In the assay of mixtures of vitamins A and E, considerable reduction in solvent volume is required prior to final measuring. Although both procedures evaluated appeared satisfactory, the Napoli, *et al.* (7), procedure was chosen because it uses ethyl ether, and the more volatile extracting solvent leads to shorter work-up time.

Elimination of Mutual Interference.—Vitamins A and E interfere with each other in the colorimetric or spectrophotometric steps when an attempt is made to apply the present official assay procedures to nonofficial preparations.

The U.S.P. method for vitamin A uses a formula originally derived by Morton and Stubbs (8) to correct for linear irrelevant absorption. The present formula (9) is: corrected $A_{(325)} = 6.815\ A_{(325)} - 2.555\ A_{(310)} - 4.26\ A_{(334)}$. Although its absorption maximum is at 292 mμ, α-tocopherol has some absorption at 310, 325, and 334 (Fig. 1). Its effect on the U.S.P. vitamin A assay is to increase slightly the uncorrected $A_{(325)}$, but to decrease corrected $A_{(325)}$, since its absorption is nonlinear in the region 310 to 334 mμ.

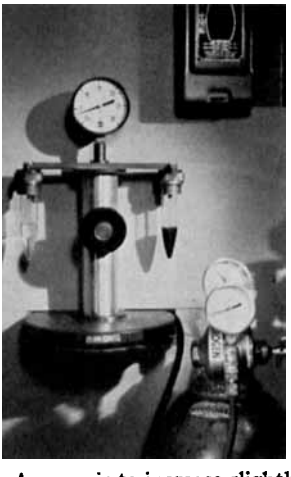

Fig. 2.—Hydrogenation apparatus.

Two general methods have been proposed for eliminating tocopherol interference so that vitamin A can be estimated accurately. Actual removal of vitamin E by partition chromatography on a column of Celite-polyethylene glycol 600 has been suggested (10). In another approach (11, 12), vitamin A is destroyed by washing an aliquot with 60% v/v sulfuric acid for use as a spectrophotometric blank.

Tocopherol reduces ferric chloride, and the ferrous ions react with dipyridyl to form a red complex. However, vitamin A also reduces ferric chloride. When vitamin A is present, vitamin E assay results tend to be too high.

Several methods have been suggested for destroying or removing vitamin A so that vitamin E can be determined accurately. These include washing with 85% v/v sulfuric acid (13), destruction of vitamin A with antimony trichloride (14), and hydrogenation (15). A number of procedures for chromatographically separating vitamins A and E have been described in the literature. These have used stannous chloride treated Floridin (16), alkaline alumina (17), and kaolin (18).

The use of hydrogenation in destroying vitamin A in the assay of vitamin E was described by Quaife and Biehler (15). Fox and Mueller (11) had used sulfuric acid to destroy the vitamin A in part of the sample to give a spectrophotometric blank used to cancel the extraneous (nonlinear) absorption interfering in the assay of vitamin A. We employed hydrogenation for this purpose instead of sulfuric acid.

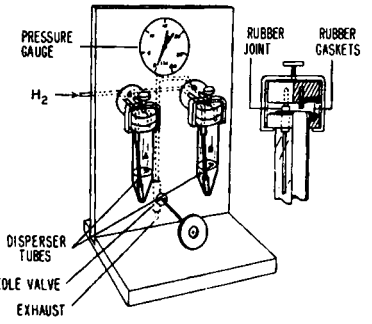

Fig. 3.—Construction and operating details of hydrogenation apparatus.

The apparatus we used is the same as that previously described (15) with differences only in external design. The apparatus and a hydrogen flow diagram are shown in Figs. 2 and 3. Hydrogen gas is bubbled through alcohol in the blank tube using a microporous dispersion tube. The gas saturated with alcohol vapor is bubbled through the sample in the sample tube. The exhaust gas passes through a pressure gauge and needle valve, allowing control of pressure in the system. In our work, 2 to 3 lb. hydrogen pressure was effective with 5 minutes hydrogenation time.

Two aliquots are taken for hydrogenation and determination of the two vitamins. Vitamin E is measured directly in one hydrogenated aliquot by ferric chloride-dipyridyl. The other hydrogenated aliquot is used for a blank in determining vitamin A spectrophotometrically; the sample for vitamin A determination is an unhydrogenated portion of this aliquot. Absorbance curves are shown in Fig. 4 for:

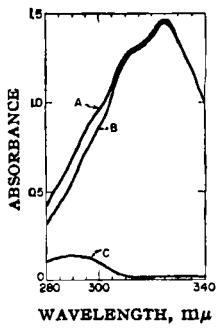

Fig. 4.—Absorbance curves for: (A) a mixture of vitamins A and E against an isopropanol blank; (B) the vitamin mixture against a hydrogenated blank; and (C) the hydrogenated aliquot against isopropanol.

(*a*) a mixture of vitamins A and E against an isopropanol blank, (*b*) the vitamin mixture against a hydrogenated blank, and (*c*) the hydrogenated aliquot against isopropanol.

Several mixtures of vitamins A and E were prepared and assayed, using a common saponification and single extraction, followed by hydrogenation to avoid interference from each vitamin during the measurement of the other. These mixtures contained both oil and dry vitamins A and E at various ratios of A to E. Results are shown in Tables V–XI. Recovery of the vitamins was based on assay values of the input materials shown in Tables III and IV.

PROPOSED PROCEDURE

In sampling for assay, use at least 10 I.U. of vitamin E since this facilitates dilution during later steps. Vitamin A concentration is less critical.

Transfer the sample for assay to a 125-ml. low actinic saponification flask. Add 3 ml. of 50% w/v KOH solution, displace the air in the flask with nitrogen, attach an air condenser, and swirl the saponification flask over a steam bath for 2 to 3 minutes. This aids in the release of vitamins from the solid matrix if present in a dry form. Add 50 ml. of ethanol through the condenser and saponify for 20 minutes. Remove from the steam bath and add 4.5 Gm. ascorbic acid U.S.P. through the condenser. (*Caution:* do not open the system until acidified!) Wash any ascorbic acid adhering to the sides of the condenser into the saponification flask with a small amount of water. Cool under tap water and transfer to a 500-ml. low actinic separator with 100 ml. of distilled water. Rinse the saponification flask with three 50-ml. portions of peroxide-free ethyl ether, combining these in the separator. Stopper, and shake vigorously to mix the layers thoroughly. Allow the layers to separate; draw off and discard the aqueous layer.

Wash the ether layer with 100-ml. portions of distilled water to neutrality as determined with phenolphthalein. Filter the ether extract through anhydrous sodium sulfate and reduce the volume to 10 to 20 ml. in a stream of nitrogen. Warm gently to aid solvent removal, but avoid overheating. Dilute the extract to 50 ml. with ethyl ether in a volumetric flask. Dilute aliquots from the master solution for assay as follows.

Vitamin E.—Prepare a solution of the unsaponifiable fraction in ethanol containing approximately 0.2 units vitamin E per milliliter. Transfer about 10 ml. of this solution to a 50-ml. centrifuge tube, and add approximately 20 mg. of catalyst (5% palladium on calcium carbonate.[1]) Stir with a glass rod, and hydrogenate for 5 minutes at 2 to 3 lb.

[1] Baker and Co., Inc., Newark 5, N. J.

TABLE III.—ASSAY RESULTS ON VITAMIN A USED IN PREPARING VITAMIN A-E MIXTURES

U.S.P. XVI u./Gm.	Morton-Stubbs Factor
Commercial Dry Vitamin A Palmitate	
560,430	1.005
561,030	1.025
Av. 560,730	1.015
Commercial Vitamin A Palmitate	
1,565,440	1.005
1,562,710	1.014
Av. 1,564,080	1.010

TABLE IV.—ASSAY RESULTS ON VITAMIN E[a] USED IN PREPARING VITAMIN A-E MIXTURES

d-α-Tocopheryl Acid Succinate, u./Gm.	*d*-α-Tocopheryl Acetate, u./Gm.	Dry *d*-α-Tocopheryl Acetate (Acacia Carrier), u./Gm.
1190	1370	354
1220	1370	354
Av. 1205	1370	354

[a] Vitamin E determined by ferric chloride-dipyridyl method on unsaponifiable fraction.

TABLE V.—MIXTURE OF 50,000 UNITS COMMERCIAL VITAMIN A PALMITATE AND 10 UNITS *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
1,612,600	...	1.012	2754	...
1,640,500	...	1.022	2730	...
Av. 1,626,550	104.0	1.017	2742	200.1
With Hydrogenation				
1,558,460	...	1.012	1391	...
1,540,050	...	1.004	1401	...
Av. 1,549,260	99.1	1.008	1396	101.9

TABLE VI.—MIXTURE OF 25,000 UNITS COMMERCIAL VITAMIN A PALMITATE AND 10 UNITS *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
1,559,580	...	0.964	2130	...
1,501,520	...	0.930	2350	...
Av. 1,530,550	97.9	0.947	2240	163.5
With Hydrogenation				
1,573,540	...	1.009	1396	...
1,564,130	...	1.038	1453	...
Av. 1,568,840	100.3	1.024	1425	104.0

TABLE VII.—MIXTURE OF 50,000 UNITS COMMERCIAL DRY VITAMIN A PALMITATE AND 10 UNITS DRY *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
538,980	...	0.967	385	...
539,990	...	0.958	466	...
Av. 539,490	96.2	0.963	426	120.3
With Hydrogenation				
544,730	...	0.989	370	...
553,810	...	0.993	357	...
Av. 549,270	98.0	0.991	364	102.8

TABLE VIII.—MIXTURE OF 25,000 UNITS COMMERCIAL DRY VITAMIN A PALMITATE AND 10 UNITS DRY *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
553,890	...	0.974	534	...
522,810	...	0.939	470	...
Av. 538,850	96.1	0.957	502	141.8
With Hydrogenation				
529,840	...	1.004	333	...
543,190	...	1.038	371	...
Av. 536,520	95.7	1.021	352	99.4

TABLE IX.—MIXTURE OF 50,000 UNITS COMMERCIAL DRY VITAMIN A PALMITATE AND 10 UNITS *d*-α-TOCOPHERYL ACID SUCCINATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
561,010	...	0.992	2856	...
527,880	...	0.954	3096	...
Av. 544,450	97.1	0.973	2976	247.0
With Hydrogenation				
551,620	...	1.055	1277	...
552,890	...	1.049	1302	...
Av. 552,260	98.5	1.052	1290	107.1

TABLE X.—MIXTURE OF 25,000 UNITS COMMERCIAL DRY VITAMIN A PALMITATE AND 10 UNITS *d*-α-TOCOPHERYL ACID SUCCINATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
467,990	...	0.919	2336	...
551,110	...	0.965	2097	...
Av. 514,050	91.7	0.942	2217	184.0
With Hydrogenation				
551,810	...	1.045	1270	...
529,900	...	0.984	1210	...
Av. 540,860	95.6	1.015	1240	102.9

pressure. Centrifuge until the solution is clear and proceed with the determination of vitamin E by ferric chloride-dipyridyl assay.[2]

Vitamin A.—Prepare a solution of unsaponifiable fraction in isopropanol containing approximately 10 units vitamin A per milliliter. Hydrogenate a portion of this solution as above. Centrifuge and use this as a reagent blank. Use an unhydrogenated portion of the isopropanol solution as the sample for assay. Determination and calculation of vitamin A from this point is by the U.S.P. procedure (2). Since vitamin E is not hydrogenated at the conditions used here, both sample and hydrogenated blank contain the same amount of vitamin E.

[2] Adaptations of official vitamin E assay procedures for "unofficial" products are discussed by Lehman (19).

Vitamin E in the blank cancels out the vitamin E in the sample, allowing accurate measurement of the vitamin A. This is illustrated in Fig. 4.

RESULTS AND DISCUSSION

The results obtained using the proposed procedure on a variety of mixtures of vitamins A and E representing combinations that might be encountered in finished pharmaceutical dosage forms are presented in Tables V–XI.

The modifications introduced into the saponification and extraction technique (use of ascorbic acid instead of hydrochloric and use of a single ether extraction instead of three or four) have not prevented quantitative recovery of both vitamins. Hydrogenation was effective both for preventing interference of vitamin A in the dipyridyl assay for vitamin E and the interference of vitamin E in the spectrophotometric assay for vitamin A.

The need for hydrogenation and its degree of effectiveness is seen by comparing the data on per cent recovery in Table XII with those in Table XIII. This comparison shows that without hydrogenation, assays for vitamin E appear to be falsely high when vitamin A levels are high, and that assays for vitamin A tend to be falsely low when vitamin E levels are high. Both defects are overcome by the hydrogenation step in the procedure as outlined.

CONCLUSIONS

The assay of vitamins A and E in mixtures has been simplified by employing a common saponifica-

TABLE XI.—MIXTURE OF 5,000 UNITS COMMERCIAL VITAMIN A PALMITATE AND 100 UNITS *d*-α-TOCOPHERYL ACETATE

Vitamin A u./Gm.	Vitamin A % of Input	Morton-Stubbs Factor	Ferric Chloride-Dipyridyl u./Gm.	Ferric Chloride-Dipyridyl % of Input
No Hydrogenation				
1,056,530	...	0.656	1390	...
1,066,490	...	0.660	1390	...
Av. 1,061,510	67.9	0.658	1390	101.5
With Hydrogenation				
1,564,850	...	1.070	1390	...
1,564,850	...	1.098	1400	...
Av. 1,564,850	100.1	1.084	1395	101.8

TABLE XII.—RECOVERY OF VITAMINS A AND E WITHOUT HYDROGENATION

Input: Vitamin A Units	Input: Vitamin E Units	% Recovery: Vitamin A	% Recovery: Vitamin E
50,000 (oil)	10 (oil, acetate)	104.0	200.1
25,000 (oil)	10 (oil, acetate)	97.9	163.5
50,000 (dry)	10 (dry, acetate)	96.2	120.3
25,000 (dry)	10 (dry, acetate)	96.1	141.8
50,000 (dry)	10 (succinate)	97.1	247.0
25,000 (dry)	10 (succinate)	91.7	184.0
5,000 (oil)	100 (oil, acetate)	67.9	101.5

TABLE XIII.—RECOVERY OF VITAMINS A AND E USING PROPOSED PROCEDURE

Input		% Recovery	
Vitamin A Units	Vitamin E Units	Vitamin A	Vitamin E
50,000 (oil)	10 (oil, acetate)	99.1	101.9
25,000 (oil)	10 (oil, acetate)	100.3	104.0
50,000 (dry)	10 (dry, acetate)	98.0	102.8
25,000 (dry)	10 (dry, acetate)	95.7	99.4
50,000 (dry)	10 (succinate)	98.5	107.1
25,000 (dry)	10 (succinate)	96.5	102.9
5,000 (oil)	100 (oil, acetate)	100.1	101.8

tion which allows good recovery of both vitamins. The key change from previous procedures is the substitution of ascorbic acid for hydrochloric acid in acidifying the saponification mixture before extraction.

Hydrogenation is effective in removing the mutual interference of vitamins A and E upon each other.

These two new improvements have been combined with one previously described (using a single large volume of ether for extraction rather than several smaller ones) in a simplified procedure for the assay of these two vitamins in mixtures. The proposed assay is valid for a number of typical mixtures.

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Pinacol Rearrangement of Phenaglycodol I

Characterization of Products Produced

By HUBERT W. MURPHY

PHENAGLYCODOL[1] is one of a series of anticonvulsant ethylene glycols described by Mills, *et al.* (1), which has been evaluated in the clinic as an anticonvulsant and neurosedative agent by Gruber and Mosier (2). This compound is characterized by a somewhat persistent bitter taste. In the hope of obtaining a product with a sufficiently improved taste to permit the convenient administration of phenaglycodol in liquid preparations, several attempts were made to prepare derivatives of this glycol.

All attempts to esterify one or both of the hydroxyl groups with acid anhydrides or acid chlorides were fruitless, even when special methods developed for tertiary hydroxyl groups were carried out. Likewise, all efforts at transesterification with a number of commonly used catalysts and attempted etherifications were unsuccessful. It was observed that a characteristic odor developed in those instances in which acidic conditions were maintained during attempted esterification. Moreover, it was difficult to recover the unreacted phenaglycodol in crystalline form from these experiments. The possibility of a pinacol rearrangement was considered, and a typical odoriferous reaction mixture was treated with 2,4-dinitrophenylhydrazine reagent. The presence of a ketone was revealed by the separation of an orange, crystalline 2,4-dinitrophenylhydrazone.

Further experimentation furnished a substantially quantitative method of converting phenaglycodol into a ketone by refluxing the glycol with 10% sulfuric acid. The ketonic liquid obtained was immediately subjected to careful fractionation at reduced pressure. The main component was 2-methyl-2-(*p*-chloro-

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[1] Marketed as Ultran by Eli Lilly and Co.