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Combined Assays for Vitamins A, D (Ergocalciferol), and E in Multivitamin Preparations with Separation by Reversed-Phase Partition Chromatography

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Abstract □ A rapid method was developed whereby vitamins A, D (ergocalciferol), and E in multivitamin products are measured in a common assay sample. The method depends on reversed-phase partition chromatography by which the vitamin alcohols are eluted in separate fractions from a column. Vitamins A and E are then determined by their UV absorption, while vitamin D is measured colorimetrically with an antimony trichloride reagent. The column consists of diatomaceous earth impregnated with dimethyl polysiloxane, with *n*-heptane as the immobile solvent, and 90% methanol followed by 95% methanol as the mobile solvents. Vitamins A and D elute in that order in the 90% methanol, and finally vitamin E elutes in the 95% methanol fraction. The method is widely applicable to various types of multivitamin and vitamin–mineral products including oil-based, water-based, and dry formulations.

Keyphrases □ Vitamins—combined analyses, vitamins A, D (ergocalciferol), and E in multivitamin preparations, reversed-phase partition chromatography □ Chromatography, reversed-phase partition—combined analyses, vitamins A, D, and E in multivitamin preparations □ Multivitamin preparations—combined analyses, vitamins A, D, and E, reversed-phase partition chromatography

The USP (1) provides assays for vitamins A and E in multivitamin preparations which utilize a common assay sample. However, duplication of procedure exists in that a similar assay step, depending on the selective degradation of vitamin A in the presence of vitamin E, is subsequently carried out in each of the two determinations. Duplication of assay steps is also involved with respect to the USP assay for vitamin D (ergocalciferol) (1). The procedure is similar to that for vitamins A and E through the initial steps of alkaline hydrolysis and extraction, but another sample is nevertheless taken and the complete analysis is carried out separately.

The USP vitamin D assay also is subject to the criticism that vitamin E, if present, carries through the analysis, causing high results for vitamin D. To correct for such interference, procedures have been proposed for the removal of vitamin E by adsorption on a column (2, 3); but this additional step lengthens what is already a rather long and tedious assay. Perhaps the main disadvantage is that the vitamin E fraction is lost, so a completely separate determination must be undertaken for vitamin E.

Incorporated in the USP vitamin D assay is a column step for the separation of vitamin D from vitamin A. This step depends on "conventional" partition chromatography; *i.e.*, the more polar solvent is the stationary phase and the less polar is the mobile phase. Conceivably such a system might also provide a separation between vitamins D and E, with vitamin E eluting first. The problem is that vitamins D and E both elute close to the solvent front, so their bands tend to overlap and a complete separation is difficult.

The reversed-phase partition column has been found

advantageous for separating lipophilic substances (4). Although several reports described its application to oil-soluble vitamins (5–9), they have not dealt with multivitamin products or have not involved the determination of more than one vitamin at a time.

The present report describes a procedure for determining vitamins A, D, and E in multivitamin preparations. The three vitamins are separated from each other by reversed-phase partition chromatography on a column. There is no duplication of assay steps, and the presence of vitamin E is no longer a problem in the determination of vitamin D. The assays for vitamins A and D parallel those in the USP (1) in many respects, but an attempt has been made to simplify those steps that are similar wherever possible.

EXPERIMENTAL

Equipment—The following were used: hot plates with magnetic stirrers; glass chromatographic tubes, 2.5 cm i.d. × 25 cm long, with stopcocks; glass chromatographic tubes with upper sections 2.5 cm i.d. × 20 cm long and lower sections 1.5 cm i.d. × 15 cm long[1], with stopcocks; rotary evaporators[2] with controlled-temperature water baths and tubes for introducing nitrogen into the evaporating flasks; and a recording spectrophotometer[3] with 1- and 2-cm cells.

Reagents and Solutions—Reagent grades of the following were used: acetic anhydride, acetyl chloride, antimony trichloride, benzene[4], chloroform, ethylene dichloride[4], ether[5], *n*-heptane, methanol, petroleum ether, potassium hydroxide, and pyrogallol.

Other reagents were 95% ethanol, USP grade; anhydrous ethanol, pure; fuller's earth[6]; and light mineral oil, NF grade.

The reversed-phase column support was diatomaceous earth impregnated with 0.5% silicone. To prepare about 1.2 kg of final product, 1-kg portions of diatomaceous earth[7] were placed in each of two 18.9-liter (5-gallon) bottles and suspended in about 11.4 liters (3 gallons) of water at 25°. After 10 min, the supernatant portions were decanted and discarded to remove the fine-particle fraction from the diatomaceous earth. The sedimentation process was repeated three more times.

The residual diatomaceous earth was collected in a percolator, vacuum dried, rinsed with methanol, and then vacuum dried for a prolonged period. The dried diatomaceous earth was transferred to a suitable basin or tub and mixed with a solution of dimethyl polysiloxane[8], 1% (w/v) in chloroform, in the proportion 1 ml of solution for each 2 g of diatomaceous earth. The mixture was dried with occasional mixing under an air stream in a hood, then washed with several liters of methanol in a percolator, and vacuum dried.

To determine vitamin D, a solution of antimony trichloride in ethylene dichloride[4], 450 g/1000 ml[4] (Solution A), or acetyl chloride in ethylene dichloride[4], 1 + 4 (v/v) (Solution B), was used. A mixture of 9 volumes of Solution A with 1 volume of Solution B was used as the final color reagent.

Standard solutions of vitamin D were prepared from USP ergocalciferol reference standard[9] or from ergocalciferol USP purchased in sealed glass ampuls[10]. Ergocalciferol USP was found to be equivalent to the USP reference standard with respect to measurements by UV spectrophotometry and by colorimetry with antimony trichloride. Stock Solution I was made in 95% ethanol, USP grade, 50 mg/100 ml. Stock Solution II was prepared by evaporating 5.0 ml of Solution I and reconstituting in 100.0 ml of ethylene dichloride[4]. Working Solution III was prepared by diluting Solution II with ethylene dichloride[4] to a final concentration of 2.5 μg/ml.

All standard solutions were refrigerated during storage and were stable up to 1 week in the case of III, up to 3–4 weeks in the case of II, and up to several months in the case of I as measured colorimetrically with antimony trichloride. (Standard solutions in ethylene dichloride may be quite unstable unless the solvent is first purified as described.) Unused portions of ergocalciferol powder were refrigerated under a nitrogen atmosphere in a desiccator and were quite stable during such storage.

Procedure—Carry out the assays while avoiding bright daylight and allow only the minimum necessary exposure to artificial light, particularly with solutions containing vitamin A.

Alkaline Reflux—Place sample without pretreatment (*e.g.*, intact capsules or tablets), equivalent to 2000 I.U. (50 μg) of vitamin D, in a 250-ml low actinic conical flask containing a 5.1-cm (2-in.) stirring bar[11]. Add 48 ml of 95% ethanol, 2 ml of 5% pyrogallol in 95% ethanol, and 10 ml of ether. Mount under a condenser and heat to boiling using a hot plate with a magnetic stirrer.

Add 20 ml of 20% (w/v) aqueous potassium hydroxide through the condenser and reflux with stirring for 45 min for oil-based formulations, for 30 min for intact tablets or powder-filled capsules, or for 20 min for powder samples or water-based formulations. Cool and, without delay, rinse the condenser with 20 ml of water. Collect in the flask and add 40 ml of petroleum ether.

Extraction into Ether–Petroleum Ether (1 + 3)—Transfer the contents of the flask to a 250-ml separator. Add 40 ml of water, 15 ml of ether, and 35 ml of petroleum ether and shake vigorously. Drain the bottom aqueous–ethanol layer into a second separator and shake with 80 ml of ether–petroleum ether (1 + 3). Discard the bottom layer. Combine the extracts in the first separator and wash with 50 ml of 40% ethanol followed by 50 ml of water. Then discard the wash solutions.

If necessary, add a few drops of 15% (w/v) aqueous sodium sulfate solution to break emulsions. Filter the ether–petroleum ether extracts through cotton into a boiling flask containing 0.4 ml of light mineral oil. (*Note:* Apply technique of pouring through neck of separator, after completely draining bottom layer, to avoid possible contamination of extracts with traces of aqueous phase.)

Reversed-Phase Column Preparation—Thoroughly mix 10 g of silicone-treated diatomaceous earth with 7.0 ml of *n*-heptane in a 250-ml beaker. Avoid air currents and complete the mixing within about 1 min to avoid appreciable loss of solvent through evaporation. Without delay, transfer the mixture in divided portions (four portions) to a chromatographic tube, 2.5 cm i.d., with stopcock, containing a small pledget of cotton. Pack each portion evenly and firmly, applying sufficient pressure to eliminate visible air pockets.

Evaporate the ether–petroleum ether extracts under vacuum, warming to not over 40°. Take up the oily residue and transfer to a 150-ml beaker, using about 25 ml of petroleum ether. Evaporate to an oily residue on a steam bath under an air current, reducing the application of heat near the end. The evaporation should be carried out as rapidly as possible, but without allowing the temperature of the final residue to exceed about 40°.

Without delay, add 0.6 ml of *n*-heptane, cover with a watch glass, and mix by swirling. Warm slightly on a steam bath if necessary to effect complete dissolution of any congealed residue. Add 1.5 g of silicone-treated diatomaceous earth and mix thoroughly with a stirring rod.

Transfer to the chromatographic tube over the first layer, packing as before. Complete the transfer by scrubbing the beaker with 1.5 g of silicone-treated diatomaceous earth to which was added 1 ml of *n*-heptane and pack into the chromatographic tube as the top layer. Cap the column with a thin pledget of cotton. Proceed with the next step without delay.

Elution from Column—With the stopcock of the chromatographic tube in the open position, introduce over the column about 15 ml of exactly 90.0% (v/v) methanol in water, previously saturated with *n*-heptane[12]. When the solvent has just receded to the top of the column,

[1] Tube was similar to that used in the second column chromatography in the USP vitamin D assay (1) but with a larger diameter bottom section.
[2] Buchler model PTFE-1G.
[3] Cary model 15, equipped with 0–1 absorbance slide wire.
[4] Purified by filtration through aluminum oxide, Brockmann activity grade I, basic, for chromatography (J. T. Baker Chemical Co., Phillipsburg, N.J.), using 110 g/3.79 liters (1 gallon) of benzene and 220 g/3.79 liters (1 gallon) of ethylene dichloride in a 3.9-cm i.d. tube and 40 g/1000 ml of antimony trichloride in purified ethylene dichloride in a 2.5-cm i.d. tube.
[5] Kept in original metal containers for satisfactory storage.
[6] Florex AA-RVM, 60–90 mesh, Floridin Co., New York, N.Y.
[7] Celite 545, Johns-Manville Corp., New York, N.Y.
[8] Medical Fluid 360, 12,500 cSt., Dow Chemical Co., Midland, Mich.
[9] USP Reference Standards, Rockville, MD 20852
[10] ICN Nutritional Biochemicals, Cleveland, Ohio.
[11] Coated with Teflon (du Pont).
[12] The eluting solvents should be stored in stoppered containers under a layer of *n*-heptane and should be dispensed in such a way as to avoid evaporation. The solutions should be maintained at room temperature and should be freshly shaken with an excess of *n*-heptane before use. For satisfactory column operation, the procedure should be conducted at a room temperature, preferably 22–28°.

repeat the addition and maintain a little liquid over the top of the column until the effluent appears at the stem. Fill the chromatographic tube with the 90.0% methanol solvent and adjust the rate of flow with the stopcock to 3–5 ml/min. Collect the first 30 ml of effluent in a graduated cylinder and reserve, storing in the dark for the *Determination of Vitamin A*.

Collect the next 90 ml of effluent, introducing more 90.0% methanol solvent over the column as necessary, and reserve for the *Determination of Vitamin D*.

Continue passing the 90.0% methanol solvent through the column, collecting and discarding an additional 20 ml of effluent. Then pour off and discard any residual 90.0% methanol solvent remaining over the column and pass *n*-heptane-saturated 95.0% methanol[12] through the column; discard the next 20 ml of effluent and collect the following 90 ml at a flow rate of 3–5 ml/min. Reserve the collected effluent for the *Determination of Vitamin E*. If the sample taken contains more than 100 I.U. of vitamin E, collect 120 ml of effluent instead of 90 ml.

Determination of Vitamin A—Transfer the first fraction of column effluent, 30 ml, containing the vitamin A to a 100-ml volumetric flask. After completing the transfer, dilute to volume with methanol and then mix. Transfer an accurately measured volume of this solution, V_1, to a volumetric flask of suitable capacity, V_2, and dilute to volume with methanol to yield a solution containing 10–15 I.U. of vitamin A/ml. With a suitable spectrophotometer, determine the absorbance of this solution at the maximum at about 324 nm, using 1-cm cells, relative to methanol as the blank. Calculate the vitamin A content in the sample using:

$$0.549\,A_{max}/LC = \text{mg of vitamin A (alcohol)} \qquad \text{(Eq. 1)}$$

where A_{max} is the absorbance at the maximum at about 324 nm, L is the cell length in centimeters, and C is the amount of sample in terms of grams, milliliters, tablets, or capsules in each milliliter of final solution[13] [*cf.*, USP (1)]. Since C is equal to the amount of sample taken times V_1/V_2, where V_1 and V_2 are as previously defined, and L is equal to 1, the calculation formula then becomes:

$$(0.549)(V_2/V_1)(A_{max}) = \text{mg of vitamin A (alcohol) in sample} \qquad \text{(Eq. 2)}$$

To convert to international units of vitamin A, instead of milligrams, the factor 0.549 is divided by 0.0003 and the calculation formula is:

$$(1830)(V_2/V_1)(A_{max}) = \text{I.U. of vitamin A in sample} \qquad \text{(Eq. 3)}$$

Determination of Vitamin D—Combine the second fraction of column effluent, 90 ml, with 0.1 ml of light mineral oil mixed with a few milliliters of ether in a 500-ml boiling flask and evaporate to a clear oily residue under vacuum, using a rotary evaporator and water bath at about 40°. Remove residual water near the end of the evaporation by adding anhydrous ethanol and reevaporate. Introduce a slow stream of nitrogen to help prevent boiling or bumping and to remove the last traces of solvent. Finally, add 10 ml of *n*-heptane and evaporate again to an oily residue, thereby removing any residual traces of ethanol. Proceed with the next step without delay.

(At this point of the analysis, a purification step with fuller's earth is described for the purpose of removing degradation products of vitamin A. Where this interfering material is present in very small amounts, as is frequently the case with fresh samples, the fuller's earth treatment may be omitted. In such cases, the residue is then reconstituted directly in ethylene dichloride instead of in benzene and the colorimetric step is carried out as described next.)

Reconstitute the oily residue in 10 ml of purified benzene. Pass the benzene solution through a column of 10 g of fuller's earth contained in a 1.5-cm i.d. tube with stopcock, with thin layers of cotton at the bottom and the top, and previously rinsed with 40 ml of purified benzene. When the column is being prepared, the flow of benzene used as a rinse should be stopped by closing the stopcock so as to retain a thin layer of benzene over the top of the column before use and the rinsings should then be discarded.

Collect the effluent from the column in a 250-ml boiling flask, allowing unrestricted gravity flow. Use an additional 100 ml of purified benzene to complete the transfer to the tube and the elution of vitamin D from the column. Evaporate the collected effluent under vacuum to an oily residue, using a rotary evaporator with a water bath at not over 40°. Proceed with the next step without delay.

Reconstitute the oily residue in 20.0 ml of purified ethylene dichloride[14], stoppering the flask to prevent evaporation of the solvent. Transfer 3.0-ml portions of the solution into each of two suitable small stoppered containers (Solutions 1 and 2). Into each of two similar containers, transfer 3.0-ml portions of ergocalciferol working standard in purified ethylene dichloride, 2.5 μg/ml (Solutions 3 and 4). To Solutions 1 and 3, add 2.0 ml of purified ethylene dichloride; to Solutions 2 and 4, add 2.0 ml of acetic anhydride–ethylene dichloride (1:1) (color inhibitor solution). Mix.

Keep the containers stoppered. Apply the antimony trichloride color test successively to the contents of each container as follows. Add with a pipet 3.0 ml of antimony trichloride color reagent. Immediately mix and transfer to a 2-cm spectrophotometer cell. Exactly 1 min after completing the delivery of the color reagent, determine the absorbance, A, of the solution at 498 nm, using a suitable spectrophotometer, relative to ethylene dichloride as the blank. Calculate the vitamin D content in the sample using:

$$(800C)(A_u/A_s) = \text{I.U. of vitamin D} \qquad \text{(Eq. 4)}$$

where C is the concentration of the ergocalciferol working standard solution in micrograms per milliliter, A_u has the value A_{498} Solution 1 − A_{498} Solution 2, and A_s has the value A_{498} Solution 3 − A_{498} Solution 4.

Determination of Vitamin E—Transfer the last fraction of methanolic effluent from the reversed-phase column, containing the vitamin E, to a suitable volumetric flask of capacity V_1 in milliliters. Complete the transfer and dilute to volume with methanol; then mix. If necessary, prepare a second dilution by transferring an accurately measured volume of the first solution, V_2, to a volumetric flask of suitable capacity, V_3, and dilute to volume with methanol to obtain a solution containing 0.12–0.18 I.U. of *d*-alpha tocopherol/ml or 0.09–0.13 I.U. of *dl*-alpha tocopherol/ml.

With a suitable spectrophotometer, record the absorbance of the solution between 350 and 240 nm, using 1-cm cells, relative to a mixture of methanol and the eluting solvent as the blank in the same proportions. Construct a baseline by drawing a straight line tangent to the curve at two points, one in the region of the minimum at about 255 nm and the other in the longer wavelength region where the curve levels off, at about 317 nm. Draw a vertical line through the maximum at about 292 nm and intersecting the tangent baseline. The length of this vertical line from the maximum to the point of intersection with the tangent baseline represents the corrected absorbance at the maximum (corr. A_{max}). Calculate the vitamin E content in the sample taken if in the *d*-form (*d*-alpha tocopherol or its ester).

For single-step dilution:

$$(14.9V_1)(\text{corr. } A_{max}/72.4) = \text{I.U. of vitamin E} \qquad \text{(Eq. 5)}$$

For two-step dilution:

$$(14.9V_1)(V_3/V_2)(\text{corr. } A_{max}/72.4) = \text{I.U. of vitamin E} \qquad \text{(Eq. 6)}$$

If the vitamin E is present in the *dl*-form, the factor 14.9 is changed to 11. The number 72.4 represents the absorptivity (1%, 1 cm) of alpha tocopherol at the maximum at about 292 nm, with tangent baseline correction.

RESULTS AND DISCUSSION

Alkaline Hydrolysis or Saponification—The sample for assay requires no pretreatment, since even intact capsules and tablets disintegrate readily in the boiling medium used for alkaline hydrolysis with the aid of mechanical stirring. The stirring also helps to prevent overheating and bumping with vitamin–mineral formulations containing much insoluble material.

[13] The factor 0.549 is for solutions of vitamin A in 2-propanol; however, substitution of methanol as the solvent, as in the present analysis, results in practically no change of the value obtained for A_{max}, so that the calculation formula is still valid.

[14] Considerable difficulty with the vitamin D assay has been associated with the use of inadequately purified ethylene dichloride, resulting in unstable vitamin D solutions and low and variable readings in the colorimetric test with antimony trichloride. These results could be caused by a relatively high impurity content in the solvent before treatment or by the use of partially inactivated aluminum oxide for the purification. If necessary, the ethylene dichloride should be retreated with aluminum oxide that has been properly maintained in a well-sealed container in a dry atmosphere.

Table I—Exposure of Vitamin D Residues[a]

Container for Evaporation	Exposure of Residue after Evaporation	Recovery of Vitamin D, %	
		UV Method	Antimony Trichloride Method
	No Mineral Oil Present		
Volumetric flask	Nitrogen[b], 0 min	100.0	
Volumetric flask	Nitrogen[b], 30 min	99.4	
Volumetric flask	Nitrogen[b], 60 min	101.2	
Beaker	Nitrogen[b], 0 min	98.1	
Beaker	Nitrogen[b], 30 min	72.8	
Beaker	Nitrogen[b], 60 min	66.8	
Beaker	Nitrogen[b], 15 min	80.2	76.7
Beaker	Nitrogen[b], 60 min	66.5	55.8
	0.1 ml of Mineral Oil Present		
Beaker	Air, 15 min	99.3	
Extraction flask	Air, 15 min	99.9	

[a] At room temperature, after evaporation of petroleum ether solutions, using nitrogen. [b] Used nitrogen stream. In beaker or extraction flask, this procedure involves also exposure to admixed air.

The alkaline hydrolysis is needed for several additional reasons. First, oil vehicles, if present, should be removed by saponification and extraction as described; otherwise they may tend to overload the sample layer in the subsequent column step. Second, esters such as vitamin A acetate and vitamin E in the form of the acid succinate, if not first converted to the alcohols by hydrolysis, tend to overlap vitamin D in the elution from the column, so there is incomplete separation. Vitamin D itself is generally already present as the alcohol. Third, the differences in the solubility properties of the esters of vitamins A and E in general and also the differences in their absorption spectra can greatly complicate the problems of separation and measurement if they are allowed to remain in their esterified forms.

Ether in the hydrolysis mixture serves to lower the reflux temperature and helps to form a protective seal of solvent vapor over the solution when heated, thereby diminishing the effects of air oxidation. Vitamin E is particularly vulnerable with respect to air oxidation when hydrolyzed to the free alcohol form in alkaline media. Therefore, pyrogallol is added as a reducing agent which preferentially oxidizes on contact with the air. The oxidation of pyrogallol is characterized by a rapid darkening of the solution, but there is no interference with the analysis since pyrogallol and its oxidation product are readily removed in the subsequent extraction step. (Addition of a little ascorbic acid may also be desirable, but this step has not been performed routinely since ascorbic acid is generally present in the multivitamin formula.)

Finally, as precautionary measures, the mixture is heated to boiling to form the protective vapor seal before adding the aqueous potassium hydroxide solution. Immediately following reflux and cooling, some petroleum ether is added to provide a protective supernatant solvent layer. Hydrolysis of the esters of vitamins A and E is quite rapid, but an additional allowance of time is required for the disintegration of intact capsules and tablets and for the complete saponification of oil vehicles. Therefore, the total time for the alkaline reflux may be as much as 45 min, but usually it is less.

Extraction Step—Emulsions are no problem with the solvent system of 40% ethanol in aqueous potassium hydroxide solution with ether–petroleum ether (1 + 3) as the extracting solvent. Maintaining the proper ethanol concentration is important, as is the use of 40% ethanol instead of water for the first wash solution. If a little emulsion remains at the solvent interface, it may be broken up readily by introducing a few drops of a saturated aqueous solution of sodium sulfate [~15% (w/v) Na_2SO_4]. There are only four shaking operations and, when carried out in this manner, the overall extraction step is not time consuming.

The evaporation of the extracts can be an important part of the analysis, since losses may occur unless the evaporation is handled carefully. *The problem is rapid degradation of the dried residue if exposed to air, particularly when heated. For added protection, a little mineral oil is added to the extracts before evaporation.* The protective effect of mineral oil is demonstrated with vitamin D in Table I.

Reversed-Phase Column—The silicone-impregnated diatoma-

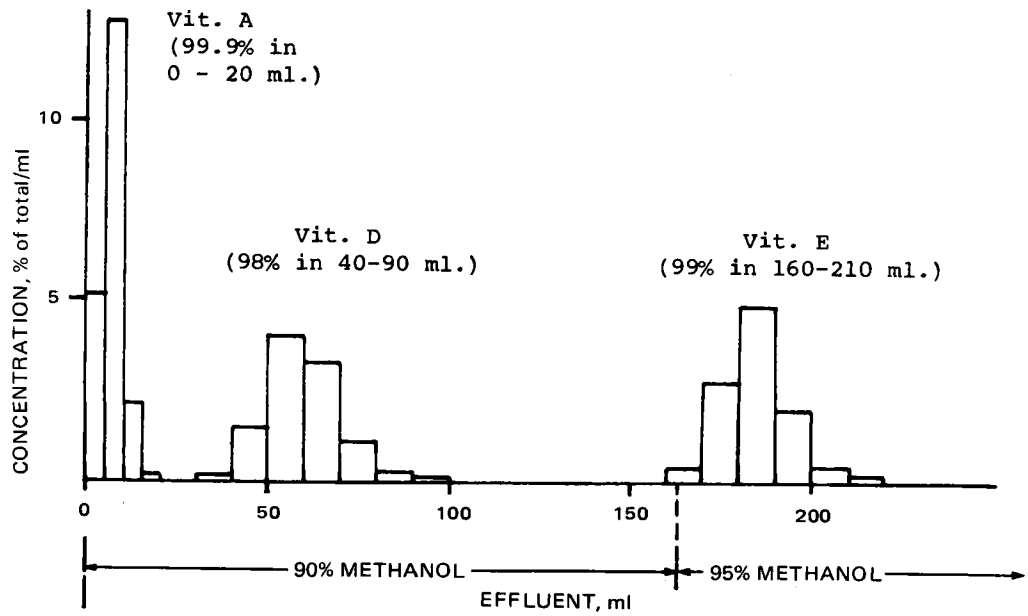

Figure 1—*Elution of vitamins A, D, and E from reversed-phase column.*

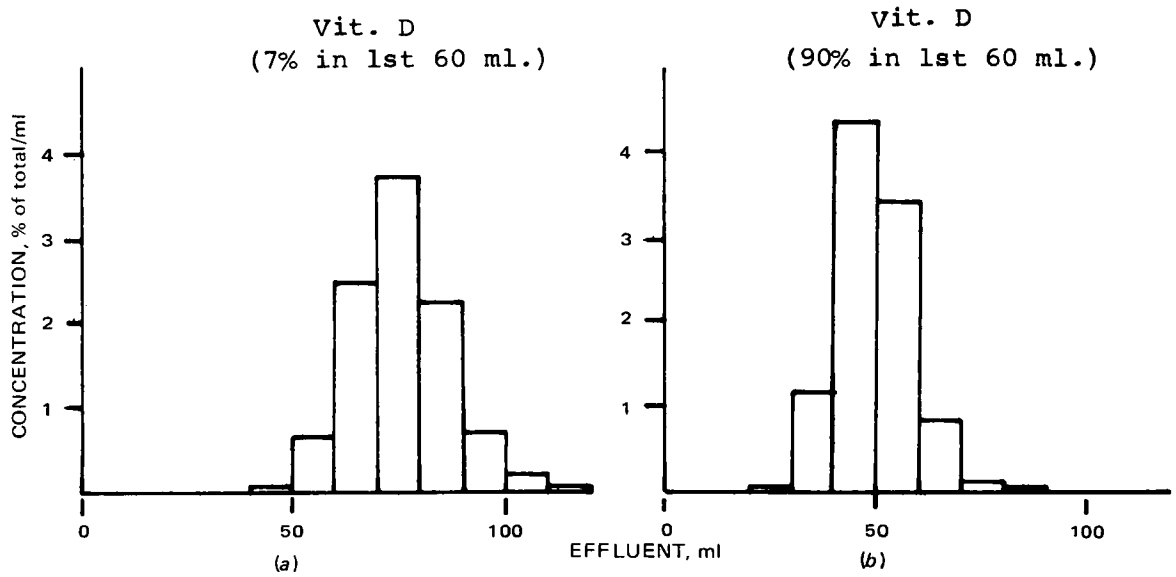

Figure 2—*Elution of vitamin D from reversed-phase column: effect of methanol concentration. Key:* (a), *89% methanol mobile solvent; and* (b), *91% methanol mobile solvent.*

ceous earth, from which the fine-particle fraction has been largely removed, has been found to be quite satisfactory as the column support. The silicone renders the diatomaceous earth readily wettable with the stationary hydrocarbon solvent but not with the mobile polar solvent, which is the desired condition in reversed-phase chromatography. Diatomaceous earth prepared in this manner is preferable to that silanized with dimethyldichlorosilane. Use of the latter results in more tailing, and the separations are not as sharp. Moreover, the silanized support is more difficult to handle during the column packing due to static charge, and it may also cause the stationary solvent to bleed from the column during the elution, a problem not encountered with the silicone-impregnated diatomaceous earth. Removal of the fine-particle fraction from the diatomaceous earth through a sedimentation process facilitates a rapid solvent flow.

Figure 1 shows the elution pattern for vitamins A, D, and E from the reversed-phase column, *i.e.*, the concentrations of the respective vitamins in successive fractions of the column effluent. According to the data from which this chart was prepared, 99.9% of the vitamin A present is eluted in the first 20 ml of effluent, 98% of the vitamin D is eluted in the portion of effluent between 40 and 90 ml, and 99% of the vitamin E is eluted in the portion between 160 and 210 ml. Larger fractions are actually collected to assure good recoveries.

To obtain consistent results, the stationary solvent (*n*-heptane) and the methanol content in the mobile solvent should be accurately measured. The importance of this step is demonstrated in Fig. 2, which depicts the effect on the elution of vitamin D if the concentration of methanol is allowed to deviate by a mere 1% from the intended 90.0% concentration. In this experiment, 7% of the vitamin D appeared in the first 60 ml of effluent with 89.0% methanol and 90% in the first 60 ml with 91.0% methanol.

To a lesser extent, variations in ambient temperature can affect the elution, with higher temperatures favoring early elution and lower temperatures favoring delayed elution. The column should be operated at 25 ± 3°.

Measurement of Vitamin A—The USP procedure for vitamin A makes use of the Morton–Stubbs (10) correction for background absorption in the spectrophotometric step of the assay. The big drawback with the method lies in the difficulty of obtaining accurate measurements on the slope of the curve at the designated wavelengths of 310 and 334 nm and the fact that any errors become greatly magnified in the final result.

The Morton–Stubbs correction has been omitted from the present procedure since the column provides added purification and removal of UV-absorbing impurities. However, the column does not serve to separate the geometric isomers of vitamin A from each other. In other words, as with the USP procedure for vitamin A, the absorbance that is finally measured represents the total amount due to all-*trans*-vitamin A, or retinol, plus any of the less active vitamin A isomers present. Although the latter are unlikely in fresh samples prepared with all-*trans*-synthetic vitamin A, they tend to form through isomerization of the all-*trans*-form in water-based formulations during aging (11). The spectrophotometric method then becomes subject to some positive error as a measure of biopotency, in which case corrective steps may be taken (12).

Measurement of Vitamin D—As with vitamin A, the measurement method is perhaps best discussed here by comparing it with the USP procedure. In several respects, the methods are similar. However, in the present method the color of the sample is compared directly with that of the standard instead of with that of a combination of sample and standard as in the USP method. Moreover, the correction based on readings of the solution at 550 nm has been dropped. These features of the USP colorimetric step appear to be unnecessary complications and may actually enhance any errors.

Other slight changes are as follows. The concentration of vitamin D in the final solution is slightly greater, and the solution is measured with 2- instead of 1-cm cells. The effect is to increase the absorbance reading. The corrected reading for the standard generally falls in the 0.360–0.400 range. The maximum for vitamin D has been observed to be at about 498 nm rather than at 500 nm as indicated in the USP.

Unfortunately, quite a few materials interfere with the antimony trichloride procedure for vitamin D, *i.e.*, materials that respond differently with and without the color inhibitor. Vitamin E is one of these, but it is removed by separation on the reversed-phase column. Degradation products of vitamin A would also interfere but are removed by the treatment with fuller's earth.

It was found advantageous to shorten the USP version of this step slightly by omitting the petroleum ether wash and by increasing the column diameter to permit a rapid flow rate by gravity flow. Furthermore, the capacity of the column for cleanup was improved by substitution of 10 g of fuller's earth for 3 g. The USP specifies 8.5–9.0% water content in the fuller's earth, and this amount was confirmed as being about optimum. A lower water content results in good

Table II—Recovery of Vitamin A in Extraction Step

Ether–Petroleum Ether Extracts	Vitamin A Found, UV Method	
	I.U. per Capsule[a]	Percent of Label Claim[a]
First two portions: 100 ml + 80 ml (usual extraction)	5597	139.9
Third portion (extra 80 ml)	3.1[b]	0.08

[a] Determinations were carried out with capsules having a high mineral content. [b] Result of 3.1 units, calculated as vitamin A, appeared to be due to extraneous background absorption rather than to actual vitamin A.

Table III—Recovery of Vitamin A through Reversed-Phase Column

Product	Type	Vitamin A Found	
		I.U. without Column	I.U. with Column
1	Capsules, with vitamins A and D	5698	5512
2	Tablets, with vitamins A, D, and E	5944	5807
3	Capsules, with vitamin A and minerals	2230	2196
4	Capsules, with vitamins A and E and minerals	6519	6353
5	Capsules, with vitamins A and E and minerals	6716	6569
5	Capsules, with vitamins A and E and minerals	6524	6313

cleanup of impurities but incomplete recovery of vitamin D from the column; a greater water content results in poor removal of impurities, although good recovery of vitamin D.

An important requirement is to use benzene free from water or contamination with polar solvents. Therefore, the benzene is treated before use by passing it through activated alumina. The USP specifies no pretreatment of the benzene.

Ergosterol and its irradiation products are not removed from the vitamin D fraction by the separation steps of either the present combined assay procedure or the USP assay for vitamin D. Ergosterol and lumisterol do not produce a significant amount of color with antimony trichloride color reagent, however, as observed by Shaw *et al.*

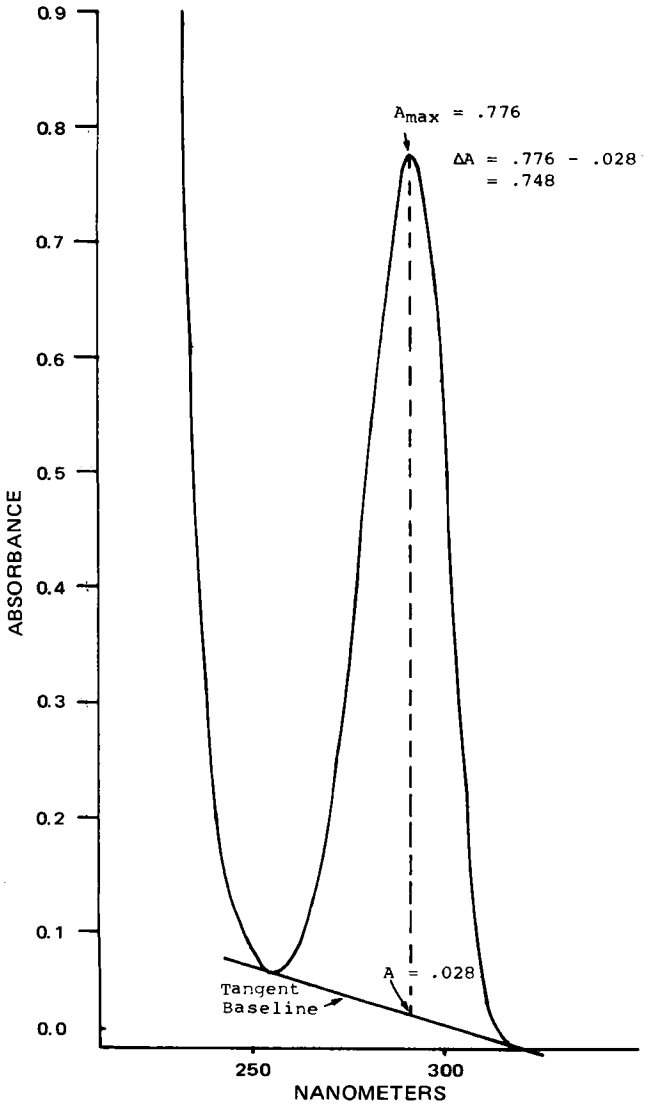

Figure 3—d-*Alpha tocopherol solution in methanol, 10.304 mg/100 ml. Absorptivity (1%, 1 cm) = 0.776/0.010304 = 75.3 (based on* A_{max}*) or 0.748/0.010304 = 72.6 (based on* ΔA_{max}*).*

(13). Precalciferol forms an equilibrium mixture with vitamin D, the rate of formation and final concentration being temperature dependent.

It has been contended that the total of vitamin D plus precalciferol, representing the potential biological activity, should be measured rather than the vitamin D content alone (14). Mixtures of the two would be typical in aged liquid preparations containing vitamin D. Fortunately in this regard, precalciferol produces the same response as vitamin D in the antimony trichloride test (13), so the assay procedure measures the total of the two.

Finally, tachysterol is not separated and would interfere if present in the original sample. Inactivation of tachysterol by reaction with maleic anhydride has been used to overcome the problem (15), but an extra step is needed. Preferably, the raw material used for the multivitamin product should be free from tachysterol. It might be monitored readily by the manufacturer by UV examination following reversed-phase column separation. As reported by Shaw *et al.* (13), tachysterol is a strong absorber in the UV region at wavelengths above 265 nm, the peak for vitamin D, so tachysterol would show up as a distortion of the vitamin D spectrum.

Some mention should be made here of material found in certain synthetic vitamin A concentrates that tends to cause extraneous color in the procedure for vitamin D. This material is not removed by the reversed-phase column, nor by the purification with fuller's earth. In the color test, the material has been observed to form a lavender background color. The absorbance is characterized by a maximum at about 580 nm, diminishing rapidly with time, and by a less pronounced maximum at about 475 nm which increases with time. Fortunately, the response is about the same with and without the color inhibitor at 498 nm, the point of measurement of vitamin D, so interference from this source has not been a serious problem.

Measurement of Vitamin E—Because of the cleanup on the reversed-phase column, it is possible to measure vitamin E by its UV absorption. Since a small amount of background absorption may still be present, a tangent baseline correction is used. The tangent baseline is illustrated in Fig. 3 as determined for a relatively pure sample of

Table IV—Recovery of Vitamin D

Product	Type	Recovery of Added Vitamin D, %
1	Water-based tablets, with vitamins A and D, Lot 1	101.1
	Lot 2	101.8
	Placebo	100.3
	Placebo	101.2
2	Capsules, with vitamins A, D, and E, Lot 1	92.5
	Lot 1	96.8
	Lot 2	93.4
3	Capsules, with vitamins A and D	98.3
4	Capsules, with vitamins A and D	101.6
5	Capsules, with vitamins A and D	100.0
6	Capsules, with vitamins A, D, and E and minerals	94.9
7	Tablets, with vitamins A, D, and E and minerals, Lot 1	96.0
	Lot 2	97.1
8	Tablets, with vitamins A, D, and E and minerals, Lot 2	92.6
9	Capsules, with vitamins A, D, and E and minerals; placebo	98.0
	Placebo	98.4

Table V—Determination of Vitamin E

Product	Type	Vitamin E, I.U. Label Claim	Found	
1	Capsules, with vitamins A, D, and E and minerals	30	31.4	
2	Capsules, with vitamins A, D, and E and minerals, Lot 1	15	15.41	
	Lot 2	15	15.66	
	Lot 3	15	16.07	
3	Tablets, with vitamins A, D, and E and minerals	30	11.12	
4	Tablets, with vitamins A, D, and E and minerals, Lot 1	10	11.12	32.6
	Lot 2	10	10.85	
	Lot 3	10	10.73	
5	Tablets, with vitamins A, D, and E and minerals	10	10.57	
6	Capsules, with vitamin E, Lot 1	400	398 (402 without column)	
	Lot 1	400	395 (404 without column)	
	Lot 2	400	406 (406 without column)	

d-alpha tocopherol[15]. This method is generally satisfactory for canceling out the effect of low levels of background.

For the calculations a standard value of 72.4 was determined, representing the absorptivity (1%, 1 cm) of alpha tocopherol (either *d* or *dl*) at 292 nm after tangent baseline correction. The standard value was obtained by collecting the middle portion of the vitamin E fraction from the reversed-phase column, with *dl*-alpha tocopherol as the starting material. The middle cut from the column was presumed to be quite pure. The collected effluent was then checked spectrophotometrically and by a gravimetric procedure to obtain the absorptivity value with tangent baseline correction for the isolated alpha tocopherol.

The average result of replicate determinations, 72.4, was in close agreement with the 72.6 value obtained with the pure sample (Fig. 3). The solvent used in this instance was methanol, but substitution of the 95% methanol eluting solvent resulted in practically no change of the absorptivity with tangent baseline correction.

The principal type of interference encountered in the UV determination of vitamin E has been background absorption due to degradation products of vitamin A. The background absorption from this source is typically characterized by a relatively broad peak near 290 nm. If not successfully removed, these impurities tend to produce a positive error for vitamin E. At low concentration, the vitamin A degradation products elute from the reversed-phase column ahead of vitamin E, largely in the vitamin D fraction but also in the discard fraction. At high concentration, as in certain aged samples, the column may become overloaded so that some of this degradation material tails into the vitamin E fraction. The answer is to reduce the amount of sample placed on the column to the point that overloading no longer occurs.

Although relatively little work was done with mixtures containing non-alpha tocopherols, they apparently also elute ahead of vitamin E (alpha tocopherol) and, consequently, do not interfere. Elution patterns obtained with such mixtures indicated little or no merging of the non-alpha tocopherols with the alpha tocopherols. Non-alpha tocopherols now may be largely phased out, however, with many newer multivitamin products being formulated to contain only the alpha form.

Recovery of Vitamin A—Good recovery of vitamin A in the extraction step is shown in Table II, *i.e.*, more than 99.9% recovery in the two portions of ether–petroleum ether normally used. The sample, a capsule formula high in mineral content, was used to demonstrate the absence of possible interference due to insoluble materials. These results for vitamin A are also indicative of good recoveries of vitamins D and E in the same extraction step, since they favor the nonpolar extracting solvent even more than does vitamin A.

In Table III, results are compared on assaying various formulations for vitamin A both with and without the column treatment. The indication is that about 2–3% of the vitamin A may be lost on the column, but the apparent loss can be greater with aged samples. In the latter case, the difference is probably due largely to purification on the column rather than to loss of vitamin A. The procedure without the column is similar to the USP vitamin A assay.

[15] Eastman Catalog No. 6340.

Recovery of Vitamin D—Percentage recoveries of known amounts of crystalline vitamin D were determined when added to placebo formulations or to the complete formulas themselves (Table IV). In each case, an accurately measured amount of vitamin D approximating the formula amount was added, the formula amount being 400 I.U. plus an excess. The average of the tabulated recoveries was 97.7%, and the standard deviation was 3.08.

Recovery of Vitamin E—Table V gives the assay results for vitamin E, comparing the amount found with the labeled amount in each case. The amount found is usually slightly more than the labeled amount, reflecting small formula excesses. With Product 6, which contains only vitamin E, assays were carried out both with and without the column separation and, as with vitamin A, the results indicate a slight loss on the column. The agreement is generally quite good, however, with apparent losses on the column usually not exceeding about 2%.

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