Modification of USP Vitamin D Assay to Remove Interference of Vitamin E

By MODEST OSADCA and ELMER DE RITTER

When tocopherol is incorporated in multivitamin products at the 10–30 mg. levels, corresponding to the daily adult requirement, serious interference is encountered in determining vitamin D by the USP method. A modification of the USP assay method is described in which the tocopherol is separated by column chromatography on secondary magnesium phosphate prior to the normal USP chromatographic purification steps. Proper conditioning and performance testing of the secondary magnesium phosphate are essential to assure good separation of tocopherol and quantitative recovery of vitamin D.

THE RECOGNITION of a daily adult requirement for tocopherol in the range of 10–30 mg. (1) has led to increased usage of vitamin E in multivitamin supplements. For example, the formulas for decavitamin capsules and tablets in the USP XVII (2) include a suitable form of α-tocopherol at a level equivalent to 15 mg. of *dl*-α-tocopheryl acetate per capsule or tablet. Vitamin D levels, on the other hand, have tended to be lowered in such products due to increased emphasis on the desirability of avoiding intakes of vitamin D in excess of the recommended daily allowance (1). As a result of these developments, the ratio of vitamin E to vitamin D in many products has increased markedly to the point where the vitamin E causes considerable overestimation of vitamin D by the USP assay method (2).

A modified vitamin D assay procedure has been developed in which tocopherol is removed from the solvent extract by column chromatography on secondary magnesium phosphate prior to the normal chromatographic purification steps in the USP method. The use of secondary magnesium phosphate for separations in the assay of vitamin E has been described by Bro-Rasmussen and Hjarde (3). Careful conditioning of the adsorbent and performance testing before use are of critical importance in the vitamin E assay and also in the vitamin D assay described below.

Mulder *et al.* (4) separated tocopherols from vitamin D by column chromatography on alkaline alumina prior to passage through the diatomaceous earth[1] and Fuller's earth[2] columns, but the oils tested contained slightly less than 1 mg. of α-tocopheryl acetate per 1,000 units of vitamin D. Multivitamin mixtures containing 10–30 mg. of vitamin E plus 400 units of vitamin D represent ratios of vitamin E to vitamin D about 25 to 75 times as great as those reported by Mulder *et al.* (4). In our experience with alkaline alumina, when quantitative recovery of vitamin D was obtained, the removal of these higher levels of vitamin E was not complete and significant interference was encountered in the vitamin D assay.

EXPERIMENTAL

Reagents and Apparatus—These are described in USP XVII (2) with the following additions.

Diethyl Ether—must be free of peroxides; Mallinckrodt's black label has been suitable.

Petroleum Ether—Merck reagent grade benzin, boiling range 30–65°; purify by passing through a silica gel column until no absorption is found at 300 mμ.

Secondary Magnesium Phosphate—The following products have been used successfully when conditioned as directed below.

(*a*) Magnesium phosphate, dibasic, pure, E. Merck A. G., Darmstadt, West Germany—distributed by Brinkmann Instruments, Inc., Westbury, L. I., N. Y. (*b*) Magnesium phosphate, C.P., dibasic—distributed by Amend Drug and Chemical Co., Inc., New York, N. Y.

Conditioning Procedure for Secondary Magnesium Phosphate—Dry about 250 Gm. for 4 hr. at 180° and cool over concentrated H_2SO_4. Boil the powder for 10 min. with 2 L. of a solution of 16 Gm. of secondary sodium phosphate (anhydrous, reagent grade) in H_2O. Cool slightly, filter through Whatman No. 1 paper on a Büchner funnel, and wash with about 1.5 L. of H_2O. Dry the secondary magnesium phosphate for 48 hr. at 180° and cool to room temperature over concentrated H_2SO_4. Grind in a mortar, screen through a 120-mesh sieve, and store in a tightly closed container. Prior to use, transfer a weighed amount of this material to a jar, add 7% H_2O, close jar, and shake thoroughly to break up all lumps and distribute the water. Store in a tightly closed jar. Let stand at least 2–3 hr. before use and do not keep for more than 2 days after adding the H_2O.

Preparation of Column—Plug the lower, constricted end of a 17–20 mm. i.d. chromatography tube about 25 cm. long with glass wool. With the stopcock closed, fill about two-thirds with petroleum ether. Add conditioned secondary magnesium phosphate portionwise, allowing it to flow slowly into the tube and settle without pressure or vacuum until a column height of 10–12 cm. is

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[1] Celite, Johns-Manville Corp., New York, N. Y.
[2] Florex, Floridin Co., Pittsburgh, Pa.

obtained. Drain off the petroleum ether until the solvent is about 3 mm. above the surface of the adsorbent.

Column Performance Check—Using a mixture of 75 mg. of α-tocopheryl acetate and 25,000 USP units of vitamin A as acetate or palmitate, obtain a sample preparation as described under *Procedure*. Transfer the 10 ml. of petroleum ether extract onto the prepared column and open the stopcock. Wash down with two 5-ml. aliquots of petroleum ether, being careful not to let the surface of the column run dry. Elute with a 6:94 (v/v) mixture of diethyl ether–petroleum ether until the vitamin A zone (seen as a green-yellow fluorescence with the aid of a long wavelength UV light) reaches the glass wool plug. This should require 35–60 ml. of eluant and all of the tocopherol which precedes vitamin A is then in the eluate receiver. Change the receiver and elute the vitamin A with a 3:7 (v/v) mixture of diethyl ether–petroleum ether. Vitamin A elution as observed with the UV light should be complete with about 50 ml. of eluant.

Check recovery of vitamin D from a second, similar column of secondary magnesium phosphate by adding 10 ml. of a petroleum ether solution containing 2,000 USP units of vitamin D and no vitamin A or vitamin E. Proceed with chromatography as described above, using the volume of 6:94 diethyl ether–petroleum ether mixture determined above to be necessary to bring the vitamin A down to the glass wool plug. Change the receiver and elute the vitamin D with 70 ml. of the 3:7 mixture of diethyl ether–petroleum ether. Evaporate this second eluate and measure the vitamin D colorimetrically as per USP XVII. Quantitative recovery of vitamin D should be obtained with this procedure.

When a new lot of secondary magnesium phosphate is received, a portion should be conditioned and checked for performance as described above. Drying times and moisture content of the adsorbent influence its activity. If the above conditions are not optimal for a particular lot of secondary magnesium phosphate, they may have to be modified until the above performance criteria are met. If vitamin A spreads all through the column and moves down too rapidly, the adsorbent is not sufficiently active. Longer drying or less water addition may then be needed. If the vitamin A does not move down or moves too slowly, addition of more water is indicated.

Procedure—Obtain a sample preparation as described on p. 892 of USP XVII except to add 50 mg. of hydroquinone instead of 2 ml. of cottonseed oil prior to saponification and to take up the residue after evaporation of the solvent hexane extract in 10 ml. of petroleum ether rather than solvent hexane. The vitamin D level in the original sample taken may be as low as 2,000 units. Transfer an aliquot of the petroleum ether extract containing 2,000–4,000 USP units of vitamin D onto the prepared secondary magnesium phosphate column. Open stopcock and allow solution to drain into column without pressure or suction. Do not let top of column run dry. Wash down with two 5-ml. portions of petroleum ether. Elute tocopherol with 6:94 mixture of diethyl ether–petroleum ether until the vitamin A zone reaches the glass wool plug. Change the receiver and elute vitamin D along with vitamin A with 70 ml. of a 3:7 mixture of diethyl ether–petroleum ether. Evaporate the eluate just to dryness under a stream of nitrogen with the flask immersed in a water bath at 40°. Dissolve the residue in 5 ml. of solvent hexane. Take a 2 or 4-ml. aliquot containing about 1,600 USP units of vitamin D for "first column chromatography" as described in USP XVII and continue to follow the USP procedure.

Table I—Comparison of USP and Modified USP Assays for Vitamin D in Presence of Vitamin E

Vitamin D, USP units	Vitamin E, mg. *dl*-α-Tocopheryl Acetate	Vitamin A, USP units	—Vitamin D (or Equivalent) Found, USP units— USP XVII	Modified USP XVII	Difference
		Model Systems			
0[a]	15	0	150	10	140
			270	20	250
0[a]	30 (*dl*-α-Tocopherol)	4,000	300	30	270
400	15	5,000	580	401	179
			700	404	296
		Multivitamin Tablets			
(Added per tablet[b])					
A 600	15	25,000	790	580	210
			820	630	190
			810	600	210
			810	610	190
B 600	15	5,000	710	580	130
		Multivitamin Drops			
(per 0.6 ml.[b])					
500	10	2,500			
		Initial	612	480	132
		2 mo. at 45°	654	450	204
		3 mo. at 45°	636	444	192

[a] Samples without vitamin D were treated as if they contained 400 USP units per specified quantity of vitamin E. [b] Vitamin D content represents the actual amount added in manufacturing; vitamin A and E values represent label claims.

Applications of Procedure—Both the USP XVII procedure for vitamin D assay and the present modified procedure have been applied to (*a*) samples of vitamin E only, (*b*) a laboratory mixture of vitamins A, D, and E, (*c*) multivitamin tablets and drops, the latter including samples aged at 45°. In all cases the equivalent of 2,000 USP units of vitamin D based on label claim was taken as the original sample and the entire 2,000 units taken for the first column chromatography in both procedures.

RESULTS

The results are summarized in Table I. When only vitamin E is carried through the USP vitamin D assay procedure, a significant amount of brown color is obtained and measured as vitamin D. The additional chromatography on secondary magnesium phosphate in the modified procedure reduces this nonspecific blank to a very low level. In the model mixture of vitamins A, D, and E, the USP method overestimated the 400 units of vitamin D by 45–74% in the presence of 15 mg. of α-tocopheryl acetate. Recoveries in the two trials of the modified procedure with this combination of vitamins were 100 and 101%. Similarly, with multivitamin products the USP method overestimates the vitamin D content to a considerable degree (130–210 USP units on a total of 500–600 units). The results by the modified procedure are close to the actual levels of addition of vitamin D.

The magnitude of the interference due to tocopherol in the USP assay for vitamin D is not sharply reproducible from one sample or product to the next. On the basis of the results in Table I, it does not appear that a generally applicable correction to the USP assay value could be calculated based on the relative proportions of tocopherol and vitamin D and the dilutions used. Preliminary removal of the tocopherol *via* column chromatography on secondary magnesium phosphate provides an effective and reliable method of removing this source of interference.

REFERENCES

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Keyphrases

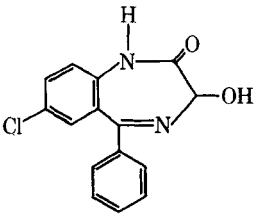
Vitamin D analysis
Vitamin E—interference elimination
Column chromatography—separation
Magnesium phosphate, secondary—column adsorbent
UV light—vitamin A fluorescence
Colorimetry—analysis

Qualitative and Quantitative Tests for Oxazepam

By EDWARD F. SALIM*, J. L. DEUBLE†, and G. PAPARIELLO†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

7-CHLORO-1,3-DIHYDRO-3-HYDROXY-5-PHENYL-2H-1,4-BENZODIAZEPIN-2-ONE; $C_{15}H_{11}ClN_2O_2$; mol. wt. 286.72. The structural formula of oxazepam may be represented as I.



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Physical Properties—Oxazepam occurs as a creamy white to pale yellow, practically odorless powder. It is practically insoluble in water, slightly

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