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〈 581 〉 VITAMIN D ASSAY

Chromatographic Method

The following pressurized liquid chromatographic procedure is provided for the determination of vitamin D, as cholecalciferol or as ergocalciferol, as an ingredient of Pharmacopeial multiple-vitamin preparations.

Throughout this assay, protect solutions containing, and derived from, the test specimen and the Reference Standard from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

USP Reference Standards 〈 11 〉— [NOTE—Use *USP Ergocalciferol RS*, or *USP Cholecalciferol RS*, for assaying pharmaceutical dosage forms that are labeled to contain vitamin D as ergocalciferol, or as cholecalciferol, respectively.] *USP Cholecalciferol RS*. *USP $\Delta^{4,6}$-Cholestadienol RS*. *USP Ergocalciferol RS*. *USP Vitamin D Assay System Suitability RS*.

Special Reagents and Solutions—

Ether— Use ethyl ether. Use within 24 hours after opening container.

Dehydrated Hexane— Prepare a chromatographic column by packing a chromatographic tube, 60 cm × 8 cm in diameter, with 500 g of 50- to 250-µm chromatographic siliceous earth, activated by drying at 150° for 4 hours (see *Column Adsorption Chromatography* under *Chromatography* 〈 621 〉). Pass 500 mL of hexanes through the column, and collect the eluate in a glass-stoppered flask.

Butylated Hydroxytoluene Solution— Dissolve a quantity of butylated hydroxytoluene in chromatographic hexane to obtain a solution containing 10 mg per mL.

Aqueous Potassium Hydroxide Solution— Dissolve 500 g of potassium hydroxide in 500 mL of freshly boiled water, mix, and cool. Prepare this solution fresh daily.

Alcoholic Potassium Hydroxide Solution— Dissolve 3 g of potassium hydroxide in 50 mL of freshly boiled water, add 10 mL of alcohol, dilute with freshly boiled water to 100 mL, and mix. Prepare this solution fresh daily.

Sodium Ascorbate Solution— Dissolve 3.5 g of ascorbic acid in 20 mL of 1 N sodium hydroxide. Prepare this solution fresh daily.

Sodium Sulfide Solution— Dissolve 12 g of sodium sulfide in 20 mL of water, dilute with glycerin to 100 mL, and mix.

Mobile Phase A— Prepare a mixture of acetonitrile, methanol, and water (25:25:1). The amount of water and the flow rate may be varied to meet system suitability requirements.

Mobile Phase B— Prepare a 3 in 1000 mixture of *n*-amyl alcohol in *Dehydrated Hexane.* The ratio of components and the flow rate may be varied to meet system suitability requirements.

Internal Standard Solution— Transfer 15 mg of USP $\Delta^{4,6}$-Cholestadienol RS, accurately weighed, to a 200-mL volumetric flask, add a 1 in 10 mixture of toluene and *Mobile Phase B* to volume, and mix.

Standard Preparation— Transfer about 25 mg of USP Ergocalciferol RS or Cholecalciferol RS, accurately weighed, to a 50-mL volumetric flask, dissolve without heat in toluene, add toluene to volume, and mix. Pipet 10 mL of this stock solution into a 100-mL volumetric flask, dilute with toluene to volume, and mix. Prepare stock solution fresh daily.

Assay Preparation—

For oily solutions— Accurately weigh a portion of the specimen to be assayed, preferably more than 0.5 g and equivalent to about 125 µg of cholecalciferol or ergocalciferol (5000 USP Units). Add 1 mL of *Sodium Ascorbate Solution*, 25 mL of alcohol, and 2 mL of *Aqueous Potassium Hydroxide Solution*, and mix.

For capsules or tablets— Reflux not less than 10 capsules or tablets with a mixture of 10 mL of *Sodium Ascorbate Solution* and 2 drops of *Sodium Sulfide Solution* on a steam bath for 10 minutes, crush any remaining solids with a blunt glass rod, and continue heating for 5 minutes. Cool, add 25 mL of alcohol and 3 mL of *Aqueous Potassium Hydroxide Solution*, and mix.

For dry preparations and *aqueous dispersions*— Accurately weigh a portion of the specimen to be assayed, preferably more than 0.5 g and equivalent to about 125 µg of cholecalciferol or ergocalciferol (5000 USP Units). Add, in small quantities and with gentle swirling, 25 mL of alcohol, 5 mL of *Sodium Ascorbate Solution*, and 3 mL of *Aqueous Potassium Hydroxide Solution.*

SAPONIFICATION AND EXTRACTION— Reflux the mixture prepared from the specimen to be assayed on a steam bath for 30 minutes. Cool rapidly under running water, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with two 15-mL portions of water, 10 mL of alcohol, and two 50-mL portions of ether. Shake the combined saponified mixture and rinsings vigorously for 30 seconds, and allow to stand until both layers are clear. Transfer the aqueous phase to a second conical separator, add a mixture of 10 mL of alcohol and 50 mL of solvent hexane, and shake vigorously. Allow to separate, transfer the aqueous phase to a third conical separator, and transfer the hexane phase to the first separator, rinsing the second separator with two 10-mL portions of solvent hexane, adding the rinsings to the first separator. Shake the aqueous phase in the third separator with 50 mL of solvent hexane, and add the hexane phase to the first separator. Wash the combined ether-hexane extracts by shaking vigorously with three 50-mL portions of *Alcoholic Potassium Hydroxide Solution*, and wash with 50-mL portions of water vigorously until the last washing is neutral to phenolphthalein. Drain any remaining drops of water from the combined ether-hexane extracts, add 2 sheets of 9-cm filter paper, in strips, to the separator, and shake. Transfer the washed ether-hexane extracts to a round-bottom flask, rinsing the separator and paper with solvent hexane. Combine the hexane rinsings with the ether-hexane extracts, add 5.0 mL of *Internal Standard Solution* and 100 µL of *Butylated Hydroxytoluene Solution*, and mix. Evaporate to dryness in vacuum by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 5.0 mL of a mixture of equal volumes of acetonitrile and methanol, or in a measured portion of the acetonitrile-methanol mixture until the concentration of vitamin D is about 25 µg per mL, to obtain the *Assay Preparation.*

Chromatographic System— Use a chromatograph, operated at room temperature, fitted with an UV detector that monitors absorption at 254 nm, a 30-cm × 4.6-mm stainless steel cleanup column packed with column packing L7 and using *Mobile Phase A*, and a 25-cm × 4.6-mm stainless steel analytical column packed with column packing L3 and using *Mobile Phase B*.

Cleanup Column System Suitability Test— Pipet 5 mL of the *Standard Preparation* into a round-bottom flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under an atmosphere of nitrogen for 45 minutes, to obtain a solution containing vitamin D and pre-vitamin D. Cool, add 10.0 mL of *Internal Standard Solution*, mix, and evaporate in vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 10.0 mL of a mixture of equal volumes of acetonitrile and methanol, and mix. Inject 500 µL of this solution into the cleanup column, and record the chromatogram as directed under *Procedure.* The chromatogram exhibits a peak exhibiting a retention time between 5 and 9 minutes, corresponding to the separation under a single peak of the mixture of vitamin D, pre-vitamin D, and $\Delta^{4,6}$-cholestadienol from other substances. Adjust the water content or other operating parameters, if necessary (see *Mobile Phase A*).

Analytical Column System Suitability Test— Transfer about 100 mg of USP Vitamin D Assay System Suitability RS to a 100-mL volumetric flask, add a 1 in 20 mixture of toluene and *Mobile Phase B* to volume, and mix. Heat a portion of this solution, under reflux, at 90° for 45 minutes, and cool. Chromatograph five injections of the resulting solution, and measure the peak

responses as directed for *Procedure:* the resolution, *R,* between *trans*-cholecalciferol and pre-cholecalciferol is not less than 1.0. and the relative standard deviation for the cholecalciferol peak response does not exceed 2.0%. [NOTE—Chromatograms obtained as directed for this test exhibit relative retention times of approximately 0.4 for pre-cholecalciferol, 0.5 for *trans*-cholecalciferol, and 1.0 for cholecalciferol.]

Calibration—

Vitamin D Response Factor— Transfer 4.0 mL of the *Standard Preparation* and 10.0 mL of *Internal Standard Solution* to a 100-mL volumetric flask, dilute with *Mobile Phase B* to volume, and mix to obtain the *Working Standard Preparation.* Store this *Working Standard Preparation* at a temperature not above 0°, retaining the unused portion for the *Procedure.* Inject 200 µL of the *Working Standard Preparation* into the analytical column, and measure the peak responses for vitamin D and for $\Delta^{4,6}$-cholestadienol. The relative retention time of $\Delta^{4,6}$-cholestadienol is about 1.3. Calculate the response factor, F_D, by the formula:

$$C_S / (R_S C_R),$$

in which C_S and C_R are the concentrations, in µg per mL, of vitamin D and $\Delta^{4,6}$-cholestadienol, respectively, in the *Working Standard Preparation*, and R_s is the ratio of the peak response of vitamin D to that of $\Delta^{4,6}$-cholestadienol.

Pre-Vitamin D Response Factor— Pipet 4 mL of the *Standard Preparation* into a round-bottom flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under a nitrogen atmosphere for 45 minutes, to obtain a solution containing vitamin D and pre-vitamin D. Cool, transfer with the aid of several portions of *Mobile Phase B* to a 100-mL volumetric flask containing 10.0 mL of *Internal Standard Solution*, dilute with *Mobile Phase B* to volume, and mix to obtain the *Working Mixture.* Inject 200 µL of this *Working Mixture* into the analytical column, and measure the peak responses for vitamin D, pre-vitamin D, and $\Delta^{4,6}$-cholestadienol. Calculate the concentration, C'_s, in µg per mL, of vitamin D in the (heated) *Working Mixture* by the formula:

$$F_D C_R R'_S,$$

in which C_R is the concentration, in µg per mL, of $\Delta^{4,6}$-cholestadienol, and R'_S is the ratio of the peak response for vitamin D to that for$\Delta^{4,6}$-cholestadienol. Calculate the concentration, C'_{PRE}, in µg per mL, of pre-vitamin D, in the *Working Mixture* by the formula:

$$C'_{PRE} = C_S - C'_S.$$

Calculate the response factor, F_{PRE}, for pre-vitamin D by the formula:

$$(F_D R'_S C'_{PRE}) / (R'_{PRE} C'_S),$$

in which R'_{PRE} is the ratio of the peak response of pre-vitamin D to that of $\Delta^{4,6}$-cholestadienol. [NOTE—Value of F_{PRE} determined in duplicate, on different days, can be used during the whole procedure.]

Procedure— Inject 500 µL of the *Assay Preparation* into the cleanup column, and collect the fraction representing 0.7 to 1.3 relative to the retention time of the mixed vitamin D peak (see *Cleanup Column System Suitability Test*) in a round-bottom flask. Add 50 µL of *Butylated Hydroxytoluene Solution*, mix, and evaporate in vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 5.0 mL of a 1 in 20 mixture of toluene and *Mobile Phase B*, and mix. Inject 200 µL of this solution into the analytical column, and measure the peak responses for vitamin D, pre-vitamin D, and $\Delta^{4,6}$-cholestadienol. Calculate the concentration, in µg per mL, of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the *Assay Preparation* by the formula:

$$(R''_D F_D + R''_{PRE} F_{PRE}) C''_R,$$

in which R''_D is the ratio of the peak response of vitamin D to that of $\Delta^{4,6}$-cholestadienol; R''_{PRE} is the ratio of the peak response of pre-vitamin D to that of $\Delta^{4,6}$-cholestadienol; and C''_R is the concentration, in µg per mL, of $\Delta^{4,6}$-cholestadienol in the *Assay Preparation.*

Chemical Method

The following procedure is provided for the determination of vitamin D as an ingredient of Pharmacopeial preparations.

Complete the assay promptly, and exercise care throughout the procedure to keep to a minimum the exposure to air and to actinic light, preferably by the use of a blanket of inert gas and low-actinic glassware.

USP Reference Standards 〈 11 〉 — [NOTE—Use *USP Ergocalciferol RS*, or *USP Cholecalciferol RS*, for assaying pharmaceutical dosage forms that are labeled to contain vitamin D as ergocalciferol, or as cholecalciferol, respectively.] *USP Cholecalciferol RS*. *USP Ergocalciferol RS*.

Special Reagents and Solutions—

Chromatographic Fuller's Earth— Use chromatographic Fuller's earth having a water content corresponding to between 8.5% and 9.0% of loss on drying.

Solvent Hexane— Use solvent hexane (see under *Reagents, Indicators, and Solutions*), redistilling if necessary so that it meets the following additional specification:

SPECTRAL PURITY—Measure in a 1-cm cell at 300 nm, with a suitable spectrophotometer, against air as the blank: the absorbance is not more than 0.070.

Ethylene Dichloride— Purify by passage through a column of granular (20 to 200 mesh) silica gel.

Potassium Hydroxide Solution— Dissolve 500 g of potassium hydroxide in water to make 1000 mL.

Butylated Hydroxytoluene Solution— Dissolve 10 mg of butylated hydroxytoluene in 100 mL of alcohol. Prepare this solution fresh daily.

Ether— Use freshly distilled ether, discarding the first and last 10% portions of the distillate.

Color Reagent— Prepare two stock solutions as follows.

SOLUTION A —Empty, without weighing, the entire contents of a previously unopened 113-g bottle of dry, crystalline antimony trichloride into a flask containing about 400 mL of *Ethylene Dichloride.* Add about 2 g of anhydrous alumina, mix, and pass through filter paper into a clear-glass, glass-stoppered container calibrated at 500 mL. Dilute with *Ethylene Dichloride* to 500 mL, and mix: the absorbance of the solution, measured in a 20-mm cell at 500 nm, with a suitable spectrophotometer, against *Ethylene Dichloride*, does not exceed 0.070.

SOLUTION B—Mix, under a hood, 100 mL of acetyl chloride and 400 mL of *Ethylene Dichloride*.

Mix 45 mL of *Solution A* and 5 mL of *Solution B* to obtain the *Color Reagent.* Store in a tight container, and use within 7 days, but discard any reagent in which a color develops.

Chromatographic Tubes—

First Column— Arrange for descending column chromatography a tube of 2.5-cm (inside) diameter, about 25 cm long, and constricted to 8-mm diameter for a distance of 5 cm at the lower end, by inserting at the point of constriction a coarse-porosity, sintered-glass disk or a small plug of glass wool. The constricted portion may be fitted with an inert, plastic stopcock.

Second Column— Select a tube that is made up of three sections: (1) a flared top section, 18 mm in (inside) diameter and approximately 14 cm long, (2) a middle section, 6 mm in (inside) diameter and approximately 25 cm long, and (3) a tapered, constricted lower exit tube approximately 5 cm long. Insert a small plug of glass wool in the upper 1-cm portion of the constricted section.

Chromatographic Columns—

First Column— To about 125 mL of isooctane contained in a screw-capped, wide-mouth bottle add 25 g of chromatographic siliceous earth, and shake until a slurry is formed. Add, dropwise and with vigorous mixing, 10 mL of polyethylene glycol 600. Replace the bottle cover, and shake vigorously for 2 minutes. Pour about half of the resulting slurry into the chromatographic tube, and allow it to settle by gravity. Then apply gentle suction, and add the remainder of the slurry in small portions, packing each portion with a 20-mm disk plunger. When a solid surface has formed, remove the vacuum, and add about 2 mL of isooctane.

Second Column— Pack the midsection of the tube with 3 g of moderately coarse *Chromatographic Fuller's Earth* with the aid of gentle suction (about 125 mm of mercury).

Standard Preparation— Dissolve about 25 mg of Reference Standard, accurately weighed, in isooctane to give a known concentration of about 250 µg per mL. Store in a refrigerator.

On the day of assay, pipet 1 mL of this solution into a 50-mL volumetric flask, remove the solvent with a stream of nitrogen, and dissolve the residue in and dilute with *Ethylene Dichloride* to volume, and mix.

Sample Preparation— Accurately weigh or measure a portion of the sample to be assayed, equivalent to not less than 125 µg but preferably about 250 µg of ergocalciferol (10,000 USP Units). If little or no vitamin A is present in the sample, add about 1.5 mg (the equivalent of 3000 USP Units) of vitamin A acetate to provide the needed pilot bands in the subsequent chromatography.

For capsules or tablets, reflux not fewer than 10 of them in 10 mL of water on a steam bath for about 10 minutes, crush the remaining solid with a blunt glass rod, and warm for 5 minutes longer.

Add a volume of *Potassium Hydroxide Solution* representing 2.5 mL for each g of the total weight of the sample, but not less than a total of 3.0 mL. Add 10 mL of *Butylated Hydroxytoluene Solution* and 20 mL of alcohol. Reflux vigorously on a steam bath for 30 minutes. Cool, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with three 10-mL portions of water and three 50-mL portions of *Ether*, adding each rinse to the separator. Add about 4 g of sodium sulfate decahydrate to the separator, and extract by shaking for 2 minutes. If an emulsion forms, extract with three 25-mL portions of *Ether.* Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with additional 50-mL portions of water until the last portion shows no pink color on the addition of phenolphthalein TS. Transfer the washed ether extract to a 250-mL volumetric flask, dilute with *Ether* to volume, and mix. Transfer the entire sample or an accurately measured aliquot containing about 250 µg to a tall-form, 400-mL beaker containing about 5 g of anhydrous sodium sulfate. Stir for 2 minutes, then decant the solution into a second 400-mL beaker. Rinse the sodium sulfate with three 25-mL portions of *Ether*, adding each rinse to the main portion. Reduce the total volume to about 30 mL by evaporation on a steam bath, and transfer the concentrate to a small, round-bottom evaporation flask. Rinse the beaker with three 10-mL portions of *Ether*, adding the rinsings to the flask. With the aid of vacuum in a water bath at a temperature not exceeding 40°, or with a stream of nitrogen at room temperature, remove the remaining solvent completely. Dissolve the residue in a small amount of *Solvent Hexane*, transfer to a 10-mL volumetric flask, dilute with *Solvent Hexane* to volume, and mix to obtain the *Sample Preparation.*

Procedure—

First Column Chromatography— Just as the 2 mL of isooctane disappears into the surface of the prepared *First Column*, pipet 2 mL of the *Sample Preparation* onto the column. As the meniscus of the *Sample Preparation* reaches the column surface, add the first of three 2-mL portions of *Solvent Hexane*, adding each succeeding portion as the preceding portion disappears into the column. Continue adding *Solvent Hexane* in portions of 5 to 10 mL until 100 mL has been added. If necessary, adjust the flow rate to between 3 and 6 mL per minute, by application of gentle pressure at the top of the chromatographic tube.

Discard the first 20 mL of effluent, and collect the remainder. Examine the column under UV light at intervals during the chromatography, and stop the flow when the front of the fluorescent band representing vitamin A is about 5 mm from the bottom of the column. (The UV lamp should provide *weak* radiation in the 300-nm region. It is frequently necessary to use a narrow aperture or screen with commercial lamps to reduce the amount of radiation to the minimum required to visualize the vitamin A on the column.)

Transfer the eluate to a suitable evaporation flask, and remove the *Solvent Hexane* completely under vacuum at a temperature not higher than 40° or with a stream of nitrogen at room temperature. Dissolve the residue in about 10 mL of *Solvent Hexane.*

Second Column Chromatography— Add the solvent hexane solution obtained as directed under *First Column Chromatography* onto the *Second Column.* Rinse the evaporation flask with a total of 10 mL of *Solvent Hexane* in small portions, adding each portion to the *Second Column* and allowing it to flow through the column, and discard the effluent. When about 1 mL of the hexane remains above the surface of the column, add 75 mL of toluene, and elute with the aid of gentle suction (about 125 mm of mercury), collecting the eluate. Evaporate the toluene under vacuum at a temperature not higher than 40°, or with a stream of nitrogen at room temperature.

Assay Preparation— Dissolve the residue obtained as directed under *Second Column Chromatography* in a small amount of *Ethylene Dichloride*, transfer to a 10-mL volumetric flask, dilute with *Ethylene Dichloride* to volume, and mix to obtain the *Assay Preparation*.

Color Development— Into each of three suitable, matched colorimeter tubes of about 20-mm (inside) diameter, and designated tubes 1, 2, and 3, respectively, pipet 1 mL of the *Assay Preparation*. Into tube 1, pipet 1 mL of the *Standard Preparation;* into tube 2, 1 mL of *Ethylene Dichloride;* and into tube 3, 1 mL of a mixture of equal volumes of acetic anhydride and *Ethylene Dichloride.* To each tube add quickly, and preferably from an automatic pipet, 5.0 mL of *Color Reagent*, and mix. After 45 seconds, accurately timed, following the addition of the *Color Reagent*, determine the absorbances of the three solutions at 500 nm, with a suitable spectrophotometer, using *Ethylene Dichloride* as the blank. Similarly, 45 seconds after making the first reading on each solution, determine the absorbances of the solutions in tubes 2 and 3 at 550 nm, in a similar manner. Designate the absorbances as A^1_{500}, A^2_{500}, A^3_{500}, A^2_{550}, and A^3_{550}, respectively, in which the superscript indicates the number of the tube and the subscript, the wavelength.

Calculation— Calculate the quantity, in µg, of vitamin D in the portion of the sample taken by the formula:

$$(C_S / C)(A_U / A_S),$$

in which C_S is the concentration of vitamin D, in µg per mL, of the *Standard Preparation; C* is the concentration of the sample (as g, capsules, tablets, etc.) in each mL of the final solution; A_U has the value of $(A^2_{500} - A^3_{500}) - 0.67(A^2_{550} - A^3_{550})$ determined from the absorbances observed on the solution from the *Assay Preparation;* and A_S has the value of $A^1_{500} - A^2_{500}$ determined on the solutions from the *Standard Preparation*.

Biological Method

The biological assay of vitamin D comprises the recording and interpretation of observations on groups of rats maintained on specified dietary regimens throughout specified periods of their lives whereby the biological response to the preparation under assay is compared with the response to USP Vitamin D Capsules RS.

USP Reference Standards 〈 11 〉 — USP Cholecalciferol RS.

Preliminary Period— Throughout the preliminary period in the life of a rat, which is not longer than 30 days and extends from birth to the first day of the depletion period, maintain litters of rats under the immediate supervision of, or according to the directions of, the individual responsible for the assay. During the preliminary period, use a dietary regimen that provides for normal development but is limited in its content of vitamin D, so that when placed upon the *Rachitogenic Diet* in the depletion period the rats develop rickets. At the end of the preliminary period, reject any rat that weighs less than 44 g or more than 60 g, or that shows evidence of injury, disease, or anatomical abnormality.

Depletion Period— Through the depletion period, which extends from the end of the preliminary period to the first day of the assay period, provide each rat ad libitum with the *Rachitogenic Diet* and water, and allow access to no other food or dietary supplement.

Rachitogenic Diet— The *Rachitogenic Diet* consists of a uniform mixture of the following ingredients in the proportions shown in the accompanying table.

Rachitogenic Diet

Ingredient	Parts by weight
Whole yellow corn, ground	76
Wheat gluten, ground	20
Calcium carbonate	3
Sodium chloride	1

When a chemical analysis of the entire ration shows a Ca:P ratio of less than 4:1 or more than 5:1, the proportion of calcium carbonate may be varied to bring the adjusted ratio to a uniform level within this range.

Assigning Rats to Groups for Assay Period— Consider a litter suitable for the assay period when individual rats in the litter show evidence of rickets such as enlarged joints and a distinctive wobbly, rachitic gait, provided that the depletion period is not less than 19 or more than 25 days. The presence of rickets may be established also from the width of the rachitic

metaphysis upon X-ray examination or by applying the *Line Test* (described below) to a leg bone of one member of each litter.

Record the weight of each rat, and assign it to a group, in which each rat will be fed a specified dose of the Reference Standard or of an assay sample that is under examination for its vitamin D potency. For each assay sample provide one or more assay groups and not less than two standard groups. The two standard groups may be used for the concurrent assay of more than one assay sample. Within an interval not exceeding 30 days, complete the assignment of rats to groups according to a design that divides litters among the groups, to achieve a complete balance.

For complete balance, whereby each litter is represented equally in every group, use 7 or more litters containing at least as many depleted rats as there are groups. From a given litter, assign one rat, selected at random, to each group on the same day. If a litter contains twice as many rats as there are groups, assign a second series of rats similarly. The last one or two litters to be assigned may be allotted to groups so that at the start of the assay period the average body weight of any completed groups will not differ by more than 8 g from that of any other group.

Assay Doses— Select two dosage levels of the *USP Cholecalciferol RS*, spaced so that the ratio of the larger to the smaller dose is not less than 1.5 or more than 2.5. Select one or two dosage levels based upon a single assumed potency for each sample. The dosage levels of the sample are equivalent to those of the standard or to a mid-level equal to the square root of the product of the two dosage levels of the standard.

Select dosage levels such that, when fed to rachitic rats, they are expected to produce degrees of calcification within the range specified under the test of data acceptability. Before feeding, the Reference Standard and/or sample may be diluted with cottonseed oil, provided that not more than 0.2 mL is fed on any one day. Store the oil solutions in well-closed bottles, protected from light, at a temperature not exceeding 10°, and use within 5 weeks.

Assign one group of rats to each dosage level of the standard and of the one or more samples.

Assay Period— During the assay period, which extends from the end of the depletion period for a fixed interval of 7 to 10 days, cage each rat individually and provide it ad libitum with the *Rachitogenic Diet* and water. Supply a *Rachitogenic Diet* prepared from the same lots of ingredients to all rats. On the first and on the third (or fourth) day of the assay period, feed each rat one-half of its total assigned dose.

Throughout the assay period, maintain as uniform environmental conditions as possible for all rats, and exclude exposure to antirachitic radiations. At the end of a fixed period of 7 to 10 days, weigh and kill each rat. From those rats that do not weigh less at the end than at the start of the assay period and that have consumed each assigned dose within 24 hours of the time it was fed, dissect out one or more leg bones for examination by the *Line Test.*

Line Test— Remove the proximal end of a tibia or the distal end of a radius, and clean adhering tissue from it, in any one assay using the same bone from all animals. With a clean, sharp blade cut a median, longitudinal section through the juncture of the epiphysis and diaphysis at the same place on each bone. Rinse both sections in purified water, immerse immediately in silver nitrate solution (1 in 50) for 1 minute, and rinse again in purified water. Expose the cut surface of bone, in water, to daylight or another source of actinic light until the calcified areas develop a clearly defined stain without marked discoloration of the uncalcified areas. The staining procedure may be modified to differentiate more clearly between calcified and uncalcified areas.

Score the degree of calcification of the rachitic metaphysis in each rat, according to a scale that allows the average response to be plotted as a straight line against the logarithm of the dose.

Acceptability— Observations are acceptable for use in calculation of the potency only from those groups in which two-thirds or more but not less than 7 rats show calcification at least as great as the lowest level and not greater than the highest level. If the average score of the standard group on the high dosage level is not greater than the average score of the standard group on the low dosage level, discard the results, and repeat the assay. If an assay sample is represented solely by assay groups that are not acceptable for measuring vitamin D potency and in each of which the average score is less than the average score of the standard group on the low dosage level or more than the average score of the standard group on the high dosage level, its assayed content of vitamin D is respectively less than that represented by the low dose or more than that represented by the high dose of the Reference Standard.

Calculation— Tabulate the scores (y), listing each litter in a separate row with treatment groups in columns. Omit any groups that do not meet the test for *Acceptability.* Equalize the number of observations in the acceptable groups by disregarding the results on all litters not equally represented in the groups or by other suitable means (see *Design and Analysis of Biological Assays* 〈111〉). Total the f scores for each of the treatment groups, where f is the number of litters, and designate each

total as T with subscripts 1 and 2 for the low and high dosage levels, respectively. Compute the slope b from the sums of T_1, i.e., ΣT_1, and of T_2, i.e., ΣT_2, for the standard and sample, provided the latter is represented at both dosage levels, from the equation:

$$b = (\Sigma T_2 - \Sigma T_1) / ifh',$$

in which i is the logarithm of the ratio of the high dose to the low dose and is the same for each preparation, and h' is the number of preparations represented by two dosage levels and included in the calculation of the value of b.

Compute the logarithm of the relative potency of each specimen under assay from the equation:

$$\log \text{(relative potency)} = M' = (\bar{y}_U - \bar{y}_S)/b = ih'T_a/2\Sigma T_b,$$

in which each mean score, $bar(y)_U$ for the assay sample and $bar(y)_S$ for the Reference Standard, is the average of the individual scores for an intermediate dosage level or of the two means for the high and the low dosage levels and where $T_b = \Sigma T_2 - \Sigma T_1$ and T_a is as defined (see *Design and Analysis of Biological Assays* 〈111〉). Convert each observed M' to its antilogarithm to obtain the relative potency of the sample. Multiply the relative potency by the assumed potency of the assay oil in Units per g, adopted at the start of the assay, to obtain its assayed content of vitamin D in USP Units per g.

Auxiliary Information— *Staff Liaison* : Lawrence Evans, III, Ph.D., Scientist
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