⟨581⟩ VITAMIN D ASSAY

ASSAY

Change to read:

Chromatographic Methods

The following liquid chromatographic procedures are provided for the determination of vitamin D as an active pharmaceutical ingredient, as a dietary supplement ingredient, or as a component in compendial dosage forms.

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.

Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard.

• **PROCEDURE 1**

This procedure uses a sample preparation without pH adjustment and involves the use of dimethyl sulfoxide to dissolve the excipients in the sample, followed by a liquid-liquid extraction of the vitamin D with hexane. The chromatographic separation is achieved using normal phase on an L8 column. It can be used to determine vitamin D alone or in combination with other vitamins and minerals in compendial dosage forms.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and *System suitability solution* are prepared as follows.

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution for tablets: Finely powder NLT 20 Tablets. Transfer a portion of the powder, not exceeding 7.5 g, equivalent to NLT 0.1 mg of vitamin D as cholecalciferol or ergocalciferol, to a centrifuge tube having a polytef-lined screw cap. Add about 2 mL of dimethyl sulfoxide and about 3 mL of *n*-hexane per each g of powdered Tablets, and shake for 45 min on a shaker in a water bath maintained at 60°. [NOTE—Set up the shaker to ensure that the contents of the container are mixed vigorously and thoroughly in order to achieve accurate recoveries.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a volumetric flask. Add 3 mL of *n*-hexane per each g of powdered Tablets to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the same volumetric flask. Repeat this extraction with three additional portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution with a concentration of 2 µg/mL of cholecalciferol or ergocalciferol. [NOTE—Dilution may not be necessary.]

Sample solution for capsules: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, not exceeding 7.5 g equivalent to NLT 0.1 mg of vitamin D as cholecalciferol or ergocalciferol, to a centrifuge tube having a polytef-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to NLT 0.1 mg of vitamin D as cholecalciferol or ergocalciferol, to a centrifuge tube having a polytef-lined screw cap.] Add about 2 mL of dimethyl sulfoxide and about 3 mL of *n*-hexane per each g of Capsule contents, and shake for 45 min on a shaker in a water bath maintained at 60°. [NOTE—Set up the shaker to ensure that the contents of the container are mixed vigorously and thoroughly in order to achieve accurate recoveries.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a volumetric flask. Add 3 mL of *n*-hexane per each g of Capsule contents to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the same volumetric flask. Repeat this extraction with three additional portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution with a concentration of 2 µg/mL of vitamin D as cholecalciferol or ergocalciferol. [NOTE—Dilution may not be necessary.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak response of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)
F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

- **PROCEDURE 2**

This procedure involves the treatment of sample with sodium bicarbonate, an antioxidant acid solution generating gas evolution; lecithin as a surfactant; and dimethyl sulfoxide, followed by methanolic sulfuric acid to create a dispersion and efficiently extract vitamin D from the matrix components into the 2,2,4-trimethylpentane phase. Separation is achieved in normal-phase mode on an L24 column. The sample preparation and *Chromatographic system* used in this procedure also are suitable to determine vitamins A and E when present in the formulation; analysts should make corresponding adjustments in sample size and detection wavelength.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, *System suitability solution*, and reagent solutions are prepared as follows.

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75: 1.25)

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution for tablets: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation. The sample amount may be adjusted depending on the presence or absence of the appropriate vitamins.] Finely powder NLT 20 Tablets. Use a portion of the powder nominally equivalent to an amount NLT 0.1 mg of vitamin D. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Sample solution for capsules: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation. The sample amount may be adjusted depending on the presence or absence of the appropriate vitamins.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to NLT 0.1 mg of the labeled amount of vitamin D, as cholecalciferol or ergocalciferol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak response of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

- **PROCEDURE 3**

This procedure is suitable for matrices with excipients that are soluble or degradable in alkaline conditions. It involves the saponification of the sample solution, followed by a liquid–liquid extraction with hexane and clean-up on a solid phase extraction (SPE) using a mixture of methylene chloride and isopropyl alcohol (99.8: 0.2) as eluent. The *Standard solution* undergoes similar treatment to compensate for losses in recovery due to isomerization. The eluate then is evaporated, and the residue is reconstituted in acetonitrile before injection into the chromatograph. Separation is achieved in reversed phase mode.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

Mobile phase: Acetonitrile and methanol (91:9)

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8: 0.2)

Extraction column: Silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent. [NOTE—Condition the column by initially washing with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of the *Extraction solvent*. Do not allow the column to dry.]

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: Dilute a volume of the *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain, and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain, and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of the *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase *Extraction column*, rinse the round-bottom flask with 1.0 mL of the *Extraction solvent*, and transfer to the column. Elute the *Extraction column* with 2.0 mL of the *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of the *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution for tablets: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 µg of cholecalciferol or ergocalciferol, to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain, and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of the *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase *Extraction column*, rinse the round-bottom flask with 1.0 mL of the *Extraction solvent*, and transfer to the column. Elute the *Extraction column* with 2.0 mL of the *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of the *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution for capsules: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 10 µg of ergocalciferol or cholecalciferol, to a

stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain, and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain, and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of the *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase *Extraction column*, rinse the round-bottom flask with 1.0 mL of the *Extraction solvent*, and transfer to the column. Elute the *Extraction column* with 2.0 mL of the *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of the *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Chromatographic system

(See *Chromatography* ⟨621⟩*, System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection volume: 15 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak response of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

• **PROCEDURE 4**

This procedure is applicable to liquid dosage forms. It involves a liquid-liquid extraction of the sample with hexane, followed by the evaporation of hexane, and reconstitution of the residue in a tetrahydrofuran and acetonitrile mixture (1:1). Separation is achieved in reversed-phase mode.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and *Diluent* are prepared as follows.

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)

Diluent: Tetrahydrofuran and acetonitrile (1:1)

Standard solution: 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Diluent*

Sample solution: Transfer an accurately measured volume of the sample, equivalent to 50 µg of cholecalciferol or ergocalciferol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer. Drain the solvent hexane extract through anhydrous sodium sulfate into a 500-mL, round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩*, System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: Two columns, 4.6-mm × 25-cm, connected in series; both with packing L1

Column temperature: 40°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak response of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)
F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

- **PROCEDURE 5**

This procedure involves the dissolution of vitamin D in toluene, and the solution is injected into the liquid chromatograph. It is applicable to simple matrices such as pure vitamins and ingredients that do not require saponification and that are soluble in toluene. Separation is achieved in normal-phase mode.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and *System suitability solution* are prepared as follows.

Dehydrated hexane: Prepare a chromatographic column by packing a chromatographic tube, 8-cm × 60-cm, with 500 g of 50- to 250-µm chromatographic siliceous earth, activated by drying at 150° for 4 h. (See *Chromatography* ⟨621⟩, *Column Chromatography*.) Pass 500 mL of hexane through the column, and collect the eluate in a glass-stoppered flask.

Mobile phase: *n*-Amyl alcohol in *Dehydrated hexane* (3 in 1000)

System suitability solution: 250 mg of USP Vitamin D Assay System Suitability RS in 10 mL of a mixture of toluene and *Mobile phase* (1:1). Heat this solution, under reflux, at 90° for 45 min, and cool. [NOTE—This solution contains cholecalciferol, precholecalciferol, and *trans*-cholecalciferol. For the stock solutions, follow these procedures: use low-actinic glassware, dissolve the samples without heating, and prepare the solutions fresh daily.]

Standard stock solution: 0.6 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in toluene

Standard solution: 120 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Mobile phase*, prepared from *Standard stock solution*

Sample stock solution: 0.6 mg/mL of cholecalciferol or ergocalciferol in toluene

Sample solution: 120 µg/mL of cholecalciferol or ergocalciferol in *Mobile phase*, prepared from the *Sample stock solution*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L3

Column temperature: 40°

Flow rate: Can be varied to meet the *System suitability requirements*

Injection volume: 5–10 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for precholecalciferol, *trans*-cholecalciferol, and cholecalciferol are 0.4, 0.5, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between *trans*-cholecalciferol and precholecalciferol

Relative standard deviation: NMT 2.0% for the peak response of cholecalciferol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak response of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

- **PROCEDURE 6**

The procedure involves the dissolution of the sample in *Mobile phase*, and the solution is injected into the liquid chromatograph. The application includes cholecalciferol and ergocalciferol dissolved in edible vegetable oil, polysorbate 80, or propylene glycol, none of which will interfere with the corresponding precursor so that it can be quantitated. Separation is achieved in normal-phase mode on an L3 column, and *System suitability* requires separation of the *trans* forms from the vitamin D precursors.

Unless specified in the individual monographs, the *Standard solutions* and *Sample solution* are prepared as follows.

Mobile phase: Hexane and pentanol (997:3)

Standard stock solution: Dissolve USP Cholecalciferol RS or USP Ergocalciferol RS in toluene, and dilute with *Mobile phase* to 50 µg/mL. [NOTE—Prepare this solution fresh daily.]

Standard solution A: 5 µg/mL from the *Standard stock solution* in *Mobile phase*. [NOTE—Store at a temperature not above 0°.]

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* to a round-bottom flask fitted with a reflux condenser. Displace the air with nitrogen, and reflux for 1 h in a water bath under a nitrogen atmosphere to obtain a solution containing cholecalciferol and precholecalciferol. Cool, transfer the solution with the aid of several portions of *Mobile phase* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.

Sample solution: Equivalent to 5 µg/mL of cholecalciferol or ergocalciferol in *Mobile phase* from an accurately measured volume of the sample

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L3

Flow rate: 1–2 mL/min

Injection volume: 10–20 µL

System suitability

Sample: *Standard solution B* (USP Cholecalciferol RS or USP Ergocalciferol RS)

[NOTE—The relative retention times for precholecalciferol and cholecalciferol are about 0.4 and 1.0, respectively. Those for pre-ergocalciferol and ergocalciferol are about 0.8 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the precholecalciferol peak and the cholecalciferol peak; NLT 1.0 between the pre-ergocalciferol peak and the ergocalciferol peak

Relative standard deviation: NMT 2.0% for the peak response of cholecalciferol or ergocalciferol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Cholecalciferol or ergocalciferol response factor

Calculate the cholecalciferol or ergocalciferol response factor, F_C:

$$F_C = C_S/r_S$$

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in *Standard solution A* (µg/mL)
r_S = peak area of cholecalciferol or ergocalciferol from *Standard solution A*

Precholecalciferol or pre-ergocalciferol response factor

Calculate the concentration, C'_S, in µg/mL, of cholecalciferol or ergocalciferol in *Standard solution B*:

$$C'_S = F_C \times r'_S$$

F_C = response factor for cholecalciferol or ergocalciferol
r'_S = peak area of cholecalciferol or ergocalciferol from *Standard solution B*

Calculate the concentration, C'_{pre}, in µg/mL, of precholecalciferol or pre-ergocalciferol:

$$C'_{pre} = C_S - C'_S$$

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in *Standard solution A* (µg/mL)
C'_S = concentration of cholecalciferol or ergocalciferol in *Standard solution B* (µg/mL)

Calculate the response factor, F_{pre}, for precholecalciferol or pre-ergocalciferol:

$$F_{pre} = C'_{pre}/r_p$$

C'_{pre} = concentration of precholecalciferol or pre-ergocalciferol (µg/mL)
r_p = peak response of precholecalciferol or pre-ergocalciferol from *Standard solution B*

Content of vitamin D

Calculate the percentage of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or as ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = \{[(F_C \times r_C) + (F_{pre} \times r_{pre})]/C_U\} \times 100$$

F_C = response factor for cholecalciferol or ergocalciferol
r_C = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
F_{pre} = response factor for precholecalciferol or pre-ergocalciferol
r_{pre} = peak area of precholecalciferol or pre-ergocalciferol from the *Sample solution*
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

- **PROCEDURE *7***

This procedure involves the saponification of the sample, followed by a liquid–liquid extraction with ether–solvent hexane, and by the evaporation of ether–hexane and reconstitution of the residue in a mixture of toluene and *Mobile phase* (1:4). It is applicable to solutions in oil and capsules containing vitamin D solutions in oil, in which the oil does not interfere with the corresponding precursor so that it can be quantitated. Separation is achieved in normal-phase mode on an L3 column, and *System suitability* requires separation of the *trans* forms from the vitamin D precursors.

Unless specified in the individual monographs, the *Standard solutions*, *Sample solution*, and reagent solutions are prepared as follows.

Dehydrated hexane: Prepare a chromatographic column by packing a chromatographic tube, 8-cm × 60-cm, with 500 g of 50- to 250-µm chromatographic siliceous earth, activated by drying at 150° for 4 h. (See *Chromatography* ⟨621⟩, *Column Chromatography*.) Pass 500 mL of hexane through the column, and collect the eluate in a glass-stoppered flask.

Mobile phase: *n*-Amyl alcohol in *Dehydrated hexane* (3 in 1000)

Butylated hydroxytoluene solution: 10 mg/mL of butylated hydroxytoluene in chromatographic hexane

Aqueous potassium hydroxide solution: 1 g/mL of potassium hydroxide in freshly boiled water. [NOTE—Prepare this solution fresh daily.]

Alcoholic potassium hydroxide solution: 3 g of potassium hydroxide in 50 mL of freshly boiled water. Add 10 mL of alcohol, and dilute with freshly boiled water to 100 mL. [NOTE—Prepare this solution fresh daily.]

Sodium ascorbate solution: 175 mg/mL of ascorbic acid in 1 N sodium hydroxide. [NOTE—Prepare fresh daily.]

Sodium sulfide solution: 12 g of sodium sulfide in 20 mL of water. Dilute with glycerin to 100 mL.

Standard stock solution: 0.5 mg/mL of USP Ergocalciferol RS or USP Cholecalciferol RS in toluene. [NOTE—Prepare this solution fresh daily.]

Standard solution A: 20 µg/mL from the *Standard stock solution* in *Mobile phase*. [NOTE—Store this solution at a temperature not above 0°.]

Standard solution B: Pipet 4 mL of the *Standard stock solution* into a round-bottomed 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 min to obtain a solution containing vitamin D and previtamin D. Cool, transfer with the aid of several portions of *Mobile phase* to a 100-mL volumetric flask, and dilute with *Mobile phase* to volume.

System suitability solution: Add 100 mg of USP Vitamin D Assay System Suitability RS to a 10-mL volumetric flask. Add a mixture (1 in 5) of toluene and *Mobile phase* to volume, and mix. Heat a portion of this solution under reflux, at 90° for 45 min, and cool.

Sample solution: Reflux NLT 10 Capsules with a mixture of 10 mL of *Sodium ascorbate solution* and 2 drops of *Sodium sulfide solution* on a steam bath for 10 min, crush any remaining solids with a blunt glass rod, and continue heating for 5 min. Cool, and add 25 mL of alcohol and 3 mL of *Aqueous potassium hydroxide solution*. Reflux the mixture on a steam bath for 30 min. 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. [NOTE—Use ether within 24 h after opening the container.] Shake the combined saponified mixture and rinsings vigorously for 30 s, 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 solvent hexane phase to the first separator, rinsing the second separator with two 10-mL portions of solvent hexane and adding the rinsings to the first separator. Shake the aqueous phase in the third separator with 50 mL of solvent hexane, and add the solvent hexane phase to the first separator. Wash the combined ether–solvent 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–solvent hexane extracts, add 2 sheets of 9-cm filter paper, in strips, to the separator, and shake. Transfer the washed ether–solvent hexane extracts to a round-bottom flask, rinsing the separator and paper with solvent hexane. Combine the solvent hexane rinsings with the ether–solvent hexane extracts, add 100 µL of *Butylated hydroxytoluene solution*, and mix. Evaporate under 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 and dilute the residue in an accurately measured volume of a mixture (1 in 5) of toluene and *Mobile phase*, until the concentration of vitamin D is about 25 µg/mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L3

Flow rate: 1–2 mL/min

Injection volume: 10–20 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for precholecalciferol, *trans*-cholecalciferol, and cholecalciferol are 0.4, 0.5, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between *trans*-cholecalciferol and precholecalciferol

Relative standard deviation: NMT 2.0% for the peak response of cholecalciferol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Cholecalciferol and ergocalciferol response factor

Calculate the cholecalciferol or ergocalciferol response factor, F_D:

$$F_D = C_S/r_S$$

C_S = concentration of USP Ergocalciferol RS in *Standard solution A* (µg/mL)
r_S = peak area of cholecalciferol or ergocalciferol from *Standard solution A*

Precholecalciferol and pre-ergocalciferol response factor

Calculate the concentration, C'_S, in µg/mL, of cholecalciferol or ergocalciferol in *Standard solution B*:

$$C'_S = F_D \times r'_S$$

F_D = response factor for cholecalciferol or ergocalciferol
r'_S = peak area of cholecalciferol or ergocalciferol from *Standard solution B*

Calculate the concentration, C'_{pre}, in µg/mL, of ▲pre-ergocalciferol:▲ (ERR 1-Sep-2020)

$$C'_{pre} = C_S - C'_S$$

C_S = concentration of USP Ergocalciferol RS in *Standard solution A* (µg/mL)
C'_S = concentration of cholecalciferol or ergocalciferol in *Standard solution B* (µg/mL)

Calculate the response factor, F_{pre}, for precholecalciferol or pre-ergocalciferol:

$$F_{pre} = C'_{pre}/r_p$$

C'_{pre} = concentration of precholecalciferol or pre-ergocalciferol (µg/mL)
r_p = peak response of precholecalciferol or pre-ergocalciferol from *Standard solution B*

Content of vitamin D

Calculate the percentage of the labeled amount of vitamin D as ergocalciferol ($C_{28}H_{44}O$) or as cholecalciferol ($C_{27}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = \{[(F_D \times r_C) + (F_{pre} \times r'_{pre})]/C_U\} \times 100$$

F_D = response factor for cholecalciferol or ergocalciferol
r_C = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
F_{pre} = response factor for precholecalciferol or pre-ergocalciferol
r'_{pre} = peak area of precholecalciferol or pre-ergocalciferol from the *Sample solution*
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

• **PROCEDURE 8**

This procedure is suitable for the determination of cholecalciferol in oils or fatty matrices. It involves two chromatographic systems, one for sample clean-up and the other for vitamin D determination. The standard, internal standard, and sample solutions are subjected to saponification, followed by a liquid–liquid extraction with a mixture of ether and hexane (1:1). The extract is evaporated and reconstituted in the *Butylated hydroxytoluene solution*.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, *Internal standard solution*, and reagent solutions are prepared as follows.

Solution A: *n*-Amyl alcohol and dehydrated hexane (3:997)

Solution B: Acetonitrile, water, and phosphoric acid (96: 3.8: 0.2)

Butylated hydroxytoluene solution: 10 mg/mL of butylated hydroxytoluene in chromatographic hexane

Aqueous potassium hydroxide solution: Dissolve 800 g of potassium hydroxide in 1000 mL of freshly boiled water, mix, and cool. [NOTE—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, and dilute with freshly boiled water to 100 mL. [NOTE—Prepare this solution fresh daily.]

Ascorbic acid solution: 100 mg/mL of ascorbic acid in water. [NOTE—Prepare this solution fresh daily.]

Internal standard solution: 5 µg/mL of USP Ergocalciferol RS in alcohol

Standard stock solution: 5 µg/mL of USP Cholecalciferol RS in alcohol

Standard solution: Transfer 2.0 mL of the *Standard stock solution* and 2.0 mL of the *Internal standard solution* to a round-bottom flask. Proceed as directed for *Sample solution 1* beginning with "Add 5 mL of…".

Sample solution 1: Transfer 4.00 g of oil to a round-bottom flask. Add 5 mL of the *Ascorbic acid solution*, 100 mL of alcohol, and 10 mL of the *Aqueous potassium hydroxide solution*, and mix. Reflux the mixture on a steam bath for 30 min. Add 100 mL of a 10-mg/mL sodium chloride solution. Cool rapidly under running water, and transfer the saponified mixture to a 500-mL separator, rinsing the saponification flask with 75 mL of a 10-mg/mL sodium chloride solution, and then with 150 mL of a mixture of ether and hexane (1:1). Shake the combined saponified mixture and rinsings vigorously for 30 s, and allow to stand until both layers are clear. Discard the lower layer. Wash the ether–hexane extracts by shaking vigorously with 50 mL of the *Alcoholic potassium hydroxide solution*, and then washing with three 50-mL portions of a 10-mg/mL sodium chloride solution. Filter the upper layer through 5 g of anhydrous sodium sulfate

on a fast filter paper into a 250-mL flask suitable for a rotary evaporator. Wash the filter with 10 mL of a mixture of ether and hexane (1:1), and combine with the extract. Evaporate the solvent at reduced pressure at a temperature not exceeding 30°, and fill with nitrogen when the evaporation is complete. Alternatively evaporate the solvent under a gentle stream of nitrogen at a temperature not exceeding 30°. Dissolve the residue in 1.5 mL of the *Butylated hydroxytoluene solution*. [NOTE—Gentle heating in an ultrasonic bath may be required. A large fraction of the white residue is cholesterol.]

Sample solution 2: To 4.00 g of oil add 2.0 mL of *Internal standard solution*, and proceed as directed for *Sample solution 1* beginning with "Add 5 mL of…".

Clean-up chromatographic system

(See *Chromatography* ⟨621⟩*, System Suitability*.)

Mode: LC

Detector: UV 265 nm

Mobile phase: *Solution A*

Clean-up column: 4.6-mm × 25-cm stainless steel; packing L10

Flow rate: 1.1 mL/min

Injection volume: 350 µL

Analysis (clean-up)

Samples: *Standard solution*, *Sample solution 1*, and *Sample solution 2*

Collect separately the eluates from 2 min before to 2 min after the retention time of cholecalciferol in a glass tube containing 1 mL of *Butylated hydroxytoluene solution* and fitted with a hermetic closure. Evaporate each tube under a stream of nitrogen at a temperature not exceeding 30°. Dissolve each residue in 1.5 mL of acetonitrile, and inject into the analytical chromatographic system below.

Analytical chromatographic system

(See *Chromatography* ⟨621⟩*, System Suitability*.)

Mode: LC

Detector: UV 265 nm

Mobile phase: *Solution B*

Analytical column: 4.6-mm × 15-cm stainless steel; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection volume: 200 µL

System suitability

Sample: *Standard solution* (after the clean-up)

Suitability requirements

Resolution: NLT 1.4 between cholecalciferol and ergocalciferol

Relative standard deviation: NMT 2.0% for the cholecalciferol peak from replicate injections

Analysis

Samples: *Standard solution*, *Sample solution 1*, and *Sample solution 2* (after the clean-up)

Calculate the content of vitamin D, in µg/g, in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U)$$

R_S = peak response of cholecalciferol relative to the internal standard in the *Standard solution*

C_S = concentration of USP Cholecalciferol RS in the *Standard solution* (µg/mL)

C_U = concentration of oil in *Sample solution 2* (g/mL)

R_U = peak response of cholecalciferol relative to the internal standard in the *Standard solution 2*, as calculated as follows:

[NOTE—If $r_{IS1} = 0$ due to no peak observed at the locus of the internal standard in the chromatogram of *Sample solution 1*, then $R_U = r_{U2}/r_{IS2}$.]

$$R_U = r_{U2}/[r_{IS2} - (r_{IS1} \times r_{U2}/r_{U1})]$$

r_{U2} = peak response of cholecalciferol from *Sample solution 2*

r_{IS2} = peak response of the internal standard from *Sample solution 2*

r_{IS1} = peak response of the internal standard from *Sample solution 1*

r_{U1} = peak response of cholecalciferol from the *Sample solution 1*

ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Cholecalciferol RS

USP Ergocalciferol RS

USP Vitamin D Assay System Suitability RS