phase to obtain a stock solution having a known concentration of about 1.25 mg per mL. Transfer 5.0 mL of this stock solution to a 50-mL volumetric flask containing 10.0 mL of *Internal standard solution*, dilute with *1% Acetic acid solution* to volume, and mix.

Resolution solution—Prepare a solution of *p*-chlorophenol in acetonitrile containing about 8.5 mg per mL. Transfer 1 mL of this solution to a 50-mL volumetric flask containing 4 mL of the stock solution used to prepare the *Standard preparation* and 10 mL of *Internal standard solution*, dilute with *1% Acetic acid solution* to volume, and mix.

Assay preparation—Weigh and finely powder not fewer than 20 Tablets. Transfer an accurately weighed portion of the powder, equivalent to about 125 mg of chlorzoxazone, to a 100-mL volumetric flask, add about 70 mL of acetonitrile, and shake by mechanical means for about 30 minutes. Dilute with acetonitrile to volume, and mix. Filter a portion of this solution, discarding the first 10 mL of the filtrate. Transfer 5.0 mL of the clear filtrate to a 50-mL volumetric flask containing 10.0 mL of *Internal standard solution*, dilute with *1% Acetic acid solution* to volume, and mix.

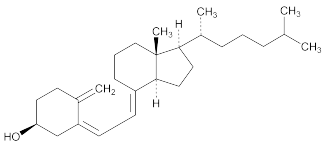
Chromatographic system (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 280-nm detector and a 4-mm × 30-cm column containing packing L1. The flow rate is about 1.5 mL per minute. Chromatograph the *Resolution solution*, and record the peak responses as directed under *Procedure*: the relative retention times are about 0.7 for phenacetin, 1.0 for chlorzoxazone, and 1.2 for *p*-chlorophenol; and the resolution, *R,* between the chlorzoxazone peak and the *p*-chlorophenol peak is not less than 2.0. Chromatograph the *Standard preparation*, and record the responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0%.

Procedure—Separately inject equal volumes (about 20 µL) of the *Standard preparation* and the *Assay preparation* into the chromatograph, record the chromatograms, and measure the area responses for the major peaks. Calculate the quantity, in mg, of $C_7H_4ClNO_2$ in the portion of Tablets taken by the formula:

$$1000C(R_U / R_S)$$

in which *C* is the concentration, in mg per mL, of USP Chlorzoxazone RS in the *Standard preparation*; and R_U and R_S are the peak response ratios of the chlorzoxazone peak to the phenacetin peak obtained from the *Assay preparation* and the *Standard preparation*, respectively.

Cholecalciferol



$C_{27}H_{44}O$ 384.64

9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3*β*,5*Z*,7*E*)-; Cholecalciferol [67-97-0].

DEFINITION

Cholecalciferol contains NLT 97.0% and NMT 103.0% of cholecalciferol ($C_{27}H_{44}O$).

IDENTIFICATION

- **A. Infrared Absorption** ⟨197K⟩
  Wavelength range: 2–12 µm
- **B. Ultraviolet Absorption** ⟨197U⟩
  Analytical wavelength: 265 nm
  Sample solution: 10 µg/mL in alcohol
  Acceptance criteria: Meets the requirements in the chapter. Absorptivities do not differ by more than 3.0%.
- **C.**
  Sample solution: 0.5 mg in 5 mL of chloroform
  Analysis: Add 0.3 mL of acetic anhydride and 0.1 mL of sulfuric acid to the *Sample solution*, and shake vigorously.
  Acceptance criteria: A bright red color is produced, and it rapidly changes through violet and blue to green.
- **D. Thin-Layer Chromatography**
  [Note—For the *Standard solution* and the *Sample solution*, follow these procedures: use low-actinic glassware, dissolve the samples without heating, and use the solutions immediately.]
  Diluent: 10 mg/mL of squalane in chloroform
  Standard solution: 50 mg/mL of USP Cholecalciferol RS in *Diluent*
  Sample solution: 50 mg/mL of Cholecalciferol in *Diluent*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Mode: TLC
  Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
  Application volume: 10 µL
  Developing solvent system: Cyclohexane and diethyl ether (1:1)
  Spray reagent: 20 mg/mL of acetyl chloride in antimony trichloride TS
  Analysis
  Samples: *Standard solution* and *Sample solution*
  [Note—Perform the development and subsequent operations in the dark.]
  Place the plate in a chamber containing and equilibrated with *Developing solvent system*. Develop until the solvent front has moved about 15 cm above the line of application. Remove the plate, allow the solvent to evaporate, and spray with *Spray reagent*.
  Acceptance criteria: The *Sample solution* shows a yellowish-orange area (cholecalciferol) having the same R_F value as the area of the *Standard solution* and may show below the cholecalciferol area a violet area, attributed to 7-dehydrocholesterol.

ASSAY

- **Procedure**
  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.]
  [Note—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 in toluene

Standard solution: 120 µg/mL of USP Cholecalciferol RS in *Mobile phase*, prepared from *Standard stock solution*
Sample stock solution: 0.6 mg/mL of Cholecalciferol in toluene
Sample solution: 120 µg/mL of Cholecalciferol in *Mobile phase*, prepared from *Sample stock solution*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L3
Injection size: 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 cholecalciferol ($C_{27}H_{44}O$) in the portion of Cholecalciferol taken:

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

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS in the *Standard solution* (µg/mL)
C_U = concentration of Cholecalciferol in the *Sample solution* (µg/mL)
Acceptance criteria: 97.0%–103.0%

SPECIFIC TESTS
• **OPTICAL ROTATION,** *Specific Rotation* ⟨**781S**⟩
Sample solution: 5 mg/mL in alcohol. [NOTE—Prepare and use the solution without delay. Use Cholecalciferol from a container opened not longer than 30 min.]
Acceptance criteria: +105° to +112°

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in hermetically sealed containers under nitrogen, and store in a cool place protected from light.
• **USP REFERENCE STANDARDS** ⟨**11**⟩
USP Cholecalciferol RS
USP Vitamin D Assay System Suitability RS

Cholecalciferol Capsules

DEFINITION
Cholecalciferol Capsules contain a solution of Cholecalciferol in an edible oil or other suitable vehicle. Cholecalciferol Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$).

IDENTIFICATION
• **A.** The retention time of the major peak for cholecalciferol of the *Sample solution* corresponds to that of *Standard solution A*, as obtained in the *Assay*.

ASSAY
• **PROCEDURE**
[NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)
System suitability solution: 250 mg of USP Vitamin D Assay System Suitability RS in 10 mL of *n*-hexane. Heat this solution under reflux, at 60° for 1 h, and cool. [NOTE—This solution contains cholecalciferol, precholecalciferol, and *trans*-cholecalciferol.]
Standard stock solution: 50 µg/mL of USP Cholecalciferol RS in *n*-hexane. [NOTE—Prepare this solution fresh daily.]
Standard solution A: 5 µg/mL of USP Cholecalciferol RS in *n*-hexane from the *Standard stock solution*
Standard solution B: Transfer a 5-mL volume of the *Standard stock solution* to a container having a polytef-lined screw cap. Displace the air with nitrogen and heat at 60° for 1 h under a nitrogen atmosphere, and cool. Quantitatively transfer the solution to a 50-mL volumetric flask, and dilute with *n*-hexane to volume.
Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. With a sharp blade or by other appropriate means, carefully open the Capsules, without loss of the shell material, and transfer as much as possible of the combined Capsule contents to a suitable container. Remove any adhering substance from the emptied Capsules and shell remains by washing with several small portions of *n*-hexane. Discard the washings, and allow the empty Capsules and shell remains to dry in a current of dry air until the odor of *n*-hexane is no longer perceptible. Weigh the empty Capsules and shell remains in the original tared weighing bottle, and calculate the average net weight per Capsule by difference. Dissolve a portion of the combined Capsule contents in *n*-hexane to prepare a cholecalciferol solution with a nominal concentration of 5 µg/mL.
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: 10 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for precholecalciferol, *trans*-cholecalciferol, and cholecalciferol are 0.5, 0.6, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1 between the precholecalciferol and *trans*-cholecalciferol peaks
Relative standard deviation: NMT 2.0% for the cholecalciferol peak
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Cholecalciferol response factor
Calculate the cholecalciferol response factor, F_C:

$$F_C = C_S/r_S$$

C_S = concentration of USP Cholecalciferol RS in *Standard solution A* (µg/mL)
r_S = peak area of cholecalciferol from *Standard solution A*
Precholecalciferol response factor
Calculate the concentration, C'_S, in µg/mL, of cholecalciferol in *Standard solution B*:

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

F_C = cholecalciferol response factor
r'_S = peak area of cholecalciferol from *Standard solution B*
Calculate the concentration, C'_{pre}, in µg/mL, of precholecalciferol in *Standard solution B*:

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