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Oil- and Water-Soluble Vitamins Oral Solution

DEFINITION

Oil- and Water-Soluble Vitamins Oral Solution contains one or more of the following oil-soluble vitamins: Vitamin A, vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), and Vitamin E; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Cyanocobalamin, Niacin or Niacinamide, Dexpanthenol or Panthenol, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin or Riboflavin-5'-Phosphate Sodium, and Thiamine Hydrochloride or Thiamine Mononitrate. It contains NLT 90.0% and NMT 250.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$), vitamin D as ergocalciferol ($C_{28}H_{44}O$) or cholecalciferol ($C_{27}H_{44}O$), vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) or alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$), ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 150.0% of the labeled amounts of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), dexpanthenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), and riboflavin ($C_{17}H_{20}N_4O_6$) or riboflavin-5'-phosphate sodium ($C_{17}H_{20}N_4NaO_9P$); and NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$).

STRENGTH

[Note—In the following assays, where more than one assay method is given for an individual ingredient the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method* 1 is not used.]

• **Vitamin A**

[Note—Use low-actinic glassware throughout this procedure.]

Diluent: Tetrahydrofuran and acetonitrile (1:1)

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

Standard solution: 0.33 mg/mL of retinol ($C_{20}H_{30}O$) from USP Vitamin A RS in *Diluent*. [Note—USP Vitamin A RS is retinyl acetate. Use it to analyze Oral Solution that contains vitamin A as retinol, retinyl acetate, or retinyl palmitate.]

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 3.3 mg of retinol, 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, and filter 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: 4.6-mm × 50-cm (prepared from two concatenated 4.6-mm × 25-cm columns); 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*

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Calculate the percentage of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$) in the portion of Oral Solution taken:

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

r_U = peak area of retinol or retinyl ester from the *Sample solution*

r_S = peak area of retinyl acetate from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A as retinol ($C_{20}H_{30}O$) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

• **Cholecalciferol or Ergocalciferol (Vitamin D)**

[Note—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. Use low-actinic glassware throughout this procedure.]

Diluent and **Mobile phase:** Prepare as directed in *Vitamin A*.

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

Sample solution: Transfer an accurately measured volume of Oral Solution, 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: Prepare as directed in *Vitamin A*.

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of vitamin D. 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 Oral Solution taken:

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

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

r_S = peak areas 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 formulation, 1.09

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **Vitamin E**

[Note—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile and ethyl acetate (1:1)

Potassium hydroxide solution: Transfer 90 g of potassium hydroxide pellets to a 100-mL volumetric flask containing 60 mL of water. Mix to dissolve, cool, and dilute with water to volume.

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

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in *Diluent*

Sample solution: Transfer an amount of Oral Solution, equivalent to 1.5 mg of alpha tocopherol, to a 125-mL conical flask fitted with a ground-glass joint, and add 25.0 mL of dehydrated alcohol. Attach a reflux condenser, and reflux in a boiling water bath for 5 min. Cautiously add 3 mL of the *Potassium hydroxide solution* through the condenser, and continue to reflux for 30 min. Remove the flask from

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the bath, and rinse the condenser with about 15 mL of water. Cool, and transfer with a minimum volume of water to a 250-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsings to the separatory funnel. Insert the stopper, shake vigorously for 1 min, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with *n*-hexane, and add the rinsing to the hexane solution in the flask. Evaporate the hexane solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 50°. Immediately add 5.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

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

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3.0 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*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Oral Solution taken:

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

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E, as alpha tocopherol, in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin E

• **Ascorbic Acid, Calcium Ascorbate,** and **Sodium Ascorbate**

(See *Vitamin C Assay ⟨580⟩*.)

[Note—For labeling purposes, consider *Method I—Titrimetric Method* as *Method 1*.]

Acceptance criteria: 90.0%–250.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$), calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$)

• **Cyanocobalamin**

[Note—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of cyanocobalamin from USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Transfer an accurately measured volume of Oral Solution, assumed to contain 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each mL of the Oral Solution taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated

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charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene protected from light in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 1*, carefully dissolving *Cystine* and *Tryptophan* in the *hydrochloric acid* before adding the next eight solutions in the resulting solution. In the resulting solution, add 100 mL of water, and dissolve *Dextrose*, *Sodium acetate*, and *Ascorbic acid*. Filter if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to 250 mL.

Table 1

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

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Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and filter with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [Note—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of *anhydrous Dextrose*, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution.* Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of the *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed in *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of the *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[1] [Note—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [Note—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes each containing 10 mL of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ± 0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [Note—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically using a standard wavelength cell or other suitable device. Before reading any tests calibrate the spectrophotometer for 0% and 100% transmittance using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minimum amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of the *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating the contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

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