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Oil- and Water-Soluble Vitamins with Minerals Oral Solution

DEFINITION

Oil- and Water-Soluble Vitamins with Minerals Oral Solution contains one or more of the following oil-soluble vitamins: Vitamin A, vitamin D as Ergocalciferol (vitamin D₂) or Cholecalciferol (vitamin D₃), and Vitamin E; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Niacin or Niacinamide, Dexpantenol 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; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: chromium, fluorine, iodine, iron, magnesium, manganese, molybdenum, and zinc. Oral Solution contains NLT 90.0% and NMT 200.0% of the labeled amounts of vitamin A (C₂₀H₃₀O) as retinol or esters of retinol in the form of retinyl acetate (C₂₂H₃₂O₂) or retinyl palmitate (C₃₆H₆₀O₂), vitamin D as ergocalciferol (C₂₈H₄₄O) or cholecalciferol (C₂₇H₄₄O), vitamin E as alpha tocopherol (C₂₉H₅₀O₂) or alpha tocopheryl acetate (C₃₁H₅₂O₃) or alpha tocopheryl acid succinate (C₃₃H₅₄O₅), ascorbic acid (C₆H₈O₆) or its salts as calcium ascorbate (C₁₂H₁₄CaO₁₂ · 2H₂O) or sodium ascorbate (C₆H₇NaO₆), and thiamine (C₁₂H₁₇ClN₄OS) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 150.0% of the labeled amounts of biotin (C₁₀H₁₆N₂O₃S), calcium pantothenate (C₁₈H₃₂CaN₂O₁₀), dexpantenol (C₉H₁₉NO₄) or panthenol (C₉H₁₉NO₄), niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O), pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆) or riboflavin-5'-phosphate sodium (C₁₇H₂₀N₄NaO₉P); NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P); and NLT 90.0% and NMT 160.0% of the labeled amounts of chromium (Cr), fluorine (F), iodine (I), and molybdenum (Mo); and NLT 90.0% and NMT 125.0% of the labeled amounts of iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn).

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 (C₂₀H₃₀O) 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*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* ($\mu\text{g/mL}$) C_U = nominal concentration of vitamin A as retinol ($C_{20}H_{30}O$) in the *Sample solution* ($\mu\text{g/mL}$) F = factor used to convert retinyl acetate, the ester form present in [USP Vitamin A RS](#), to retinol, 0.872**Acceptance criteria:** 90.0%–200.0% 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 for *Vitamin A*.**Standard solution:** 5 $\mu\text{g/mL}$ 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 150.0 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 for *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* ($\mu\text{g/mL}$) C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* ($\mu\text{g/mL}$) F = correction factor to account for the average amount of previtamin D present in the formulation, 1.09**Acceptance criteria:** 90.0%–200.0% 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 1 min. Cautiously add 3 mL of *Potassium hydroxide solution* through the condenser, and continue to reflux for 30 min. Remove the flask from 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%–200.0% 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%–200.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$)

• BIOTIN, Method 1

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

Dehydrated mixtures, yielding formulations similar to the media described herein, may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of [USP Biotin RS](#) in 50% alcohol. Store this solution in a refrigerator.

Standard solution: On the day of the assay, prepare 0.1 ng/mL of biotin from the *Standard stock solution* in water.

Sample solution: Dilute an accurately measured portion of the Oral Solution with water to an assumed concentration of 0.1 ng/mL.

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 make 1000 mL. Add 20 g of

activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to make 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

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 make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 20 g of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to [Table 1](#), and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* 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 plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

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

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. 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 of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$ until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer 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 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_S$, adding together the two transmittances (Σ_S) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M' , of the *Sample solution*.

Calculate the quantity, in ng, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of ng of biotin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin, in ng, in the portion of Oral Solution taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\bar{M}$, is the assayed log-potency of the test material (see [Design and Analysis of Biological Assays \(111\)](#), [The Confidence Interval and Limits of Potency](#)). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, Method 2**

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

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 $\mu\text{g/mL}$ of [USP Biotin RS](#) in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: 0.6 $\mu\text{g/mL}$ of biotin, from the Oral Solution in water. Adjust the solution with either dilute acetic acid or 0.1 N sodium hydroxide to a pH of 6.0–7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL each of the *Sample solution* and *Standard*

solution into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a copolymer of *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.]²

Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See [Chromatography \(621\), System Suitability](#).)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and a portion of *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) 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 biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of [USP Biotin RS](#) in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• 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 *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 B₁₂ activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 1*.

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: Prepare as directed for *Biotin, Method 1*.

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 make 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 primary calcium sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. 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 2](#), carefully dissolving *Cystine* and *Tryptophan* in the *hydrochloric acid* before adding the next eight solutions. 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 make 250 mL.

Table 2

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

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 make 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 *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 for *Culture medium*.