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Water-Soluble Vitamins with Minerals Capsules

DEFINITION

Water-Soluble Vitamins with Minerals Capsules contain one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Dexpanthenol or Panthenol, Niacin or Niacinamide, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, 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: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Capsules contain NLT 90.0% and NMT 150.0% of the labeled amounts 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 ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), dexpanthenol or panthenol ($C_9H_{19}NO_4$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 125.0% of the labeled quantities of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled quantities of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V). They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They may contain other labeled added substances that are generally recognized as safe, in quantities that are unobjectionable.

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.]

• 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%–150.0% 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.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of [USP Biotin RS](#) in dimethyl sulfoxide

Standard solution: 5 µg/mL of [USP Biotin RS](#) prepared by diluting the *Standard stock solution* with water

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

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

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements**Relative standard deviation:** NMT 3.0%**Analysis****Samples:** *Standard solution* and *Sample solution*

Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules 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 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 ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, Method 2**

[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, dilute the *Standard stock solution* with water to a concentration of 0.1 ng/mL of [USP Biotin RS](#).

Sample solution: Proceed as directed in *Biotin, Method 1* through “calculate the average net weight per Capsule”. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with diluted alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a 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 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 NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in 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 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 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, and 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 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. 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
<i>p</i> -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°, 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 mg of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121°, 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.] Make a transfer of 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 ±0.5° 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 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) as the difference, $y = 2.00 - \Sigma$. 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 , adding together the two transmittances 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 fall 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*.

Determine the quantity, in μg , of [USP Biotin RS](#) corresponding to the biotin in each mg of the portion of Capsules taken:

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

R = number of μg of biotin assumed to be present in each mg of the portion of the Capsules 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 ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

• **CYANOCOBALAMIN, Method 1**

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

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 $\mu\text{g}/\text{mL}$ of [USP Cyanocobalamin \(Crystalline\) RS](#) in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 $\mu\text{g}/\text{mL}$ of [USP Cyanocobalamin \(Crystalline\) RS](#) from *Standard stock solution* diluted with water

Sample solution: Proceed as directed in *Biotin, Method 1* through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to a nominal amount of 100 μg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

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

Mode: LC

Detector: 550 nm

Column: 4.6-mm $\times$ 15-cm; 5- μm packing L1

Flow rate: 0.5 mL/min

Injection volume: 200 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) in the portion of Capsules taken:

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

r_U = peak response of cyanocobalamin from the *Sample solution*

r_S = peak response of cyanocobalamin from the *Standard solution*

C_S = concentration of [USP Cyanocobalamin \(Crystalline\) RS](#) in the *Standard solution* ($\mu\text{g}/\text{mL}$)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* ($\mu\text{g}/\text{mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **CYANOCOBALAMIN, Method 2**

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

Standard cyanocobalamin stock solution: 1.0 $\mu\text{g}/\text{mL}$ of [USP Cyanocobalamin \(Crystalline\) RS](#) in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard cyanocobalamin stock solution* with water to a measured volume. On the day of the incubation period as described in the *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: Proceed as directed in *Biotin, Method 1* through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL 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 *Calcium Pantothenate, Method 2*.

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 in *Calcium Pantothenate, Method 2*.

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. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

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

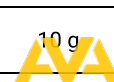
Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected 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 according to [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 the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of between 5.5 and 6.0, and dilute with Purified Water to 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 pass, 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 *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*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *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 (exhaust line temperature), 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*.² [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.] Make a transfer of 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 *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 minute amounts of vitamin B₁₂ 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 *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 *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 *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 contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance 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.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ) as the difference, $y = 2.00 - \Sigma$. 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 , adding together the two transmittances 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 the 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*.

Determine the quantity, in μg , of [USP Cyanocobalamin \(Crystalline\) RS](#) corresponding to the cyanocobalamin in each mg of the portion of Capsules taken:

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

R = number of μg of cyanocobalamin assumed to be present in each mg of the portion of the Capsules 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 cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **Folic Acid, Method 1**

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

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [NOTE—The methanol and water content may be varied (between 1% and 3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve.

Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of [USP Folic Acid RS](#) in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through “calculate the average net weight per Capsule.” Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of *Internal standard solution* to obtain a nominal concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

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

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm $\times$ 30-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 15 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of [USP Folic Acid RS](#) in the *Standard solution* (mg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• Folic Acid, Method 2

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

Reagent: Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of [USP Folic Acid RS](#) in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to a nominal amount of 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

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

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

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

r_U = peak area of folic acid from the *Sample solution*

r_S = peak area of folic acid from the *Standard solution*

C_s = concentration of [USP Folic Acid RS](#) in the *Standard solution* ($\mu\text{g/mL}$)

C_u = nominal concentration of folic acid in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$)

• **DEXPANTHENOL OR PANTHENOL**

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

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: 800 $\mu\text{g/mL}$ of [USP Dexpanthenol RS](#) in water. Store in a refrigerator protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 $\mu\text{g/mL}$ of dexpanthenol from *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as possible to a beaker. Remove any contents adhering to the empty Capsule 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 until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents, nominally equivalent to 1.2 mg of dexpanthenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a nominal concentration of 1.2 $\mu\text{g/mL}$ of dexpanthenol or 2.4 $\mu\text{g/mL}$ of panthenol.

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 about 500 mL of 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 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.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to $75 \pm 5^\circ$, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 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 200 mL. Store under toluene in a refrigerator.

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

Riboflavin–thiamine hydrochloride–biotin solution: 20 $\mu\text{g/mL}$ of riboflavin, 10 $\mu\text{g/mL}$ of thiamine hydrochloride, and 0.04 $\mu\text{g/mL}$ of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 $\mu\text{g/mL}$ of p-aminobenzoic acid, 50 $\mu\text{g/mL}$ of niacin, and 40 $\mu\text{g/mL}$ of pyridoxine hydrochloride in neutral 25% alcohol. 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 10 drops of hydrochloric acid per L of solution. Store under toluene.

Salt solution B: Dissolve 20 mg/mL 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 10 drops of hydrochloric acid per L of the solution. Store under toluene.

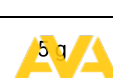
Pyridoxal–calcium pantothenate solution: Dissolve 200 $\mu\text{g/mL}$ of pyridoxal hydrochloride and 1.875 $\mu\text{g/mL}$ of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: Dissolve 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed according to [Table 3](#), and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL.

Table 3

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
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
<i>p</i> -Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium: Prepare as directed for the *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*³ on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes and in the same order add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes and in the same order add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the Standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the *Sample solution*. To obtain the individual responses, divide the potency of each tube by the amount of the *Sample solution* added to it. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for assay, in terms of dexpanthenol, by multiplying the mean response by the appropriate dilution factor.

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• CALCIUM PANTOTHENATE, Method 1

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of [USP Calcium Pantothenate RS](#) in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through “calculate the average net weight per Capsule.” Transfer the equivalent to 0.6 mg/mL of calcium pantothenate, from mixed Capsule contents, in *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system