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Water-Soluble Vitamins with Minerals Oral Solution

DEFINITION

Water-Soluble Vitamins with Minerals Oral Solution contains one or more of the following water-soluble vitamins: 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; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: iodine, iron, magnesium, manganese, and zinc. Oral Solution contains NLT 90.0% and NMT 250.0% of the labeled amount of 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 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 ($C_{17}H_{20}N_4NaO_9P$); NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$); NLT 90.0% and NMT 160.0% of the labeled amount of iodine (I); 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.]

• CYANOCOBALAMIN

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

Standard stock solution: 1.0 µg/mL 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 ng/mL 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: 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.

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: 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 nicotinic acid 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 *L*-Cystine and *L*-Tryptophan in the hydrochloric acid before adding the next eight solutions. To 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

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 $\pm 0.5^\circ$. 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 $\pm 0.5^\circ$. 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 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 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 $\pm 0.5^\circ$ for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating 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.

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_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 the *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 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 cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) in the portion of Oral Solution taken:

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

R = μg of cyanocobalamin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of Oral Solution taken:

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

N = nominal amount of cyanocobalamin 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, Vitamin B₁₂ Activity](#)). 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%–450.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: Methanol and 0.2 M monobasic sodium phosphate (3:97). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1 .

Standard solution: 80 $\mu\text{g}/\text{mL}$ of [USP Calcium Pantothenate RS](#) in *Mobile phase*

System suitability solution: 80 $\mu\text{g}/\text{mL}$ of [USP Racemic Panthenol RS](#) in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 $\mu\text{g}/\text{mL}$ of calcium pantothenate from Oral Solution in the *Mobile phase*

Chromatographic system

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

Mode: LC

Detector: UV 210 nm

Column: 4.0-mm $\times$ 10-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 20 μL

System suitability

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

Suitability requirements

Resolution: NLT 1.5 between panthenol and calcium pantothenate, *System suitability solution*

Tailing factor: NMT 2.0 for both the calcium pantothenate and the panthenol peaks, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$) 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 calcium pantothenate from the *Sample solution*

r_S = peak area of calcium pantothenate from the *Standard solution*

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

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

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$)

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of [USP Calcium Pantothenate RS](#), previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 $\mu\text{g}/\text{mL}$ of [USP Calcium Pantothenate RS](#). Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of the *Standard stock solution* with water to obtain a concentration of 0.01–0.04 $\mu\text{g}/\text{mL}$ of calcium pantothenate, the exact concentration being such that the responses obtained as directed in *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 mg of calcium pantothenate in a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with

water to volume, and filter. Dilute a measured volume of this solution quantitatively, and stepwise if necessary, with water to obtain a solution with about the same concentration as 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 charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator 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 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 1000 mL. Store under toluene in a refrigerator 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 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/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 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. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to [Table 2](#), and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 2

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
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 the 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*,² incubating for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for the *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 the *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. 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 the *Culture medium*.