⟨481⟩ RIBOFLAVIN ASSAY

ASSAY

• **CHEMICAL METHODS, PROCEDURE 1**

The following procedure is suitable for preparations in which riboflavin is a constituent of a mixture of several ingredients. In using the procedure, keep the pH of solutions below 7.0, and protect the solutions from direct sunlight at all stages.

Standard riboflavin stock solution: To 50.0 mg of USP Riboflavin RS, previously dried and stored protected from light in a desiccator over phosphorus pentoxide, add about 300 mL of 0.02 N acetic acid, and heat the mixture on a steam bath with frequent agitation until the riboflavin has dissolved, then cool. To this solution add 0.02 N acetic acid to make 500 mL; then mix. Store the solution under toluene in a refrigerator.

Dilute an accurately measured portion of this solution by using 0.02 N acetic acid to a concentration of 10.0 µg/mL of the dried USP Riboflavin RS to obtain the *Standard riboflavin stock solution*. Store the solution under toluene in a refrigerator.

Standard solution: Dilute with water 10.0 mL of *Standard riboflavin stock solution* in a 100-mL volumetric flask to volume, and mix. Each mL represents 1.0 µg of USP Riboflavin RS. Prepare a fresh *Standard solution* for each assay.

Sample solution: Place an amount of the material to be assayed in a flask of suitable size, and add a volume of 0.1 N hydrochloric acid equal in mL to NLT 10 times the dry weight of the material in grams, but the resulting solution will contain NMT 100 µg/mL of riboflavin. If the material is not readily soluble, comminute the material so that it may be evenly dispersed in the liquid. Agitate vigorously, and wash down the sides of the flask with 0.1 N hydrochloric acid.

Heat the mixture in an autoclave at 121°–123° for 30 min, and cool. If clumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture, with vigorous agitation, with sodium hydroxide solution[1] to a pH of 6.0–6.5, then add hydrochloric acid solution[1] immediately until no further precipitation occurs (usually at a pH of approximately 4.5, which is the isoelectric point of many of the proteins present). Dilute the mixture with water to make a measured volume that contains about 0.11 µg of riboflavin in each mL, and filter through paper known not to adsorb riboflavin. To an aliquot of the filtrate add, with vigorous agitation, sodium hydroxide solution[1] to produce a pH of 6.6–6.8, dilute the solution with water to make a final measured volume that contains approximately 0.1 µg of riboflavin in each mL, and if cloudiness occurs, filter again.

Instrumental conditions

(See *Fluorescence Spectroscopy* ⟨853⟩.)

Mode: Fluorescence

Excitation wavelength: 444 nm

Emission wavelength: 530 nm

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*

To each of four or more tubes (or reaction vessels) add 10.0 mL of the *Sample solution*. To each of two or more of these tubes add 1.0 mL of the *Standard solution*, and mix; to each of two or more of the remaining tubes add 1.0 mL of water, and mix. To each tube add 1.0 mL of glacial acetic acid, mix, then add, with mixing, 0.50 mL of potassium permanganate solution (1 in 25), and allow to stand for 2 min. To each tube add, with mixing, 0.50 mL of hydrogen peroxide solution, whereupon the permanganate color is destroyed within 10 s. Shake the tubes vigorously until excess oxygen is expelled. Remove any gas bubbles remaining on the sides of the tubes after foaming has ceased by tipping the tubes so that the solution flows slowly from end to end.

Measure the fluorescence of all tubes, designating the average reading from the tubes containing only the *Sample solution* as I_U, and designating the average from the tubes containing both the *Sample solution* and the *Standard solution* as I_S. Then to each of one or more tubes of each kind add, with mixing, 20 mg of sodium hydrosulfite, and within 5 s again measure the fluorescence, designating the average reading as I_B.

Calculate the quantity, in mg, of riboflavin ($C_{17}H_{20}N_4O_6$) in each mL of the *Sample solution* taken:

$$\text{Result} = [0.0001 \times (I_U - I_B)]/(I_S - I_U)$$

I_U = average reading of the tubes containing only the *Sample solution*

I_B = average reading of fluorescence of the tubes after adding 20 mg of sodium hydrosulfite

I_S = average reading of the tubes containing both the *Sample solution* and *Standard solution*

Calculate the quantity, in mg, of riboflavin ($C_{17}H_{20}N_4O_6$) in each capsule or tablet.

• **CHEMICAL METHODS, PROCEDURE 2**

This procedure is suitable for the determination of riboflavin as a dietary ingredient or active pharmaceutical ingredient. [NOTE—Conduct the entire *Analysis* without exposure to direct sunlight.]

Standard solution: Transfer 50 mg of USP Riboflavin RS to a 1000-mL volumetric flask containing 50 mL of water. Add 5 mL of acetic acid and sufficient water to make 800 mL. Heat on a steam bath, protected from light, with frequent agitation until dissolved. Cool to 25°, and dilute with water to volume. Dilute this solution with water to bring it within the operating sensitivity of the fluorometer used.

Sample solution: Transfer 50 mg of Riboflavin to a 1000-mL volumetric flask containing 50 mL of water. Add 5 mL of acetic acid and sufficient water to make 800 mL. Heat on a steam bath, protected from light, with frequent agitation until

[1] The concentrations of the hydrochloric acid and sodium hydroxide solutions used are not stated in each instance, because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

dissolved. Cool to 25°, and dilute with water to volume. Dilute this solution with water to bring it to the same concentration as that of the *Standard solution*.

Blank: Prepare as directed for the *Sample solution*, except omit the test specimen.

Instrumental conditions

(See *Fluorescence Spectroscopy* ⟨853⟩.)

Mode: Fluorescence

Excitation wavelength: 444 nm

Emission wavelength: 530 nm

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*

Measure the fluorescence intensity of the *Standard solution*. Immediately after the reading, add to the solution 10 mg of sodium hydrosulfite, stirring with a glass rod until dissolved, and at once measure the fluorescence again. [NOTE—Depending on the final concentration of riboflavin in the solution, it may be necessary to increase the amount of sodium hydrosulfite to suppress the fluorescence activity completely.] The difference between the two readings represents the fluorescence intensity (I_S) due to the *Standard solution*. Similarly, measure the fluorescence intensity (I_U) due to the *Sample solution*. Perform the blank determination, and make any necessary correction.

Calculate the percentage of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Riboflavin taken:

$$\text{Result} = (I_U/I_S) \times (C_S/C_U) \times 100$$

I_U = fluorescence of the *Sample solution*
I_S = fluorescence of the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (µg/mL)
C_U = concentration of riboflavin in the *Sample solution* (µg/mL)

The following liquid chromatographic procedures are provided for the determination of riboflavin as an active pharmaceutical ingredient, a dietary supplement ingredient, or a component in the dietary supplements or pharmaceutical dosage forms. Use the appropriate USP Reference Standards.

Throughout these procedures, protect solutions containing and derived from the test specimen and the Reference Standards from the atmosphere and light, preferably by the use of low-actinic glassware.

Chromatographic Methods, Procedure 1

This procedure can be used to determine riboflavin in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of riboflavin from the formulation by the *Diluent*, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking, or using a vortex mixer, until all of the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution for capsules: Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the 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. Transfer a portion of the capsule contents, equivalent to 2.5 mg of riboflavin, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Sample solution for tablets: Finely powder NLT 30 tablets. Transfer a portion of the powder, equivalent to 2.5 mg of riboflavin, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and appropriate *Sample solution*
Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

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

r_U = peak response of riboflavin from the appropriate *Sample solution*
r_S = peak response of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the appropriate *Sample solution* (mg/mL)

• **CHROMATOGRAPHIC METHODS, PROCEDURE 2**
This procedure can be used to determine riboflavin in:
- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of riboflavin from the formulation by the *Extraction solvent*, heat, and mechanical shaking.
Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.
Extraction solvent: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume. Mix the resulting solution with methanol (3:1).
Solution A: 6.8 g of sodium acetate per 1000 mL of water
Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.
Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.
Standard solution: Dilute 5.0 mL of the *Standard stock solution* with *Extraction solvent* to 25.0 mL.
Sample solution for capsules: Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the 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. Weigh the empty capsule shells in the tared weighing bottle, and calculate the net weight of the capsule contents. Transfer a portion of the capsule contents, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. Add 100.0 mL of *Extraction solvent*, and mix for 20 min using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.
Sample solution for tablets: Finely powder NLT 20 tablets. Transfer a portion of the powder, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. Add 100.0 mL of *Extraction solvent*, and mix for 20 min using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and appropriate *Sample solution*

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

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

r_U = peak response of riboflavin from the appropriate *Sample solution*
r_S = peak response of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the appropriate *Sample solution* (mg/mL)

- **CHROMATOGRAPHIC METHODS, PROCEDURE 3**

This procedure can be used to determine riboflavin in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of riboflavin from the formulation by mixtures of organic solvents, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solutions*, *Sample solutions*, and reagent solutions are prepared as follows.

Diluent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 0.08 mg/mL of USP Riboflavin RS in *Diluent*, with heating if necessary

Standard solution for capsules/tablets: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Standard solution for oral solution: 8 µg/mL of USP Riboflavin RS in *Diluent*, diluted from the *Standard stock solution*

Sample solution for capsules: 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 0.4 mg of riboflavin, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution for oral solution: Equivalent to 8 µg/mL of riboflavin from oral solution in the *Diluent*

Sample solution for tablets: Weigh and finely powder NLT 20 tablets. Transfer a portion of the powder, equivalent to 0.4 mg of riboflavin, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, 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: Appropriate *Standard solution* and appropriate *Sample solution*

For capsules and tablets, calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

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

r_U = peak response of riboflavin from the appropriate *Sample solution*

r_S = peak response of riboflavin from the *Standard solution for capsules/tablets*
C_S = concentration of USP Riboflavin RS in the *Standard solution for capsules/tablets* (mg/mL)
C_U = nominal concentration of riboflavin in the appropriate *Sample solution* (mg/mL)

For oral solution, calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

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

r_U = peak response of riboflavin from the *Sample solution for oral solution*
r_S = peak response of riboflavin from the *Standard solution for oral solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution for oral solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution for oral solution* (mg/mL)

- **CHROMATOGRAPHIC METHODS, PROCEDURE 4**

This procedure can be used to determine riboflavin in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is a newly added procedure as part of the USP monograph modernization efforts. The procedure uses hydrophilic interaction liquid chromatography (HILIC), and the sample preparation involves the extraction of riboflavin from the formulation by the *Diluent*, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

Diluent: Methanol, glacial acetic acid, and water (50:1:49)
Solution A: 50 mM ammonium formate; adjust with ammonium hydroxide to a pH of 9.0.
Solution B: Acetonitrile
Mobile phase: Gradient elution. See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	11	89
8	17	83
15	23	77
20	30	70
21	50	50
24	50	50
25	11	89
30	11	89

Standard solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 160 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all of the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution for capsules: 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 2.5 mg of riboflavin, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 68°, heat for 10 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 10 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate.

Sample solution for tablets: Finely powder NLT 30 tablets. Transfer a portion of the powder, equivalent to 2.5 mg of riboflavin, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 10 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 10 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 267 nm
Column: 4.6-mm × 15-cm; 3.5-µm packing L68
Flow rate: 1.2 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and appropriate *Sample solution*
Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

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

r_U = peak response of riboflavin from the appropriate *Sample solution*
r_S = peak response of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the appropriate *Sample solution* (mg/mL)

ADDITIONAL REQUIREMENTS

• **USP Reference Standards** ⟨11⟩
USP Riboflavin RS