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⟨411⟩ FOLIC ACID ASSAY

INTRODUCTION

The following liquid chromatographic procedures are provided for the determination of folic acid as an active pharmaceutical ingredient, a dietary supplement ingredient, or a component of dietary supplements or pharmaceutical dosage forms.

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

ASSAY

• **Procedure 1**

This procedure can be used to determine folic acid in the following:

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

This procedure involves the extraction of analytes from the formulation by using an *Internal standard solution* that contains methylparaben, tetrabutylammonium hydroxide, and pentetic acid in alcoholic phosphate buffer and by mechanically shaking to release the analytes from the matrices.

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

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 (1%–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 the *Internal standard solution*

Sample solution for Tablets: Crush not less than 30 Tablets until finely powdered. Transfer a portion of powder, equivalent to 0.4 mg of folic acid, to a 50-mL amber-colored centrifuge tube. Add 25.0 mL of the *Internal standard solution*, shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Sample solution for Capsules: Weigh not less than 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 an amount of the Capsule contents to a suitable centrifuge tube, and add a volume of the *Internal standard solution* to obtain a 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: 4.6-mm × 25-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 appropriate *Sample solution*

Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of the *Sample* taken:

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

R_U = internal standard ratio (peak response of folic acid/peak response of the internal standard) from the appropriate *Sample solution*

R_S = internal standard ratio (peak response of folic acid/peak response of the internal standard) from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

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

• **PROCEDURE 2**

This procedure can be used to determine folic acid in the following:

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

This procedure involves the extraction of analytes from the formulation by using an extracting solution that contains either a mixture of edetate disodium and ammonium hydroxide or a mixture of methanol, glacial acetic acid, and ethylene glycol, and by mechanically shaking to release the analytes from the matrices.

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

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 for Tablets: Mix 5.0 mL of the *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 milliliters of the filtrate.

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

Sample solution for Tablets: Transfer a portion of finely powdered Tablets, equivalent to 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 milliliters of the filtrate.

Sample solution for Capsules: Weigh not less than 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 an amount of the Capsule contents, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered 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). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters 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*

Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of the *Sample* taken:

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

r_U = peak response of folic acid from the appropriate *Sample solution*

r_S = peak response of folic acid from the appropriate *Standard solution*

C_S = concentration of USP Folic Acid RS in the appropriate *Standard solution* (µg/mL)

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

• **Procedure 3**

This procedure can be used to determine folic acid in the following:

- Active pharmaceutical ingredient
- Dietary ingredient

This procedure involves the dissolution of analytes and internal standard in *Mobile phase*.

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

3 N phosphoric acid: 98 g/L of phosphoric acid in water

6 N ammonium hydroxide: Dilute 40 mL of ammonium hydroxide with water to 100 mL.

Mobile phase: Transfer 2.0 g of monobasic potassium phosphate to a 1000-mL volumetric flask, and dissolve in 650 mL of water. Add 15.0 mL of a solution of 0.5 M tetrabutylammonium hydroxide in methanol, 7.0 mL of *3 N phosphoric acid*, and 270 mL of methanol. Cool to room temperature, adjust with *3 N phosphoric acid* or *6 N ammonium hydroxide* to a pH of 5.0, and dilute with water to volume. Recheck the pH before use.

Internal standard solution: 2 mg/mL of methylparaben in *Mobile phase*. Dissolve the methylparaben first with methanol (about 4% of the final volume), and dilute with *Mobile phase* to volume.

Standard stock solution: 1 mg/mL of USP Folic Acid RS in *Mobile phase*. Dissolve the folic acid with the aid of 10% ammonium hydroxide (about 1% of the final volume), and dilute with *Mobile phase* to volume.

Standard solution: Transfer 4.0 mL of the *Standard stock solution* and 4.0 mL of the *Internal standard solution* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.

Sample stock solution: Transfer 100 mg of folic acid to a 100-mL volumetric flask, and dissolve in 40 mL of *Mobile phase* and 1 mL of 10% ammonium hydroxide. Dilute with *Mobile phase* to volume.

Sample solution: Transfer 4.0 mL of the *Sample stock solution* and 4.0 mL of the *Internal standard solution* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.

Chromatographic system

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

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.2 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 3.6 between methylparaben and folic acid

Relative standard deviation: NMT 2.0% for the ratios of the folic acid peak response to the internal standard peak response

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of the *Sample* taken:

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

R_U = internal standard ratio (peak response of folic acid/peak response of the internal standard) from the *Sample solution*

R_S = internal standard ratio (peak response of folic acid/peak response of the internal standard) from the *Standard solution*

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

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

Change to read:

• **Procedure 4**

This procedure can be used to determine folic acid in the following:

- Folic Acid Tablets
- Folic Acid Injection

This procedure involves the dissolution of analytes in a *Diluent* that contains 2 mL of ammonium hydroxide and 1 g of sodium perchlorate per 100 mL of water.

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

Mobile phase: Transfer 35.1 g of sodium perchlorate and 1.40 g of monobasic potassium phosphate to a 1-L volumetric flask. Add 7.0 mL of 1 N potassium hydroxide and 40 mL of methanol, dilute with water to volume, and mix. Adjust with 1 N potassium hydroxide or phosphoric acid to a pH of 7.2.

Diluent: Aqueous solution containing 2 mL of ammonium hydroxide and 1 g of sodium perchlorate per 100 mL

System suitability solution: 0.2 mg/mL each of USP Folic Acid RS and ▲USP Leucovorin Calcium RS▲ (IRA 1-Nov-2022) in *Diluent*. [Note—Before use, pass through a filter of 1-µm or finer pore size.]

Standard solution: 0.20 mg/mL of USP Folic Acid RS in *Diluent*

Sample solution for Tablets: Equivalent to 0.2 mg/mL of folic acid from not less than 20 powdered Tablets in *Diluent*. Shake gently to aid dissolution, and filter, discarding the first few milliliters of the filtrate.

Sample solution for Injection: Dilute with *Diluent* an accurately measured volume of Injection, quantitatively and stepwise, to obtain a solution having a concentration close to that of the *Standard solution* and between 0.20 and 0.80 mg/mL.

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: 25 µL

System suitability

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

Suitability requirements

Resolution: NLT 3.6 between ▲leucovorin▲ (IRA 1-Nov-2022) and folic acid, *System suitability solution*

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

Analysis

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

Calculate the percentage of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

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

r_U = peak response of folic acid from the *Sample solution for Tablets*

r_S = peak response of folic acid 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 for Tablets* (mg/mL)

Calculate the quantity, in milligrams, of folic acid ($C_{19}H_{19}N_7O_6$) in each milliliter of Injection taken:

$$\text{Result} = (r_U/r_S) \times (C_S/V_U) \times V$$

r_U = peak response of folic acid from the *Sample solution for Injection*

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

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

V_U = volume of Injection taken for preparation of the *Sample solution for Injection* (mL)

V = total volume used to dilute the Injection (mL)

ADDITIONAL REQUIREMENTS

Change to read:

• **USP REFERENCE STANDARDS ⟨11⟩**

USP Folic Acid RS

▲USP Leucovorin Calcium RS▲ (IRA 1-Nov-2022)

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
<411> FOLIC ACID ASSAY	Natalia Davydova Scientific Liaison	NBDS2020 Non-botanical Dietary Supplements

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