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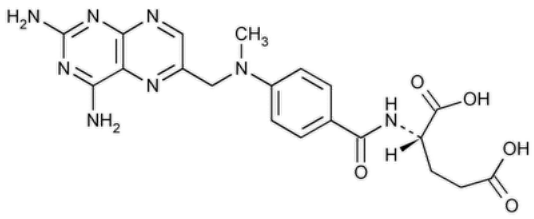
Methotrexate



$C_{20}H_{22}N_8O_5$ 454.45

L-Glutamic acid, *N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-;
L-(+)-*N*-(*p*-{[(2,4-Diamino-6-pteridinyl)methyl]methylamino}benzoyl)glutamic acid;
(*S*)-2-(4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzamido)pentanedioic acid;
4-Amino-10-methylfolic acid CAS RN®: 59-05-2; UNII: YL5FZ2Y5U1.

DEFINITION

Methotrexate contains NLT 98.0% and NMT 102.0% of methotrexate ($C_{20}H_{22}N_8O_5$), calculated on the anhydrous basis.

[**Caution—**Great care should be taken to prevent inhaling particles of methotrexate and exposing the skin to it.]

IDENTIFICATION

- **A. Spectroscopic Identification Tests ⟨197⟩, *Infrared Spectroscopy*:** 197A or 197K. Do not dry specimens.
- **B. Spectroscopic Identification Tests ⟨197⟩, *Ultraviolet-Visible Spectroscopy*:** 197U

Sample solution: 10 µg/mL of Methotrexate in 0.1 N hydrochloric acid VS

Acceptance criteria: Meets the requirements

ASSAY

Change to read:

- **Procedure**

Buffer: 3.4 g/L of anhydrous monobasic sodium phosphate in water. Adjust with ▲1 N sodium hydroxide▲ (ERR 1-Oct-2024) to a pH of 5.8.

Solution A: Acetonitrile and *Buffer* (1:19)

Solution B: Acetonitrile and *Buffer* (1:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
30	50	50
34	50	50
35	100	0
40	100	0

Standard stock solution: 1.0 mg/mL of USP Methotrexate RS prepared as follows. Transfer a known amount of USP Methotrexate RS to a suitable volumetric flask, dissolve in dimethyl sulfoxide equivalent to 5% of the final volume, and dilute with *Solution A* to volume.

Standard solution: 0.2 mg/mL of USP Methotrexate RS from the *Standard stock solution*, in *Solution A*

Sample stock solution: 1.0 mg/mL of Methotrexate prepared as follows. Transfer a known amount of Methotrexate to a suitable volumetric flask, dissolve in dimethyl sulfoxide equivalent to 5% of the final volume, and dilute with *Solution A* to volume.

Sample solution: 0.2 mg/mL of Methotrexate from the *Sample stock solution*, in *Solution A*

Chromatographic system

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

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 15°

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.6

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methotrexate ($C_{20}H_{22}N_8O_5$) in the portion of Methotrexate taken:

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

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

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

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

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

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **Residue on Ignition ⟨281⟩:** NMT 0.1%
- **Organic Impurities, Procedure 1: Related Compounds**

Solution A, Solution B, Mobile phase, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Standard stock solution A: Use the *Standard solution* from the *Assay*.

Standard solution A: 0.1 µg/mL of USP Methotrexate RS in *Solution A*, from *Standard stock solution A*

Standard stock solution B: 0.1 mg/mL of USP Methotrexate Related Compound B RS, 0.2 mg/mL of USP Methotrexate Related Compound C RS, and 0.1 mg/mL of USP Methotrexate Related Compound E RS prepared as follows. Transfer known quantities of USP Methotrexate Related Compound B RS, USP Methotrexate Related Compound C RS, and USP Methotrexate Related Compound E RS to a suitable volumetric flask. Dissolve in dimethyl sulfoxide equivalent to 5% of the final volume. Add 75% of the flask volume of *Solution A* and sonicate to dissolve, if needed. Dilute with *Solution A* to volume.

Standard solution B: 0.4 µg/mL of USP Methotrexate Related Compound C RS, 0.2 µg/mL of USP Methotrexate Related Compound B RS, and 0.2 µg/mL of USP Methotrexate Related Compound E RS from *Standard stock solution B*, in *Solution A*

System suitability solution: 0.1 mg/mL of USP Methotrexate System Suitability Mixture RS, 0.2 µg/mL of USP Methotrexate Related Compound B RS, 0.4 µg/mL of USP Methotrexate Related Compound C RS, and 0.2 µg/mL of USP Methotrexate Related Compound E RS prepared as follows. Transfer a known quantity of USP Methotrexate System Suitability Mixture RS to a suitable volumetric flask, and dissolve in dimethyl sulfoxide equivalent to about 2% of the final volume. Add *Standard stock solution B* equivalent to 0.2% of the final volume, and dilute with *Solution A* to volume.

System suitability

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

Suitability requirements

Resolution: NLT 1.7 between methotrexate related compound B and methotrexate related compound C; NLT 10.0 between methotrexate related compound C and methotrexate; and NLT 5.0 between methotrexate related compound I and methotrexate related compound H, *System suitability solution*

Relative standard deviation: NMT 5.0%, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Calculate the percentage of methotrexate related compound B and methotrexate related compound C in the portion of Methotrexate taken:

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

r_U = peak response of methotrexate related compound B or methotrexate related compound C from the *Sample solution*

r_S = peak response of methotrexate related compound B or methotrexate related compound C from *Standard solution B*

C_S = concentration of USP Methotrexate Related Compound B RS or USP Methotrexate Related Compound C RS in *Standard solution B* (mg/mL)

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

Calculate the percentage of methotrexate related compound E free base in the portion of Methotrexate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of methotrexate related compound E from the *Sample solution*

r_S = peak response of methotrexate related compound E from *Standard solution B*

C_S = concentration of USP Methotrexate Related Compound E RS in *Standard solution B* (mg/mL)

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

M_{r1} = molecular weight of methotrexate related compound E free base, 325.33

M_{r2} = molecular weight of methotrexate related compound E, 343.56

Calculate the percentage of methotrexate related compound H, methotrexate related compound I, and any unspecified impurity in the portion of Methotrexate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (1/F) \times 100$$

r_U = peak response of methotrexate related compound H, methotrexate related compound I, or any unspecified impurity from the *Sample solution*

r_S = peak response of methotrexate from *Standard solution A*

C_S = concentration of USP Methotrexate RS in *Standard solution A* (mg/mL)

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

F = relative response factor for each individual impurity (see *Table 2*)

Acceptance criteria: See *Table 2*.

Table 2

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
Methotrexate related compound B	0.72	—	0.3
Methotrexate related compound C	0.77	—	0.5
Methotrexate	1.00	—	—
Methotrexate related compound E free base	1.46	—	0.3
Methotrexate dimethylamide[a] and methotrexate related compound I[b]	1.54	0.71	0.2[c]
Methotrexate related compound H[d]	1.65	1.0	0.2
Any unspecified impurity	—	1.0	0.10
Total impurities	—	—	1.0

[a] (*S*)-2-(4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzamido)-5-(dimethylamino)-5-oxopentanoic acid.

[b] (*S*)-4-(4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzamido)-5-methoxy-5-oxopentanoic acid.

[c] If present, methotrexate dimethylamide and methotrexate related compound I may not be completely resolved by the method. These peaks are integrated together to determine conformance. If present and resolved, the relative retention time of methotrexate dimethylamide is 1.51.

[d] (*S*)-2-(4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzamido)-5-methoxy-5-oxopentanoic acid.

• **Organic Impurities, Procedure 2: Enantiomeric Purity**

Solution A: 7.1 g/L of anhydrous dibasic sodium phosphate in water

Solution B: 6.9 g/L of monobasic sodium phosphate in water

Solution C: *Solution A* and *Solution B* (5:6). Adjust with 2 N sodium hydroxide TS to a pH of 6.9.

Mobile phase: *n*-Propanol and *Solution C* (2:23)

System suitability solution: 0.02 mg/mL each of USP Methotrexate RS and USP *R*-Methotrexate RS in *Mobile phase*

Sample solution: 0.2 mg/mL of Methotrexate in *Mobile phase*

Diluted sample solution: 2 µg/mL of Methotrexate from the *Sample solution*, in *Mobile phase*

Chromatographic system

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

Mode: LC

Detector: UV 302 nm

Column: 4.0-mm × 15-cm; 7-µm packing L75

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *System suitability solution*

[Note—The relative retention times for methotrexate and *R*-methotrexate are 1.0 and 1.95, respectively.]

Suitability requirements

Resolution: NLT 1.3 between methotrexate and *R*-methotrexate

Relative standard deviation: NMT 5.0% for the methotrexate peak

Analysis

Samples: *Sample solution* and *Diluted sample solution*

Calculate the percentage of *R*-methotrexate in the portion of Methotrexate taken:

$$\text{Result} = [r_U/(r_S \times 100)] \times 100$$

r_U = peak area of *R*-methotrexate from the *Sample solution*

r_S = peak area of methotrexate from the *Diluted sample solution*

Acceptance criteria: NMT 3.0%

SPECIFIC TESTS

• **Water Determination ⟨921⟩, *Method I*:** NMT 12.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight, light-resistant containers.

• **USP Reference Standards ⟨11⟩**

USP *R*-Methotrexate RS

[Note—May be available as a free acid or a disodium salt.]

(*R*)-2-(4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzamido)pentanedioic acid.

$C_{20}H_{22}N_8O_5$ 454.45

Sodium (*R*)-2-(4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzamido)pentanedioate.

$C_{20}H_{20}N_8Na_2O_5$ 498.41

USP Methotrexate System Suitability Mixture RS

This mixture contains:

Methotrexate

Methotrexate dimethylester hydrochloride;

(*S*)-Dimethyl-2-(4-{[(2,4-diaminopteridin-6-yl)methyl](methyl)amino}benzamido) pentanedioate hydrochloride.

$C_{22}H_{26}N_8O_5 \cdot x\text{HCl}$

Methotrexate related compound I;

(*S*)-4-(4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzamido)-5-methoxy-5-oxopentanoic acid.

$C_{21}H_{24}N_8O_5$ 468.47

Methotrexate related compound H;

(*S*)-2-(4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzamido)-5-methoxy-5-oxopentanoic acid.

$C_{21}H_{24}N_8O_5$ 468.47

USP Methotrexate RS

USP Methotrexate Related Compound B RS

(*S*)-2-{4-[(2,4-Diaminopteridin-6-yl)methylamino]benzamido}pentanedioic acid.

$C_{19}H_{20}N_8O_5$ 440.42

USP Methotrexate Related Compound C RS

(*S*)-2-(4-{[(2-Amino-4-oxo-1,4-dihydropteridin-6-yl)methyl](methyl)amino}benzamido)pentanedioic acid.

$C_{20}H_{21}N_7O_6$ 455.42

USP Methotrexate Related Compound E RS

4-{[(2,4-Diaminopteridin-6-yl)methyl](methyl)amino}benzoic acid, hemihydrochloride.

$C_{15}H_{15}N_7O_2 \cdot ½HCl$ 343.56

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

Topic/Question	Contact	Expert Committee
METHOTREXATE	Documentary Standards Support	SM32020 Small Molecules 3

Chromatographic Database Information: Chromatographic Database

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