

CALCIUM L-5-METHYLTETRAHYDROFOLATE

New specifications prepared at the 65th JECFA (2005) and published in FNP 52 Add 13 (2005). At the 65th JECFA (2005) the Committee had no safety concerns for the use of the substance in dry crystalline or microencapsulated form as an alternative to folic acid used in dietary supplements, foods for special dietary uses and other foods.

SYNONYMS

L-5-Methyltetrahydrofolic acid, calcium salt
L-Methyltetrahydrofolate, calcium salt
L-Methylfolate, calcium
L-5-MTHF-Ca

DEFINITION

Calcium L-5-methyltetrahydrofolate (L-5-MTHF-Ca) is a synthetic derivative of folic acid, the predominant, naturally occurring form of folate. It is synthesized by reduction of folic acid to tetrahydrofolic acid followed by methylation and diastereoselective crystallization (in water) of L-5-MTHF as its calcium salt. The product contains variable amounts of water of crystallization.

Chemical name

N-{4-[[[(6S)-2-amino-3,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridiny]methyl]amino]benzoyl}-L-glutamic acid, calcium salt

C.A.S. number

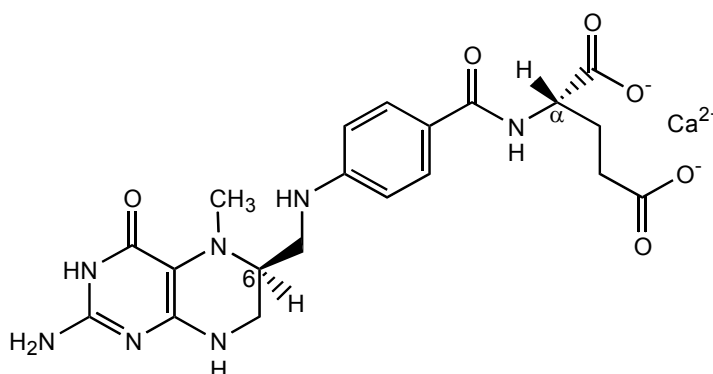
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Chemical formula

C₂₀H₂₃CaN₇O₆ (anhydrous form)

Structural formula

(anhydrous form)



Formula weight

497.5 (anhydrous form)

Assay

95.0 – 102.0% (anhydrous basis)

DESCRIPTION

White to light yellowish, almost odourless, crystalline powder

FUNCTIONAL USES

Nutrient supplement

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)

Sparingly soluble in water and very slightly soluble or insoluble in most organic solvents; soluble in alkaline solutions

Infrared absorption

The infrared absorption spectrum of a potassium bromide dispersion of the

sample corresponds to that of a L-5-MTHF-Ca standard (see Appendix).

<u>Calcium</u>	Dilute 30 g of acetic acid (glacial) to 100 ml with water. Dissolve 5.3 g of $K_4Fe(CN)_6$ in 100 ml of water. To 5 ml of the acetic acid solution, add 20 mg of the sample and then 0.5 ml of the potassium ferrocyanide solution. Mix and add 50 mg of ammonium chloride. A white crystalline precipitate is formed.
<u>Liquid chromatography</u>	Retention time matches that of a reference standard (see under TESTS)
PURITY	
<u>Water</u> (Vol. 4)	Not more than 17.0% (Karl Fischer method) (Note: Allow sufficient time (15 min) for release of bound water.)
<u>Calcium</u>	7.0 - 8.5% (anhydrous basis) Accurately weigh 500 mg of sample and transfer to a 500-ml conical flask. Add 150 ml of water to dissolve the sample and 20 ml of a pH 10 buffer (NH_3/NH_4Cl). Using eriochrome black T as indicator, titrate (continuous stirring) with standardized 0.1 M EDTA until the colour changes from violet to blue/green. Each 1.0 ml of 0.1 M EDTA corresponds to 4.008 mg of calcium. Calculate the calcium content on the anhydrous basis.
<u>Other folates and related substances</u>	Not more than 2.5% See description under TESTS
<u>D-5-Methylfolate</u>	Not more than 1.0% See description under TESTS
<u>Total viable aerobic count</u> (Vol. 4)	Not more than 1000 CFU/g
<u>Lead</u> (Vol. 4)	Not more than 2 mg/kg Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, "Instrumental methods".

TESTS

PURITY TESTS

<u>Other folates and related substances</u>	Using a L-5-MTHF-Ca reference standard, Quantitate other folates and related substances by HPLC. The suitability of the applied HPLC system is checked daily by a "system suitability test" (see below). <u>Reference standard solution</u> : Accurately weigh 50 mg of L-5-MTHF-Ca (L-5-methyltetrahydrofolic acid, calcium salt (Merck Eprova AG, CH-8200 Schaffhausen, Switzerland) into a 100-ml volumetric flask. Dissolve in a small quantity of water and dilute to volume. <u>Sample solution</u> : Prepare as for the reference standard using 50 mg of the sample. <u>Mobile phase solutions</u> A: Dissolve 7.80 g of $NaH_2PO_4 \cdot 2H_2O$ (0.05 mol) in 1000 ml of water and adjust the pH to 6.5 with 32% NaOH. Filter and degas the solution. B: Dissolve 5.07 g of $NaH_2PO_4 \cdot 2H_2O$ (0.03 mol) in 650 ml of water and 350 ml of methanol (chromatography grade) and adjust the pH to 8.0 with 32%
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NaOH. Filter and degas the solution.

Chromatography Conditions

Column: Hypersil-ODS, 5 µm; 250 x 4 mm (Thermo Hypersil Keystone or equivalent)

Flow rate: 1.1 ml/min

Gradient:

<i>Time (min)</i>	<i>% Mobile phase A</i>	<i>% Mobile phase B</i>	<i>Remark</i>
0	100	0	Start
0 - 14	100 – 45	0 – 55	Linear gradient
14 – 17	45 – 0	100	Linear gradient
17 – 22	0	100	Hold
22 – 31	100	0	Reconditioning

Temperature: Room temperature

Injection volume: 10 µl

Detection: UV (280 nm)

Run time: 22 min

Retention times given below are approximate:

<i>Folates and related substances</i>	<i>Retention time (min)</i>
4-Aminobenzoylglutamic acid (ABGA)	3.1
4α-Hydroxy-5-methyltetrahydrofolic acid (HOMeTHFA)	4.3
D-Pyrazino-s-triazine derivative (D-Mefox)	6.1
L-Pyrazino-s-triazine derivative (L-Mefox)	6.3
Tetrahydrofolic acid (THFA)	8.5
7,8-Dihydrofolic acid (DHFA)	11.2
Folic acid (FA)	11.4
5,10-Methylenetetrahydrofolic acid (CH ₂ THFA)	11.7
5-Methyltetrahydrofolic acid (5-MTHF)	13.6
5-Methyltetrahydropteroic acid (MeTHPA)	15.1
N ² -Methylamino-5-methyltetrahydrofolic acid (DiMeTHFA)	17.6

Sample analysis: Inject the reference standard solution and the sample solutions immediately after preparation, using the conditions described above.

(Note: After analysis, flush the column with methanol/water 85:15 and store it under the same conditions.)

Calculate the content of each folate (other than 5-MTHF) and related substance, X_i (%), listed in the above table according to the following formula:

$$X_i (\%) = A_i \times W_S \times S \times (RF)_i / A_S \times W$$

where

A_i = the peak area for each folate (other than 5-MTHF) and related substance

A_S = the peak area for the L-5-MTHF-Ca Standard

W_S = the weight (mg) of L-5-MTHF-Ca Standard

W = the weight (mg) of the sample

S = the percent of L-5-MTHF in the L-5-MTHF-Ca Standard, calculated as

free acid

$(RF)_i$ = Response Factor for the i-th substance (absorbance at 280 nm in the applied eluent system relative to that of L-5-MTHF)

<i>Other folates and related substances</i>	<i>RF</i>
ABGA	0.93
HOMeTHFA	1.11
L-Mefox and D-Mefox	1.11
DHFA	0.98
FA	0.86
MeTHPA	0.68
THFA	1.00
CH ₂ THFA	1.00
DiMeTHFA	1.00

If there are any unidentified impurities, apply a RF of 1.00.

Calculate the total amount of "Other folates and related substances" by summing the X_i for all impurities.

System suitability test procedure

Mixed folates solution: Weigh 25 mg each of ABGA, HOMeTHFA, L-Mefox, DHFA, FA and MeTHPA (all available from Merck Eprova AG) into a 100-ml volumetric flask. Add a small quantity of water to dissolve the mixture; add some sodium hydrogen carbonate and sodium carbonate to aid the dissolution, and fill to the mark with water.

System suitability test solution (SST solution): Weigh accurately 50 mg of a L-5-MTHF-Ca sample containing DiMeTHFA into a 100-ml volumetric flask. (Available from Merck Eprova AG). Add 1 ml of the Mixed folates solution and a small quantity of water to dissolve, mix and dilute to volume with water.

System suitability test: Inject 10 μ l of the SST solution immediately. The resolution between L-5-MTHF and MeTHPA must be at least 5.

D-5-Methylfolate

D-5-Methylfolate is quantitated by HPLC using a chromatographic system which allows separation of the D- from the L-stereoisomer. The suitability of the applied HPLC system is checked daily by a "system suitability test" (see below).

Sample preparation: Accurately weigh 50 mg of the sample into a 100 ml volumetric flask. Dissolve in water and dilute to volume with water.

Mobile phase: Mix 970 ml of 0.03 M NaH₂PO₄ (obtained by dissolving 4.68 g of NaH₂PO₄ · 2H₂O in water and diluting with water to 1000 ml) with 30 ml of acetonitrile (chromatography grade) and adjust the pH to 6.8 with 32% NaOH. Filter and degas the solution.

Chromatography Conditions

Column: Chiral Protein HSA, 5 μ m, 150 x 4 mm (ChromTech or equivalent)

Flow rate: 1 ml/min

Temperature: 40°

Injection volume: 10 μ l

Detection: UV (280 nm)

Run time: 22 min

Solvent: Water

Sample analysis: Inject the sample solution immediately after preparation using the conditions described above. Determine the areas under peak for L-5-MTHF (retention time: ca. 11 min) and D-5-MTHF (retention time: ca. 15 min).

Calculation

Determine the ratio of the peak area for the D-isomer (A_D) to the sum of the peak areas for the D- and L-isomers (A_T), and calculate the D-5-MTHF content as follows:

$$\text{D-5-MTHF (\%)} = 100A_D/A_T$$

System suitability test procedure

System suitability test solution (SST solution): Weigh and transfer into a 200-ml volumetric flask the following: 1.0 mg of HOMeTHFA, 1.5 mg ABGA, 2.0 mg each of L-Mefox and MeTHPA, 3.0 mg of FA, 4.0 mg of DHFA, 10 mg of L,D-5-MTHF and D,D-5-MTHF (L-5-MTHF and D-5-MTHF carrying D-glutamic acid substitution), and 50 mg of racemic 5-MTHF-Ca (L-5-MTHF and D-5-MTHF carrying L-glutamic acid substitution) (all available from Merck Eprova AG). Add a small quantity of water to dissolve the mixture; add some sodium hydrogen carbonate to aid the dissolution, and fill to the mark with water. Immediately inject into the HPLC system.

The resolution between L-5-MTHF and D-5-MTHF must be at least 2.

METHOD OF ASSAY

Calculate the percentage of L-5-MTHF-Ca in the sample from the content of 5-MTHF-Ca (L- and D-diastereoisomers), determined in the test for "Other folates and related substances", and the content of D-5-MTHF-Ca, determined in the test for D-5-Methylfolate, and correcting for water content, as follows:

$$\text{L-5-MTHF-Ca (\%)} = 100 \times A_T \times W_S \times S \times (100 - D) \times 1.083 / A_S \times W \times (100 - \%H_2O)$$

where

A_T is taken from the calculation for the D-5-Methylfolate analysis

D = the percentage of D-5-Methylfolate in the sample

A_S , W, W_S , and S are taken from the determination of Other folates and related substances

$\%H_2O$ = water content (%)

1.083 is the ratio of the formula weight of 5-MTHF-Ca to that of 5-MTHF.

Appendix

Infrared spectra of
Calcium L-5-Methyl-
tetrahydrofolate