Chapter 19

Quantitation of 5-Methyltetrahydrofolate in Cerebrospinal Fluid Using Liquid Chromatography-Electrospray Tandem Mass Spectrometry

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Abstract

We describe a simple stable isotope dilution method for accurate and precise measurement of cerebrospinal fluid (CSF) 5-methyltetrahydrofolate (5-MTHF) as a clinical diagnostic test. 5-MTHF is the main biologically active form of folic acid and is involved in regulation of homocysteine and DNA synthesis. Measurement of 5-MTHF in CSF provides diagnostic information regarding diseases affecting folate metabolism within the central nervous system, in particular inborn errors of folate metabolism. Determination of 5-MTHF in CSF (50 μL) was performed utilizing high performance liquid chromatography coupled with electrospray positive ionization tandem mass spectrometry (HPLC-ESI-MS/MS). 5-MTHF in CSF is determined by a 1:2 dilution with internal standard (5-MTHF-$^{13}C_5$) and injected directly onto the HPLC-ESI-MS/MS system. Each assay is quantified using a five-point standard curve (25–400 nM) and has an analytical measurement range of 3–1000 nM.

Key words 5-Methyltetrahydrofolate, Cerebral folate deficiency, Methylation, Mass spectrometry

1 Introduction

5-Methyltetrahydrofolate (5-MTHF) is the predominant form of folate in cerebrospinal fluid (CSF). Testing for 5-MTHF in CSF is useful to determine a deficiency of folate in the central nervous system. Low 5-MTHF levels are associated with inborn errors of metabolism affecting folate metabolism and in dietary deficiency of folate. Disorders associated with low folate include anemia, developmental delay, seizures, depression, dementia, cerebral folate deficiency, and Kearns-Sayre syndrome [1]. More recent reports indicate that there is reduced uptake of 5-MTHF across the blood–brain barrier due to the presence of auto-antibodies to the folate receptor at the choroid plexus. Patients with cerebral folate deficiency (CFD) are characterized by normal plasma folate in the

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presence of decreased concentration of 5-MTHF in CSF [2, 3]; patients with CFD have neurological complications. The following is a simple high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for determination of cerebrospinal fluid 5-MTHF as a clinical diagnostic test.

2 Materials

2.1 Samples

Human lumbar CSF—Specimen drawn any time during the day will be acceptable. No patient preparation is required. Optimal volume of 1.0 ml (minimum 0.25 mL) CSF should be collected in tube 2 (or 1, 4 or 5) of the collection kit provided by the testing laboratory or in a regular CSF collection tube. If the CSF is clear, the sample should be immediately frozen at the bedside on dry ice. If blood contaminated, the sample should be placed on wet ice, centrifuged within 5 min, and the clear CSF transferred to another vial and frozen on dry ice as soon as possible. CSF is stored at –80 °C until time of testing.

2.2 Solvents and Reagents

1. Magnesium chloride × $6H_2O$, ACS grade.
2. Sodium phosphate dibasic anhydrous, ACS grade.
3. Mobile Phase A (0.1 % formic acid in HPLC-grade water): In a hood add 1 mL of formic acid to a 1 L volumetric flask, bring to volume with water, and mix. Stable at room temperature, 18–24 °C, up to 3 months.
4. Mobile Phase B (0.1 % formic acid in methanol): In a hood, add 1 mL of formic acid to a 1 L volumetric flask, bring to volume with methanol, and mix. Stable at room temperature, 18–24 °C, up to 3 months.
5. 10× Artificial CSF (aCSF): 1450 mM NaCl, 27 mM KCl, 10 mM $MgCl_2$, 12 mM $CaCl_2$, 20 mM Na_2HPO_4.
   (a) Weigh the following and combine in 100 mL volumetric flask containing 50 mL water: 8.474 g NaCl, 0.201 g KCl, 0.203 g $MgCl_2$, 0.176 g $CaCl_2$, 0.284 g Na_2HPO_4.
   (b) Bring to volume with water.
   (c) Add small magnetic stir bar and mix on magnetic stirrer until dissolved.
   (d) Adjust pH to 7.4 with 85 % phosphoric acid.
   (e) Store at 2–8 °C for up to 1 year.
6. 1× aCSF: Add 1 mL 10× aCSF to a 10 mL volumetric flask and bring to volume with water. 1× aCSF is stable for up to 8 h at 2–8 °C and must be made fresh daily.

2.3 Internal Standards and Standards

1. Primary standard: 5-MTHF ((6S)-5-Methyl-5,6,7, 8-tetrahydrofolic acid, calcium salt) (Schircks Laboratories).
2. Primary internal standard (I.S.): 5-MTHF-$^{13}C_5$ (Calcium-L-Mefolinate-^{13}C5) (Merck Eprova).
3. 5-MTHF Standard Stock Solution (1 mM): Add 49.8 mg 5-MTHF to 100 mL volumetric flask, bring to volume with water containing 1 mg/mL ascorbic acid. Wrap flask with foil and sonicate for 5 min. Store in 125 μL aliquots at −80 °C for up to 4 years (*see* **Note 1**).
4. 5-MTHF-$^{13}C_5$ I.S. Stock Solution (1 mM): Add 5 mg 5-MTHF-^{13}C5 to 10 mL volumetric flask, bring to volume with water containing 1 mg/mL ascorbic acid. Wrap flask with foil and sonicate for 5 min. Store in 125 μL aliquots at −80 °C for up to 4 years (*see* **Note 1**).
5. I.S. Working Solution (5-MTHF-$^{13}C_5$ prepared in water containing ascorbic acid and dithiothreitol): Add 2 μL 1 mM 5-MTHF-$^{13}C_5$ to 2 mL of water containing 40 mg ascorbic acid and 18 mg dithiothreitol in a 2 mL screw-top tube and mix by vortex. Working internal standard may be stored in the refrigerator at 0–10 °C for up to 8 h. Volume of internal standard may be increased to process the number of specimens within the assay.

2.4 Calibrators and Controls

1. Calibrators: 5-MTHF Working Standard Curve, dilute stock solution 1 mM 5-MTHF as follows:
   (a) Dilution A (100 μM): Add 100 μL of 1 mM 5-MTHF stock solution to 900 μL of 1× aCSF and mix well by vortex.
   (b) Dilution B (10 μM): Add 100 μL Dilution A to 900 μL of 1× aCSF and mix well by vortex.
   (c) Dilution C (1 μM): Add 100 μL Dilution B to 900 μL of 1× aCSF and mix well by vortex.
   (d) Working Standard Curve (25–400 nM): Add 400 μL of Dilution C to 600 μL of 1× aCSF and mix well by vortex. Perform four additional serial dilutions by adding 500 μL of previous standard to 500 μL of 1× aCSF. This will provide a calibration curve of (400, 200, 100, 50, 25 nM). Working standard curve may be stored in the refrigerator at 4 °C for up to 8 h (*see* **Note 2**).
2. Normal Control: (5-MTHF = 80–240 nM target value):
   (a) Prepare 10 mL pooled CSF.
   (b) Assay pooled CSF to quantitate the native concentration of 5-MTHF.

(c) Spike or dilute pooled CSF with diluted stock standard or water to obtain a final concentration of 80–240 nM 5-MTHF. Store in 80 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).

3. Abnormal Control: (5-MTHF = 20–40 nM target value):
   (a) Prepare 10 mL pooled CSF.
   (b) Assay pooled CSF to quantitate the native concentration of 5-MTHF.
   (c) Spike or dilute pooled CSF with diluted stock standard or water to obtain a final concentration of 20–40 nM 5-MTHF. Store in 80 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).

2.5 Analytical Equipment and Supplies

1. Shimadzu Prominence liquid chromatograph system with ABSciex 4000QTRAP® with Analyst software version 1.6.2.
2. Analytical Column: Phenomenex Synergi-Hydro, 4 µm, 150 × 3 mm.
3. Guard Column: Phenomenex Security Guard, 5 µm, 4 × 3 mm.
4. 1.5 mL microcentrifuge tubes.

3 Methods

3.1 Sample Preparation

1. To labeled 1.5 mL microcentrifugal units, pipette 50 µL sample (calibrators, controls, patient CSF).
2. Add 50 µL of 5-MTHF-$^{13}C_5$ I.S. Working Solution.
3. Cap and vortex mix tubes at maximum speed for 3 s.
4. Centrifuge for 10 min at 14,000 × *g*.
5. Transfer 90 µL of prepared sample into corresponding work list position in 96-well microtiter plate and cover with silicone cover.
6. Place completed 96-well microtiter plate onto refrigerated autosampler (4 °C).
7. Inject 10 µL of sample onto HPLC-ESI-MS/MS. Representative HPLC-ESI-MS/MS ion chromatograms for 5-MTHF and I.S. are shown in Figs. 1 and 2 (*see* **Note 3**).

3.2 Data Analysis

1. Instrumental operating parameters are given in Table 1.
2. Data are analyzed using Analyst software version 1.6.2 (AB Sciex).
3. Standard curves are generated based on linear regression of the analyte/I.S. peak-area ratio (y) versus analyte concentration (x) using the primary ions indicated in Table 2.

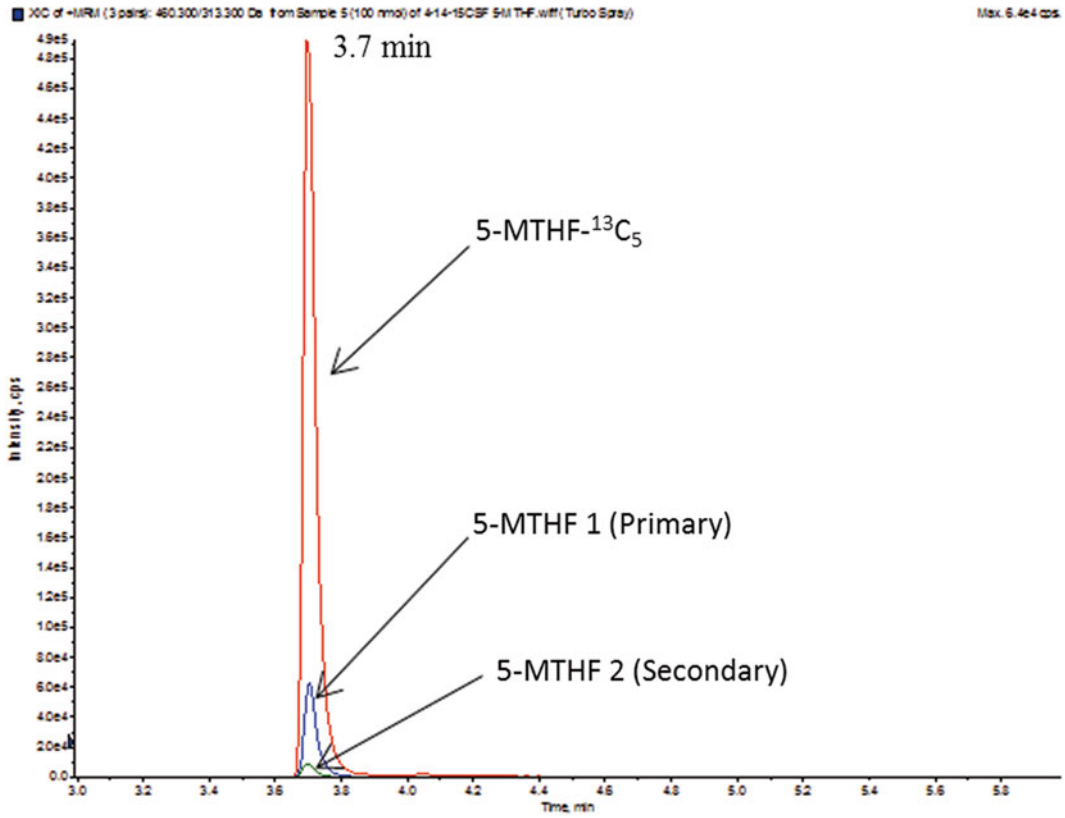

Fig. 1 HPLC-ESI-MS/MS ion chromatogram of 5-MTHF 100 nM standard. [5-MTHF 1 (*m/z* 460.3 > 313.3), 5-MTHF 2 (*m/z* 460.3 > 194.5), and 5-MTHF-13C5 (*m/z* 465.3 > 313.3)]

4. Acceptability of each run is confirmed if the calculated control concentrations fall within two standard deviations of the target values. Inter-day precision was evaluated by repeated analysis of bi-level QC material analyzed in duplicate over a period of 20 different days.
5. Liquid chromatography retention time window limits for 5-MTHF and 5-MTHF-$^{13}C_5$ are set at 3.7 (±0.2) min.
6. The assay has a lower limit of quantitation of 3 nM for 5-MTHF, with imprecision of <6 % over the entire range. *See* **Note 4** for information regarding ion suppression studies. *See* Table 3 for age-specific reference range [4].

4 Notes

1. Individual sets of 5-MTHF Standard and Internal Standard Stock Solutions and controls can be pre-aliquoted and frozen until use in each analytical run. For each set pipette specified

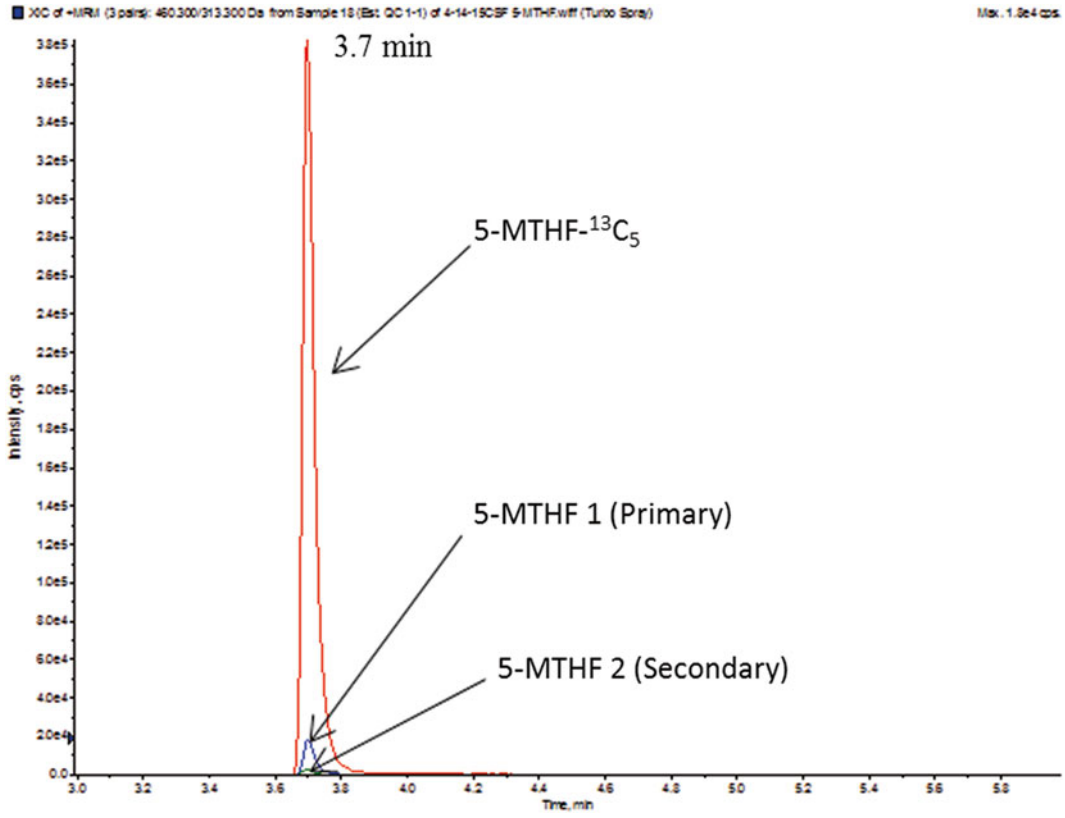

Fig. 2 HPLC-ESI-MS/MS ion chromatogram of 5-MTHF abnormal QC (30 nM). [5-MTHF 1 (*m/z* 460.3 > 313.3), 5-MTHF 2 (*m/z* 460.3 > 194.5), and 5-MTHF-13C5 (*m/z* 465.3 > 313.3)]

volume of stock standards/control solution into 1.5 mL microfuge tubes and freeze at −80 °C until use. Thaw completely before use. Stable for 4 years at −80 °C.

2. A new standard curve should be prepared with each analytical run to optimize method performance.
3. The controls are analyzed at the beginning of analysis, every five unknowns and at the end of the assay as analysis verification.
4. Ion suppression effects were evaluated by sample infusion method. No significant interferences or ion suppression was identified.

Table 1
HPLC-ESI-MS/MS operating conditions

(a) HPLC (5-MTHF)[a]		
Column temp	40 °C	
Flow rate	0.375 mL/min	
Gradient	Time (min)	Mobile phase A (%)
	0	100
	1.5	0
	2	0
	2.1	100
(b) MS/MS tune settings[b]		
Entrance potential (V)	10	
Curtain gas (psi)	20	
CAD gas	Medium	
Ion spray (V)	5500	
Temp (°C)	500	
GS 1 (psi)	50	
GS 2 (psi)	50	
Resolution Q1 and Q3	Unit	

[a]Optimized for Shimadzu prominence liquid chromatography system equipped with Phenomenex Synergi-Hydro, 4 μm, 150×3 mm analytical column; Mobile phase A: 0.1 % formic acid in water; Mobile phase B: 0.1 % formic acid in methanol
[b]Optimized for ABSciex 4000QTRAP®. Tune settings may vary slightly between instruments

Table 2
HPLC-ESI-MS/MS operating conditions

	MRM transition					
Compound	**Q1 (*m/z*)**	**Q3 (*m/z*)**	**Dwell time (ms)**	**DP (V)**	**CE (V)**	**CXP (V)**
5-MTHF 1	460.3[a]	313.3[a,b]	150	96	29	18
5-MTHF 2	460.3[a]	194.5[a,c]	150	96	48	8
5-MTHF-$^{13}C_5$	465.3[a]	313.1[a,b]	150	96	29	18

[a]Optimized *m/z* may change based on tuning parameters and instrument used
[b]Primary ion for 5-MTHF quantitation
[c]Secondary ion used for MRM ratio confirmation

Table 3
Age-specific reference range for CSF 5-MTHF

Age	5-MTHF (nmol/L)
0–1 year ($n = 12$)	63–129
2–3 years ($n = 32$)	44–122
4–18 years ($n = 19$)	42–81

Table modified from reference [3]

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