DEVELOPMENT AND VALIDATION OF A SIMPLE LC-MS/MS METHOD FOR THE QUANTIFICATION OF MTHF (N5-MÉTHYL-5, 6, 7, 8-TÉTRAHYDROFOLATE) IN HUMAN CEREBROSPINAL FLUID USING DEUTERATED MTHF AS INTERNAL STANDARD.

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Introduction and Clinical interest

Cerebral folate deficiency (CFD) has been described as any neurological condition associated with low cerebrospinal fluid (CSF) MTHF but normal folate status in plasma.
N5-methyl-5, 6, 7, 8-tétrahydrofolate (MTHF) is the most abundant circulating form of folate in biological fluids. The main passage of MTHF from the circulating blood to the CSF is made by active transport across epithelial cells of the choroid plexus. This process is accomplished by folate receptor-1 (FR-1, synonymous to FRα; gene *FOLR1*) mediated endocytosis and the proton-coupled folate transporter (PCFT) action. CFD syndromes can occur at any age and the most common cause is the presence of serum autoantibodies directed against FR-1. Less frequent causes of CFD are *FOLR1* gene defects and inborn errors of metabolism such as mitochondrial disorders and others.
Early diagnosis and treatment (with high doses folinic acid) of CFD syndromes can often prevent neurodevelopmental complications due to CNS folate deficiency.
Reference values are shown below in *Table 1*.
For recent review, read Ramaekers V. et al[1].

Objective

Our goal was to develop a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to determine MTHF in CSF and validate the assay.

Materials and methods

An High-pressure liquid chromatography (Alliance, model HT 2795, Waters) using a reverse phase C18 column coupled to a tandem mass spectrometer (Micromass Quattro micro, Waters) was used for the isocratic elution, the detection and quantification.
All calibrators and quality controls were prepared in artificial CSF as seen in Hubbard K.E.et al[2].
Stable isotopic MTHF-d_3 was used as internal standard (IS) and was eluted at the same time.
An example of chromatogram is shown below.

Sample preparation:

No pre-treatment was required, 50 µL of ascorbic acid 20g/L IS solution was mixed with 50 µL of sample solution.

LC conditions:

- Injection volume: 25 µL
- Column: LiCrospher RP-18 5µ 150mm x 4,6mm (Grace)
- Column temperature: 40°C
- Mobile phase: 40% methanol, 60% formic acid 0,1%
- Flow rate: 0,4mL/'
- Run time: 10'

MS/MS conditions:

- Positive electrospray ionization
- Mode of detection: multiple reaction monitoring
  Cone voltage 23V
  Collision energy 20eV
  MTHF transition: 460,46 → 313,46
  MTHF-d_3 transition: 463,46 → 316,46

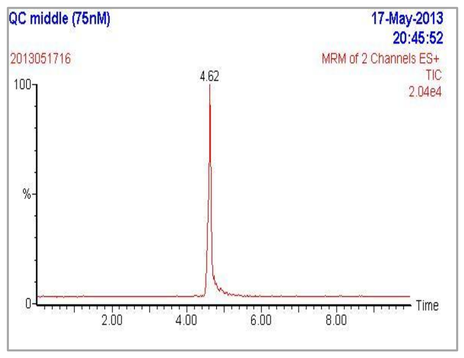


Table 1 (Mangold S et al[3])

Age (years)	Normal range (nM)	Low CSF MTHF (nM)	Very low CSF MTHF (nM)
0 -1,99	64 – 182	34,5 – 63	< 34,5
2 – 4,99	63 – 111	27 – 62	< 27
≥ 5	41 - 117	22 - 40	< 22

Validation results

Specificity:

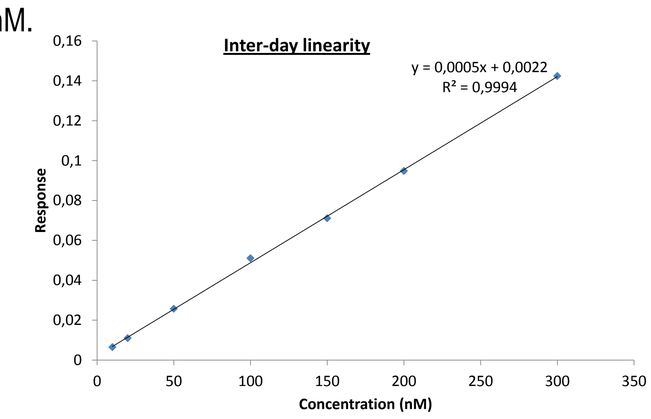
No interference was seen between 10 blank solution chromatograms and 10nM calibrator solution chromatograms.

Linearity:

The assay was linear over the concentration range of 10-300nM.

R^2_{J1}: 0,99984146
R^2_{J2}: 0,99970205
R^2_{J3}: 0,99444413
$R^2_{Inter\text{-}day}$: 0,99940532

Lower limit of detection (LLOD): 1nM
Lower limit of quantification (LLOQ): 10nM



Precision:

The intra- and inter-day precision in terms of relative standard deviation (RSD %) was within 7, 2%.

RSD%	QC low (15 nM)	QC middle (75 nM)	QC high (250 nM)
J1	7,182530955	2,777856	1,29661556
J2	5,52693789	3,5524903	3,1231947
J3	6,374333731	3,06911991	2,53009046
Inter-day	6,971419357	4,87952207	3,53927361

Accuracy: within 13,75% in term of relative error (RE %) (homemade solutions used because no available external quality control)

Target concentrations (nM)	Measured concentrations (nM)	RE (%)
8	9.1	13.75
12	13.6	13.33
15	14.3	4.67
18	18.5	2.78
36	39.3	9.17
60	59.6	0.67
75	75.9	1.20
120	119.2	0.67
180	184.6	2.56
250	254.7	1.88

Stability:

We observed good stability for calibrator and quality control solutions kept at -20°C after1 month.
We observed good stability for quality control solutions after four freeze thaw cycles except for low concentrations (good stability after only two cycles).
Furthermore, we observed rapid degradation of samples kept at 4°C. That's the reason we recommend to keep the CSF samples on ice and freeze it (-20°C) as soon as possible before measurement.
We still need to study the stability over time of CSF samples at -20°C.

Carry-over effect:

No one was observed.

CONCLUSIONS

This method fulfills all the regulatory requirements for specificity, linearity, LLOQ, precision and accuracy for the determination of MTHF in human CSF.

In practice for the CSF samples:

CONSERVATION AND SHIPPING TEMPERATURE : **-20°C**

- No need for antioxidant in the tube.
- Minimal volume required for one injection: 60µL
- Place the tube ON ICE AT THE BEDSIDE
  - If the sample is not blood contaminated, transfer it at -20°C as soon as possible.
  - If the sample is blood contaminated, first centrifuge the tube (preferably at 4°C) then transfer the clear CSF sample to another tube, and place it at -20°C as soon as possible.

References

[1]Ramaekers V, Sequeira J M, Quadros E V. Clinical recognition and aspects of the CFD syndromes. *Clin Chem Lab Med* 2013; 51(3):497-511
[2]Hubbard K.E., Wells A, Owens TS, Tagen M, Fraga CH, Stewart CF. Determination of dopamine, serotonin, and their metabolites in pediatric csf by isocratic HPLC coupled with EC detection. *Biomedical Chromatography 2010; 24(6):626-631*
[3]Mangold S, Blau N, Opladen T, Steinfeld R, Wessling B, Zerres K, Häusler M. Cerebral folate deficiency: a neurometabolic syndrome? *Molecular Genetics and Metabolism 2011; 104(3):369-372*

Conflicts of interest: none.