

Analysis of 5-methyltetrahydrofolate in serum of healthy children

Thomas Opladen ^{a,b}, Vincent Th. Ramaekers ^b, Gerhard Heimann ^b, Nenad Blau ^{a,*}

^a *Division of Clinical Chemistry and Biochemistry, University Children's Hospital Zürich, Switzerland*

^b *Division of Pediatric Neurology, Department of Pediatrics, University Hospital Aachen, Rheinisch Westfälische Technische Hochschule, Aachen University, Aachen, Germany*

Received 30 June 2005; received in revised form 22 August 2005; accepted 22 August 2005

Available online 28 November 2005

Abstract

5-Methyltetrahydrofolate (5MTHF) is the active one-carbon donor and the principal circulating form of plasma folates. It is involved in a number of metabolic and neurodevelopmental processes and analysis of cerebrospinal fluid (CSF) 5MTHF is of great importance in the diagnosis of cerebral folate deficiency (CFD). Serum 5MTHF levels and the 5MTHF serum/CSF ratio may be important additional parameters for the understanding of CFD. We developed a HPLC method for the measurement of 5MTHF in serum and established reference values for the pediatric population. Serum samples from 64 healthy children were extracted with Sep-Pak C18 cartridges and 5MTHF was separated by RP-HPLC and quantified by electrochemical detection. 5MTHF was separated from other folates and detected after 8.7 min with linearity of up to 1600 nmol/L. The detection limit was 4.5 nmol/L and recovery during solid-phase extraction for low and high concentrations of 5MTHF was 66 and 62%, respectively. Within-run imprecision (13.5%) was slightly higher than run-to-run imprecision (8.5%). 5MTHF levels in healthy children were found to be age-dependent, decreasing from 158.0 nmol/L in newborns to 60.1 nmol/L in children older than 16 years. The method we describe is sensitive, selective, and reliable for the analysis of 5MTHF from 400 μ L of serum.

© 2005 Elsevier Inc. All rights reserved.

Keywords: 5-Methyltetrahydrofolate; 5MTHF; Cerebral folate deficiency; HPLC; Electrochemical detection; Serum; Children; Folates

Introduction

Folic acid is a water-soluble vitamin that functions as one-carbon donor in various metabolic cycles. It is involved in the biosynthesis of thymidylates and purines, the methionine synthesis via homocysteine remethylation, the methylation of phospholipids, the serine and glycine interconversion, and the metabolism of histidine and formate. It is therefore essential for growth, reproduction, and maintenance of normal body function. The natural form is referred to as folate, in serum it consists mainly of 5-methyltetrahydrofolate (5MTHF) and 10-formyltetrahydrofolate in their polyglutamate derivatives and intracellular the main component exists of reduced folates [1]. Systemic folate deficiency is associated with megaloblastic anemia,

high blood levels of homocysteine, and neural tube defect in newborns [2,3].

Measurement of CSF 5MTHF by HPLC with electrochemical (EC) detection is a well established method [4] and age-related reference values for the pediatric population have been reported previously [5]. However, there is no information about the serum 5MTHF levels in children, a parameter that may be of importance in patients with cerebral folate deficiency (CFD). CFD is a recently recognized neurological disorder found in a number of children with psychomotor retardation, spastic paraplegia, cerebellar ataxia and dyskinesia. These patients have very low 5MTHF in CSF and normal blood folates and benefit from folic acid substitution [6,7].

5MTHF was first measured by microbiological and radioisotope dilution assay [8,9], later by HPLC using either EC, ultraviolet (UV) or fluorescence detection [10–14]. EC detection is the most sensitive but also most vulnerable

* Corresponding author. Fax: +411 266 7169.

E-mail address: nenad.blau@kispi.unizh.ch (N. Blau).

method. In this study, we developed an easy to use, sensitive and reliable HPLC method with EC detection that allows measurement of 5MTHF in serum. 5MTHF reference values were established for healthy children at different ages.

Materials and methods

5MTHF reference standard (L-mefolate calcium salt, 99.45% purity) was kindly provided by Eprova AG, Switzerland. Sodium acetate, EDTA, and acetic acid were purchased from Merck (Darmstadt, Germany), ascorbic acid and methanol from Fluka (Sigma–Aldrich, Switzerland). All chemicals were HPLC grade. An HPLC system including a solvent module Gold 128 (Beckman-Coulter, Inc.) with autosampler (Midas, Spark, The Netherlands) and electrochemical detection CLC-100 (Chromsystems, Munich, Germany) was used. The analytical column Spherisorb ODS-1, 5 μm , 250 \times 4.6 mm (Stagroma AG, Switzerland) was guarded by a pre-column Spherisorb C8, 5 μm , 40 \times 4.6 mm (Stagroma AG, Switzerland). Sample results were analyzed by 32 Karat Software (Beckman-Coulter, Inc.).

Preparation of standard solutions

All solutions were prepared on ice, in light-protected tubes, and with double deionizer water, after degassing and aerating with nitrogen. A 0.2 mmol/L 5MTHF stock solution was prepared by dissolving 9.95 mg of solid 5MTHF standard in a 2.8 mmol/L ascorbic acid solution. Working solutions (6.25, 12.5, 25, 50, 100, 400, 800, and 1600 nmol/L) were prepared by diluting stock solution with eluent (for details see HPLC analysis). Aliquots were covered with nitrogen and frozen at -20°C and are stable for one year.

Control persons and sample collection

Blood was collected from children presenting at the outpatient clinic at the University Children's Hospital Aachen for minor surgical intervention or routine check-ups. None had a history of acute or recent infections, chronic or syndrome diseases, malignancies, medication treatment or malabsorption. After informed parental consent blood samples were taken during a routine venous puncture. Blood was collected in tubes without additives and put on ice immediately. After centrifugation at 1500g for 15 min at 4°C , the serum fraction was separated and frozen at -20°C . Samples were processed within 3 months. The study was approved by the Ethics Committee of the Aachen University.

Sample preparation

Four hundred microliter serum was thawed immediately before measurement and mixed with 600 μl ice-cold 28 $\mu\text{mol/L}$ ascorbic acid solution to protect against oxidation. After activation of a Sep-Pak C18 cartridge (360 mg,

Waters, Milford Massachusetts) with 2 ml methanol and 10 ml of 14 $\mu\text{mol/L}$ ascorbic acid solution, 1 ml of diluted serum was applied. The cartridge was washed with 3 ml of 14 $\mu\text{mol/L}$ ascorbic acid solution and 5MTHF was eluted with 1.5 ml methanol in a light-protected tube. The eluates were evacuated under vacuum (Speed Vac, SVC 100, Savant) and dried samples were dissolved in 100 μl of 14 $\mu\text{mol/L}$ ascorbic acid solution diluted 1:2000.

HPLC analysis

5MTHF was separated using 50 mmol/L sodium acetate in 22.5% (v/v) methanol with 67 $\mu\text{mol/L}$ EDTA, pH 4.4–4.6 (adjusted with 100% anhydrous acetic acid) as a mobile phase. Forty microliter of sample (standard or serum extract) was injected. The flow-rate was maintained at 1 ml/min and the detector was set in the oxidation mode at a potential of +300 mV. Before each series of samples a calibration curve with 6.25, 12.5, 25, 50, 100, and 400 nmol/L standard solutions was run.

Performance evaluation

Recovery was calculated using serum samples spiked with 20 and 100 nmol/L 5MTHF prior to solid phase extraction. Intra-assay reproducibility was assessed by repeated analyses of the same sample on the same day. For the inter-assay reproducibility the same sample was analyzed on 10 consecutive days. The detection limit was calculated from a signal-to-noise ratio greater than 3:1 (6-sigma signal-to-noise calculation) or 6:1 (ASTM signal-to-noise calculation). Linearity was tested up to 1600 nmol/L by using diluted stock solutions. Statistical analyses were performed using SPSS software for Windows (Version 11.5.1).

Results

Sample preparation and chromatography

Solid-phase extraction by Sep Pak C18 cartridges resulted in a recovery of $66 \pm 7.9\%$ for 20 nmol/L 5MTHF, and $62 \pm 7.2\%$ for 100 nmol/L 5MTHF ($n = 10$). 5MTHF eluted around 8.7 min (Fig. 1) and the applied potential of 300 mV in oxidation mode was optimal with regard to sensitivity and selectivity (Fig. 2). Other folate metabolites eluted at different times (10-formyltetrahydrofolate at 4.6 min, tetrahydrofolate at 5.8 min, both detected at 300 mV and 5-formyltetrahydrofolate at 6.7 min, detected at 1 V) and can be clearly separated from 5MTHF (data not shown).

Linearity, reproducibility, and limit of detection

5MTHF detection was linear between 6.25 and 1600 nmol/L with a regression coefficient (R^2) of 0.999. The inter-assay coefficient of variation (CV) assessed from 10

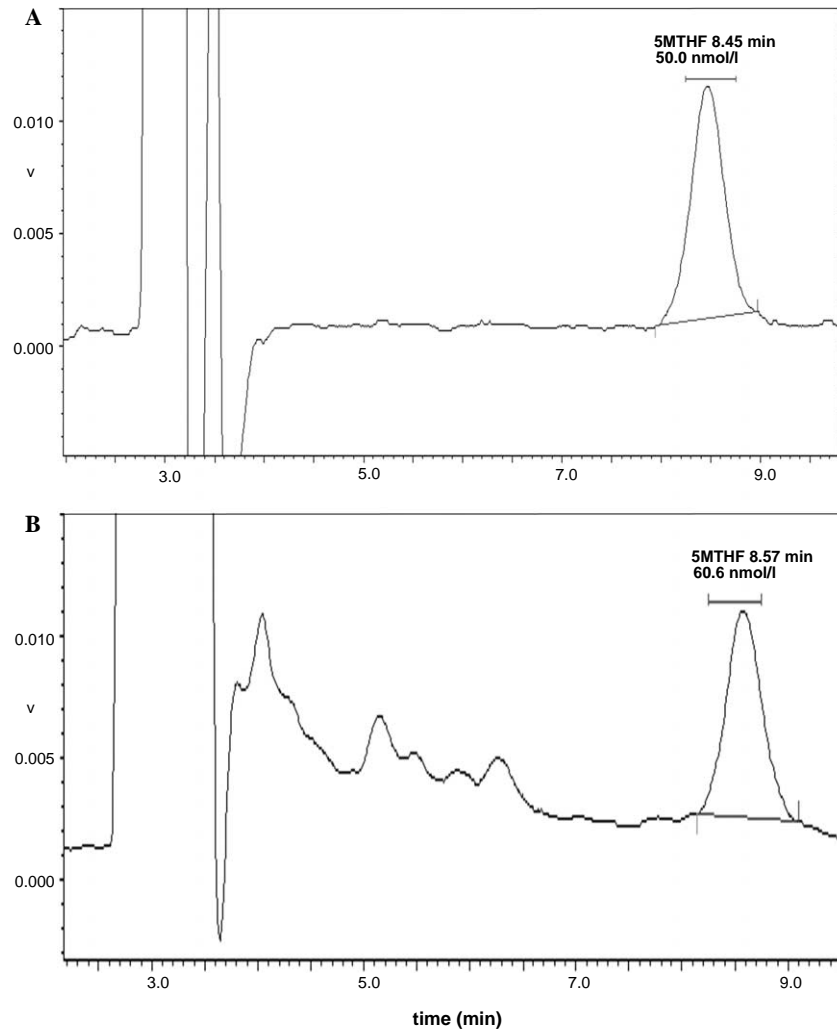


Fig. 1. HPLC of 5MTHF. (A) 50 nmol/L standard solution, (B) serum sample containing 60.6 nmol/L 5MTHF. Vitamin C elutes after 3 min.

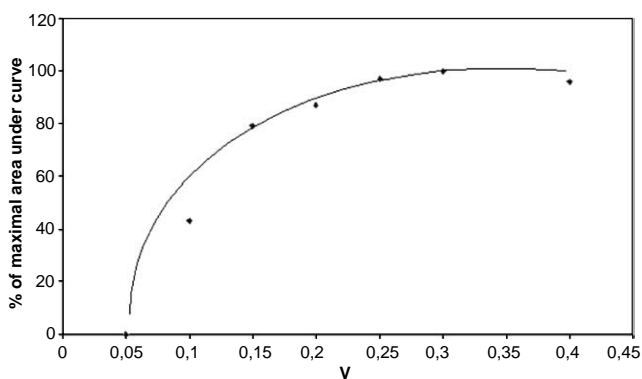


Fig. 2. Voltogram of 5MTHF generated using a 100 nmol/L standard solution. The best sensitivity and selectivity for the detection of 5MTHF was found at an applied potential of 300 mV in oxidation mode. Values expressed as percentage of maximal area under curve (AUC).

measurements on 10 consecutive days was 8.5% (mean = 57.8 nmol/L; SD = 4.9). CV of ten repeated analyses of the same sample on the same day (intra-assay) was 13.5% (mean = 83.5; SD = 11.3). The detection limit was 4.5 nmol/L.

5MTHF serum levels in healthy controls

Serum samples were obtained from 64 healthy children, 33 girls and 31 boys, aged of 1 week to 20 years. Serum 5MTHF levels were highest in the first year of life (158.0 nmol/L, range 77.4–256.8 nmol/L), followed by a continuous decrease until the age of 16 years (60.1 nmol/L range 35.6–100.9 nmol/L) (Table 1). Analysis of variance (ANOVA) revealed significant differences between age groups 1 and 2 ($p < 0.03$) but not between female and male controls (data not shown).

Discussion

Folic acid and its biologically active metabolite 5MTHF play an important role in cell function, division, and differentiation, and is therefore essential during brain development in childhood [15]. Cerebral folate deficiency (CFD) is a recently recognized neurological disorder found in a number of children with psychomotor retardation, spastic paraplegia, cerebellar ataxia, and dyskinesia. These patients have very low 5MTHF in CSF but normal

Table 1
Sixty-four serum samples from healthy children aged 1 week to 21 years were analyzed

Group	Age (years)	N	Mean 5MTHF (nmol/L)	Range 5MTHF (nmol/L)	
				Lowest value	Highest value
1	0–1.25	8	158.0*	77.4	256.8
2	3–10.99	31	97.0*	29.9	239.3
3	11–15.99	22	91.0	26.4	219.7
4	16–21	3	60.1	35.6	100.9

Results are expressed as mean and range with lowest and highest values, corrected for the reported 5MTHF recovery (details see text). The difference between the mean of age-groups 1 and 2 is significant ($p < 0.03$).

* $p < 0.03$.

blood folates, and they benefit from folic acid substitution [6,7,16]. Because the 5MTHF transport across the blood–CSF barrier seems to be disturbed in CFD, serum 5MTHF levels and serum/CSF ratios may be important for further understanding of the pathophysiology of this new syndrome. We now developed a HPLC method that allows the analysis of serum samples and we present age-related reference values for 5MTHF in children. For ethical reasons pairing serum/CSF samples from healthy children was not possible.

A number of HPLC applications are described in the literature to measure 5MTHF in biological samples. Since 5MTHF is oxygen-sensitive, EC detection provides a selective and sensitive detection method. Kohashi et al. [10] and Lankelma et al. [14] presented previously low nanomolar detection limits which allow measurement of endogenous plasma levels of 5MTHF. In contrast, UV detection with limits of around 40 nmol/L is not sensitive enough [13,14]. Using fluorescence detection, Belz et al. [13] described the detection limit for 5MTHF measurement in spiked serum at 0.2 nmol. Similar sensitivity was reported by Chladek et al. [17] for plasma samples (1.7 nmol/L). Bagley and Selhub [19] and Kok et al. [18] presented two further sensitive approaches for 5MTHF using HPLC with multi-channel electrochemical detection or with tandem mass-spectrometry. These methods are, however, rather cost intensive or require highly equipped laboratories [18,19]. All these studies lack confirmation in large normal control groups.

The method presented here is based on the use of C18 solid-phase extraction of 400 μ L of serum samples and subsequent RP-HPLC and EC detection. It is linear up to the micromolar range and the detection limit allows 5MTHF analysis down to the nanomolar range. Because of the relatively low amount of serum needed it is suitable for the pediatric population. We investigated samples from healthy, untreated children and young adults and found, similar as for CSF folates, 5MTHF concentrations in serum to be age-dependent. Higher values during the early period of life may indicate an increased need for folate at this time [15]. The age-dependent serum levels correlate well with known CSF 5MTHF values [5] and can be used to calculate the serum/CSF ratio in paired samples. Furthermore, since CFD patients benefit from folic acid substitution

[6,7,16], analysis of serum 5MTHF can also be used for therapeutic monitoring.

Acknowledgments

This work was supported in part by a research grant from the Medical Faculty Aachen, by the Dr. Emil-Alexander Huebner Foundation, and by the Swiss National Science Foundation Grant No. 310000-107500.

References

- [1] N.M.J. van der Put, H.W.M. van Straaten, F.J.M. Trijbels, B.H.J. Blom, Folate, homocysteine and neural tube defects: an overview, *Exp. Biol. Med.* 226 (4) (2001) 243–270.
- [2] M.S. Yerby, Clinical care of pregnant women with epilepsy: neural tube defects and folic acid supplementation, *Epilepsia* 44 (Suppl. 3) (2003) 33–40.
- [3] S.J. Moat, D. Lang, I.F. McDowell, Z.L. Clarke, A.K. Madhavan, M.J. Lewis, J. Goodfellow, Folate, homocysteine, endothelial function and cardiovascular disease, *J. Nutr. Biochem.* 15 (2) (2004) 64–79.
- [4] K. Hyland, R. Surtees, Measurement of 5-methyltetrahydrofolate in cerebrospinal fluid using hplc with coulometric electrochemical detection, *Pteridines* 3 (1992) 149–150.
- [5] N. Blau, M. Duran, M. Blaskovics, K.M. Gibson (Eds.), *Physician's Guide to Laboratory Diagnosis of Metabolic Diseases*, second edition, Springer, Berlin, 2005.
- [6] V.T. Ramaekers, N. Blau, Cerebral folate deficiency, *Dev. Med. Child Neurol.* 46 (12) (2004) 843–851.
- [7] V.T. Ramaekers, M. Hausler, T. Opladen, G. Heimann, N. Blau, Psychomotor retardation, spastic paraplegia, cerebellar ataxia and dyskinesia associated with low 5-methyltetrahydrofolate in cerebrospinal fluid: a novel neurometabolic condition responding to folic acid substitution, *Neuropediatrics* 33 (6) (2002) 301–308.
- [8] R.T. Dunn, L.B. Foster, Radioassay of serum folate, *Clin. Chem.* 19 (10) (1973) 1101–1105.
- [9] K.R. Thien, J.A. Blair, R.J. Leeming, W.T. Cooke, V. Melikian, Serum folates in man, *J. Clin. Pathol.* 30 (5) (1977) 438–448.
- [10] M. Kohashi, K. Inoue, H. Sotobayashi, K. Iwai, Microdetermination of folate monoglutamates in serum by liquid chromatography with electrochemical detection, *J. Chromatogr. A* 382 (1986) 303–307.
- [11] H. Ghandour, P.J. Bagley, D. Shemin, N. Hsu, P.F. Jacques, L. Dworin, A.G. Bostom, J. Selhub, Distribution of plasma folate forms in hemodialysis patients receiving high daily doses of l-folinic or folic acid, *Kidney Int.* 62 (6) (2002) 2246–2249.
- [12] W. Luo, H. Li, Y. Zhang, C.Y.W. Ang, Rapid method for the determination of total 5-methyltetrahydrofolate in blood by liquid chromatography with fluorescence detection, *J. Chromatogr. B Biomed. Sci. Appl.* 766 (2002) 331–337.
- [13] S. Belz, C. Frickel, C. Wolfrom, H. Nau, G. Henze, High-performance liquid chromatographic determination of methotrexate, 7-hydroxymethotrexate, 5-methyltetrahydrofolic acid and folic acid in serum and cerebrospinal fluid, *J. Chromatogr. B Biomed. Appl.* 661 (1) (1994) 109–118.
- [14] J. Lankelma, E. Van Der Kleijn, M.J. Jansen, Determination of 5-methyltetrahydrofolic acid in plasma and spinal fluid by high-performance liquid chromatography, using on-column concentration and electrochemical detection, *J. Chromatogr. A* 182 (1) (1980) 35–45.
- [15] J.M. Greenblatt, L.C. Huffman, A.L. Reiss, Folic acid in neurodevelopment and child psychiatry, *Prog. Neuropsychopharmacol. Biol. Psychiatry* 18 (4) (1994) 647–660.
- [16] F.J. Hansen, N. Blau, Cerebral folate deficiency: life-changing supplementation with folic acid, *Mol. Genet. Metab.* 84 (2005) 371–373.
- [17] J. Chladek, L. Sispera, J. Martinkova, High-performance liquid chromatographic assay for the determination of 5-methyltetrahydrofolate

- in human plasma, *J. Chromatogr. B Biomed. Sci. Appl.* 744 (2) (2000) 307–313.
- [18] R.M. Kok, D.E. Smith, J.R. Dainty, J.T. Van Den Akker, P.M. Fin-
glas, Y.M. Smulders, C. Jakobs, K. De Meer, 5-Methyltetrahydrofolic
acid and folic acid measured in plasma with liquid chromatography
tandem mass spectrometry: applications to folate absorption and
metabolism, *Anal. Biochem.* 326 (2) (2004) 129–138.
- [19] P.J. Bagley, J. Selhub, Analysis of folate form distribution by affinity
followed by reversed-phase chromatography with electrical detection,
Clin. Chem. 46 (3) (2000) 404–411.