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Analysis of 5-methyltetrahydrofolate in serum of healthy children

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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 μL of serum.

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 folinic 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

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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-mefolinate 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 × 4.6 mm (Stagroma AG, Switzerland) was guarded by a pre-column Spherisorb C8, 5 μm, 40 × 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 1500*g* 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 μmol/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 μmol/L ascorbic acid solution, 1 ml of diluted serum was applied. The cartridge was washed with 3 ml of 14 μmol/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 μmol/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 μmol/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 ± 7.9% for 20 nmol/L 5MTHF, and 62 ± 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