

Determination of 5-methyltetrahydrofolate in cerebrospinal fluid of paediatric patients: Reference values for a paediatric population

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Received 17 February 2006; received in revised form 3 March 2006; accepted 4 March 2006

Available online 19 April 2006

Abstract

Background: Cerebral folate deficiency (CFD) has been described as a neurological syndrome associated with low 5-methyltetrahydrofolate (5-MTHF) values in cerebrospinal fluid (CSF) with normal folate concentrations in plasma. Our aim was to analyse CSF 5-MTHF concentrations in a paediatric control population and in patients with various neurological disorders.

Methods: We studied plasma and CSF samples from 63 paediatric controls (age range: 2 days to 18 years, average: 3.8 years) and from 165 patients (age range: 1 day to 22 years, average: 5.0 years) with severe epileptic encephalopathies of unknown origin, movement disorders, Rett syndrome and mitochondrial diseases. CSF 5-methyltetrahydrofolate was analysed by reverse phase HPLC with fluorescence detection (excitation: 295 nm and emission: 355 nm).

Result: A negative correlation between 5-MTHF values and age of controls was observed ($r = -0.468$; $p < 0.0001$) and reference values were therefore stratified into 3 age groups. Regarding patients, 122 out of 165 showed normal CSF 5-MTHF values while 43 showed decreased values ranging from profound to mild deficiencies. Increased CSF total protein values were associated with the presence of low 5-MTHF concentrations ($\chi^2 = 7.796$; $p = 0.005$).

Conclusions: The application of this method has been useful for the establishment of reference values and for diagnosis of CFD in paediatric patients. Furthermore, increased CSF total protein concentrations should be considered as a marker of a possible CFD.

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Keywords: Cerebral folate deficiency; 5-Methyltetrahydrofolate; Blood–CSF brain barrier; Total proteins; Neurotransmitters; Paediatric patients

1. Introduction

Cerebral folate deficiency (CFD) has been described as a neurological syndrome associated with low 5-methyltetrahydrofolate (5-MTHF) values in cerebrospinal fluid (CSF) with normal folate concentrations in plasma [1]. Recently, it has been demonstrated that CFD in some patients is caused by autoantibodies against high affinity folate receptor (FR1) [2]. Furthermore, CFD has also been associated with several

disorders, such as Rett syndrome, Aicardi–Goutieres and Kearns–Sayre syndrome (KSS) [3,4].

Folate is involved in almost 100 metabolic reactions [4], and CFD might be associated with impaired biosynthesis of some essential molecules. Previous articles have reported a possible association between low 5-MTHF values and impaired tetrahydrobiopterin biosynthesis caused by a reduction in guanosine triphosphate concentrations (folate is involved in the purine biosynthetic pathway). Consequently, since tetrahydrobiopterin is the essential cofactor in the rate-limiting steps of dopamine and serotonin biosynthesis, a reduction of biogenic amine metabolites (especially 5-hydroxyindoleacetic acid) has been previously reported [1].

Our aim was to standardize an HPLC procedure with fluorescence detection for 5-MTHF analysis in CSF and to establish reference values in a paediatric population. Moreover,

Abbreviations: CFD, cerebral folate deficiency; 5-MTHF, 5-methyltetrahydrofolate; CSF, cerebrospinal fluid; KSS, Kearns–Sayre syndrome; 5-HIAA, 5-hydroxyindoleacetic acid; HVA, homovanilic acid; NP, neopterin; BP, biopterin.

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we analyzed CSF samples from paediatric patients with various neurological disorders.

2. Materials and methods

2.1. Control subjects

For reference values, plasma and CSF samples from 63 paediatric controls (age range: 2 days to 18 years, average: 3.8 years) were simultaneously analysed. In this control population, lumbar puncture was performed for the diagnosis of viral or bacterial meningitis, encephalitis, and other neurological conditions of non-metabolic origin. Exclusion criteria were plasma folate deficiency, clinical profile compatible with CFD, presence of bacterial meningitis, traumatic or tumoral disorders of the central nervous system, intracerebral haemorrhage, CSF blood contamination and increased total protein concentration in CSF.

2.2. Patients

We studied plasma and CSF samples from 165 patients (age range: 1 day to 22 years, average: 5.0 years) with severe epileptic encephalopathies of unknown origin, movement disorders, Rett syndrome and mitochondrial diseases. Plasma and CSF samples from patients and controls were collected on the same day between 8:00 and 10:00 a.m., following a previously reported protocol [5]. For 5-MTHF determination, CSF samples were taken and immediately frozen at -80°C until analysis.

Samples from patients and controls were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. Informed consent was obtained from parents, and the ethics committee of the Hospital Sant Joan de Déu approved the study.

2.3. Biochemical studies

For 5-MTHF analysis, CSF samples were diluted 1:2 in 5 mg/mL of ascorbic acid diluted in phosphate buffer 5 mmol/L (pH=2.3), centrifuged at $1500\times g$ (10 min), and filtered through 0.45- μm nylon filters (Millipore, Bedford, MA, USA; reference: SLHVR04NK). 5-MTHF calibrator (Sigma Chemical Co, St Louis, MO, USA; Reference: M-0132) was diluted to attain a final concentration of 22 nmol/L. 20 μL of sample or calibrator was injected onto the HPLC. 5-Methyltetrahydrofolate was analysed by reverse phase HPLC with fluorescence detection (Waters, MA, USA) following a previously reported procedure [6] with some modifications. Briefly, the mobile phase consisted of phosphate buffer (5 mmol/L, pH2.3) mixed with acetonitrile (93:7 v/v) for 5 min followed by a linear gradient (curvature=6) to achieve a proportion of 83:17 (v/v). Fluorescent conditions were excitation 295 nm and emission 355 nm. 5-MTHF was separated in a nucleosil C-18 column (150 mm \times 0.4 mm, 5 μm particle size; Teknokroma, Barcelona, Spain). Chromatographic data were processed with the Breeze 3.3 GP software (Waters, MA, USA). Flow rate was 0.8 mL/min.

Biogenic amine metabolites in CSF (5-hydroxyindoleacetic (5-HIAA), homovanilic acids (HVA), pterins (neopterin (NP), and biopterin (BP)) were measured by ion pair HPLC with electrochemical and fluorescence detection following previously reported procedures [5]. CSF total protein was analysed by automated procedures in an Architect c8000 system (Abbott Laboratories, IL, USA). Increased total protein concentration in CSF was considered when values were higher than the upper limit of our reference intervals: 1.20 g/L in newborns (1–7 days), 0.80 g/L (8–30 days), 0.65 g/L (1–2 months) and 0.40 g/L (3 months to 18 years).

To establish reference values for the CSF/plasma folate ratio (a marker of folate transport across blood–CSF barrier), plasma folate concentrations were analyzed by automated chemiluminescent immunoassays (ADVIA Centaur, Bayer, Tarrytown, NY, USA).

Kolmogorov–Smirnov test was applied to assess data distribution. Since data followed a Gaussian distribution, Pearson test was applied to search for correlation among the different variables in the study. Student's *t*-tests were applied to compare 5-MTHF results between the different age groups. Chi-square test was applied to search for association between qualitative variables (folate deficiency and the presence of increased CSF total protein concentrations). Statistical calculations were performed with the SPSS 12.0 program.

3. Results

Within-run imprecision was 2.70% (56.4 nmol/L) and between-run imprecision was 1.92% (16.4 nmol/L). The procedure was linear over the range of 0.5–250 nmol/L.

Reference values and patient data are reported in Table 1. A negative correlation between 5-MTHF values and age of controls was observed ($r=-0.468$; $p<0.0001$) (Fig. 1) and reference values were therefore stratified into 3 age groups. Student's *t*-test showed significant differences among these groups (Table 1). Regarding patients, 122 out of 165 showed normal CSF 5-MTHF values (data not shown), while 43 showed

Table 1
Reference values of 5-MTHF in a paediatric population and patients with CFD

	5-MTHF (nmol/L)	Plasma folate (nmol/L)	CSF/plasma
<i>Controls</i>			
A 0–1 year (<i>n</i> =12)	103 (20.4) 63–129	32 (9.58) 20–52	3.5 (1.2) 1.6–6.3
B 2–3 years (<i>n</i> =32)	72 (19.1) 44–122	34 (13.5) 12–64	2.4 (0.9) 1.2–4.4
C 4–18 years (<i>n</i> =19)	56 (10.7) 42–81	18.1 (4.9) 12–27	3.2 (0.7) 1.8–4.3
<i>Patients with low 5-MTHF</i>			
0–1 year (<i>n</i> =5)	42 (13.5) 27–61	27 (14.4) 16–44	1.6 (0.9) 0.6–2.2
2–3 years (<i>n</i> =8)	31 (12.9) 2–41	25 (13.6) 5–38	1.4 (1.1) 0.4–3.6
4–18 years (<i>n</i> =30)	31 (9.6) 0.6–42	12 (7.9) 5–44	3.0 (1.3) 0.06–4.6

Results were expressed as average (S.D.) range. Student's *t*-test: group A vs. B: 5-MTHF, $p<0.0001$. Group B vs. C: 5-MTHF, $p=0.002$.

5-MTHF: 5-methyltetrahydrofolate. CSF: cerebrospinal fluid.

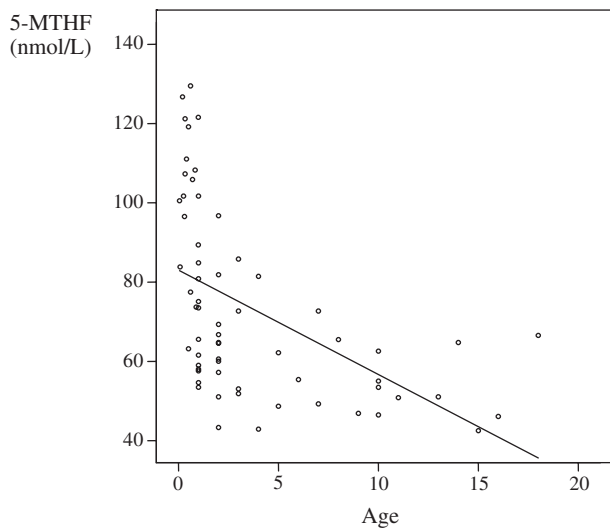


Fig. 1. Negative correlation between 5-MTHF and age in the control group.

decreased values ranging from profound to mild deficiencies (Table 1). Among these, 5 presented plasma folate deficiency and 8 decreased CSF/plasma folate ratio (suggesting a transport defect across blood–CSF barrier), while the other 30 showed neither plasma folate deficiency nor decreased CSF/plasma folate ratio. Clinically, among these 43 patients with CSF 5-MTHF deficiency, 4 were diagnosed with a mitochondrial disorder (3 presenting a KSS with CSF 5-MTHF values of 0.6, 7.8 and 24.2 nmol/L, respectively), 8 had Rett syndrome, 4 had clinical signs compatible with CFD caused by autoantibodies, and the other 27 had severe epileptic encephalopathy and movement disorders of unknown origin. Taking all patients together ($n=165$), 11 out of the 43 patients who presented with decreased 5-MTHF values in CSF showed CSF total protein concentration higher than the upper limit of our reference intervals, while only 10 out of 122 with normal 5-MTHF values showed such increased CSF total protein values ($\chi^2=7.796$; $p=0.005$). No correlation was observed (Pearson test) between 5-MTHF and BP, NP 5-HIAA, and HVA after correction for age in controls and patients.

4. Discussion

We present here an HPLC with fluorescence detection procedure [6] whose metrological data suggest that the quality of the procedure is appropriate for diagnosis of CFD. To date, reference values for CSF 5-MTHF and CSF/plasma folate ratio in paediatric patients have scarcely been reported [1]. As previously reported [1], CSF 5-MTHF concentrations were age-related, while CSF/plasma values were not age-related.

Besides CFD caused by autoantibodies [2], decreased CSF 5-MTHF values may be associated with other neurological conditions. According to our results, patients with mitochondrial disorders (especially those with KSS) presented a profound 5-MTHF deficiency. In these patients, an impaired 5-MTHF transport across choroid plexus has been proposed as the main mechanism explaining these profound deficiencies [7]. Moreover, patients with other neurological disorders such as Rett

syndrome may also present decreased 5-MTHF values. All these data suggest that monitoring of 5-MTHF values is advisable in these disorders, since therapeutic administration of folinic acid may reverse some of the neurological signs [3,8,9].

We found 8 patients with decreased CSF/plasma folate ratio, suggesting a transport defect across blood–CSF brain barrier. Among these, 3 were those with KSS, supporting a choroid plexus dysfunction in this syndrome, and 4 with clinical features resembling CFD (motor regression after a normal period of development, seizures and choreoatetotic movements). A further 5 patients presented plasma folate deficiency together with low CSF 5-MTHF values, highlighting the importance of the simultaneous determination of plasma folate status in order to identify systemic folate deficiency as a cause of decreased CSF 5-MTHF concentrations. Taken together, 43 out of 165 patients studied presented low CSF 5-MTHF values, suggesting that CFD was a relatively common observation in our paediatric patients. Although a final diagnosis was not achieved in all of these cases, the early detection of CFD would allow starting folinic acid supplementation, since it seems that patients treated early with this drug respond better than those with a late folinic acid treatment [9].

Increased CSF total protein concentration has been related with neurological diseases associated with either increased permeability of blood–CSF barrier or increased intrathecal immunoglobulin synthesis [10]. In KSS, it has been suggested that the accumulation of mutated mtDNA in the choroid plexus could be associated with the characteristically high CSF protein values and the CSF 5-MTHF deficiency [7]. This led us to analyse a possible association between the presence of increased CSF total protein values with decreased CSF 5-MTHF in our patients, resulting in a statistically significant association between the two factors. Therefore, CSF 5-MTHF measurement seems advisable in patients presenting increased total protein concentrations, especially in those with a possible blood–CSF barrier dysfunction. The simultaneous measurement of albumin concentrations both in blood and CSF would provide more information about the blood–CSF barrier status than total protein values, and this index has been applied to estimate impaired protein transport across blood/CSF barrier [11]. Unfortunately, we could not measure plasma and CSF albumin in the present study, but our results suggest that the calculation of albumin plasma/CSF ratio should be included when low CSF 5-MTHF and increased total protein values are detected.

Due to the elevated number of reactions in which folate is involved, the consequences of CFD may be very variable; involvement of decreased biogenic amine turnover has been suggested as a possible pathophysiological mechanism [4]. However, according to our data, no association was demonstrated between 5-MTHF values and pterins or neurotransmitter metabolites. Even patients with a profound folate deficiency showed normal biogenic amines values, and therefore, other pathogenic mechanisms are probably related with CFD.

In conclusion, the application of this method has been useful for the establishment of reference values and for the diagnosis of CFD in paediatric patients. Furthermore, increased CSF total

protein concentrations should be considered as a marker of a possible CFD. No relationship was observed between 5-MTHF values and pterin and neurotransmitter concentrations.

Acknowledgements

This work was supported by FIS grants REDEMETH G03/054, INERGEN C03/05 and Marató TV3 (014010).

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