

BRIEF COMMUNICATION

Detection of Sepiapterin in CSF of Patients with Sepiapterin Reductase Deficiency

Sepiapterin reductase (SR) deficiency was recently described in patients with a severe biogenic amine deficiency presenting without hyperphenylalaninemia and it was suggested that the tetrahydrobiopterin (BH₄) pathway may be different in different cells and tissues. We now developed a HPLC method for the measurement of yellow fluorescing sepiapterin for the rapid diagnosis of SR deficiency. Sepiapterin was elevated in CSF from two patients with SR deficiency (5.6 and 11.4 nmol/L) when compared with healthy controls (<0.5 nmol/L). Our data further support the hypothesis that sepiapterin is an intermediate in the salvage pathway of BH₄ and that it accumulates in the brain of patients with SR deficiency. © 2002 Elsevier Science (USA)

Tetrahydrobiopterin (BH₄) deficiency is a rare form of hyperphenylalaninemia caused by autosomal recessive mutations in the genes encoding for the enzymes involved in the biosynthesis or regeneration of BH₄ (1). BH₄ acts as a cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases (2) and any defect in its metabolism affects both phenylalanine and biogenic amine neurotransmitter homeostasis. Furthermore, BH₄ is essential for the production of the nitric oxide (NO) free radical by all the isoforms of NO synthase, and patients with BH₄ deficiency have reduced production of NO in the brain (3). Patients with BH₄ deficiencies present with mental retardation, convulsions (grand mal or myoclonic attacks), disturbance of tone and posture, oculogyric crises, drowsiness, irritability, abnormal movements, recurrent hyperthermia without infections, hypersalivation, and swallowing difficulties. Two forms of BH₄ deficiency present without hyperphenylalaninemia: Dopa-responsive dystonia due to autosomal dominant mutations in the GTP cyclohydrolase I gene and the recently discovered sepiap-

terin reductase (SR) deficiency (4,5). In contrast to classical forms of BH₄ deficiencies which can be detected via the newborn screening for phenylketonuria (PKU) these disorders can be diagnosed only by CSF investigations of dopamine and serotonin metabolites (5-hydroxyindoleacetic acid and homovanillic acid) and pterins (neopterin and biopterin) (6). More accurate diagnosis is possible by the measurement of neopterin and biopterin in cytokine-stimulated fibroblasts or by the enzyme activity measurement in stimulated cells for the GTP cyclohydrolase I and in the nonstimulated cells for SR (7).

Diagnosis of SR deficiency is not simple due to a) the absence of hyperphenylalaninemia and b) the fact that the pterins profile in CSF is the same as in patients with defective dihydropteridine reductase (high dihydrobiopterin and biopterin). However, because in SR patients dihydropteridine reductase activity is normal, dihydrobiopterin probably originates from sepiapterin formed in the salvage pathway. The two-step reduction of the intermediate 6-pyruvoyltetrahydropterin to BH₄ can be catalyzed by two additional reductases, carbonyl and aldose reductase. We developed a new approach for the rapid diagnosis of SR deficiency by measurement of sepiapterin in CSF.

PATIENTS AND METHODS

Patients. Patients with the SR deficiency are registered in the international BIODÉF database (www.bh4.org/biodef1.html). Diagnosis was established by the measurement of pterins and SR activity in cytokine-stimulated and in nonstimulated cultured skin fibroblasts, respectively, as published previously (5). Initial analysis of CSF revealed high

dihydrobiopterin and biopterin concentrations and a severe biogenic amine neurotransmitter deficiency (8). The procedures used were in accordance with the current revision of the Helsinki Declaration of 1975. Appropriate informed consents were obtained from all subjects.

Chemicals. Pterins (xanthopterin, sepiapterin, 3-hydroxysepiapterin) were obtained from Dr. Schircks Laboratories (Jona, Switzerland) and Sep-Pak C18 cartridges were from Waters Associates (Milford, MA).

Sample concentration on the Sep-Pak C18. Sepiapterin and its analogue are very sensitive to light and oxygen. When not protected both pterins were oxidized to a nonfluorescent 7,8-dehydro compound (6-lactylpterins). Therefore, samples and standard solutions should be kept in dark vials at -20°C . Prior to use cartridges were activated with 2 ml of methanol and 10 ml of water as previously described by Ferre and Jacobson (9). Then, 0.5 ml of fresh CSF (stored at -20°C protected from light) was applied, and the column was washed with 3 ml of water and eluted with 1.5 ml of methanol. The methanol fraction was evaporated in a Speed Vac Concentrator SVC-100H (Savant Instruments, Formingdale, NY) for 3–4 h protected from light. The residue was dissolved in 100 μl of water (degassed with nitrogen) and used for the HPLC of pterins.

HPLC of the yellow fluorescing pterins. The following equipment was used: precolumn (10×4.6 mm) Li Chrosorb RP8, 5 μm ; analytical column (125×4.6 mm) Li Chrosorb RP8, 5 μm (both from Stagma AG, Herrenberg-Kayh, Germany); System Gold HPLC (Beckman Instruments, Fullerton, CA); and Model L-7480 fluorimeter (Hitachi, Tokyo, Japan) with settings 425/530 nm, time constant, 0.1.

The following solvents were used: A, isopropanol:methanol:acetic acid:water (0.5:0.5:0.05:98.95; v/v); B, isopropanol:methanol:acetic acid (49:49:2). All solvents were degassed with nitrogen. The flow rate was maintained at 1.0 ml/min using the following gradient program: solvent A (100%), 2 min; solvent B (0–2%), 5 min; solvent B (2%), 2 min; solvent B (2–20%), 10 min; solvent B (20%), 1 min; solvent B (40%), 5 min; solvent A (100%), 10 min.

An external standard mixture of xanthopterin (X), 3-hydroxysepiapterin (HS), and sepiapterin (S), each 200 nmol/L in water, was stored in small dark vials at -20°C .

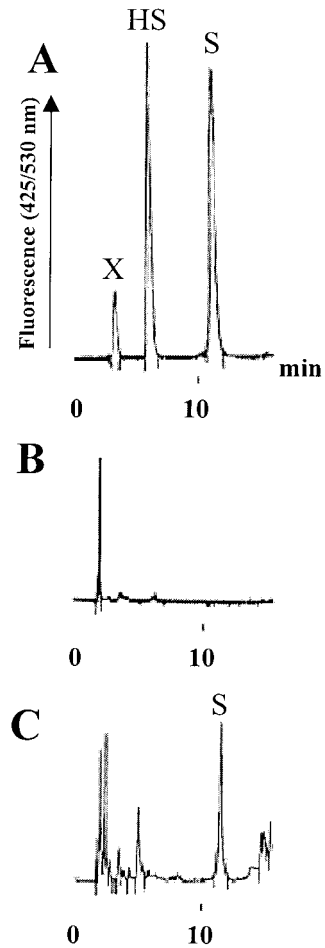


FIG. 1. HPLC of the yellow fluorescing pterins. (A) Standard mixture containing xanthopterin (X), 3'-hydroxysepiapterin (HS), and sepiapterin (S), 200 nmol/L of each; (B) control CSF; (C) CSF from the patient with SR deficiency. For details see Patients and Methods.

RESULTS

CSF analysis. Chromatograms of a standard mixture and CSF from a control person and the patient with SR deficiency are shown in Fig. 1. Normal CSF contains no xanthopterin, 3'-hydroxysepiapterin, or sepiapterin (<0.5 nmol/L). In contrast, in CSF from two patients with SR deficiency, sepiapterin was significantly increased (5.6 and 11.4 nmol/L). There was no 3'-hydroxysepiapterin and only traces of xanthopterin were detectable. We also investigated two CSF samples from patients with 6-pyruvoyltetrahydropterin synthase deficiency; however, we found no 3'-hydroxysepiapterin (data not shown). In contrast to the patient investigated

by Niederwieser *et al.*, (10), our patients were already on treatment with BH₄.

Urine analysis. HPLC of yellow fluorescing pterins revealed numerous unknown peaks in both control urines and samples from patients with SR deficiency; however, there was no sepiapterin detectable (data not shown). Using the same system with the longer run time urinary riboflavin can be measured (RT, 25 min).

DISCUSSION

SR deficiency is a recently recognized form of BH₄ deficiency (5,11). Affected patients present with severe dopamine and serotonin deficiency without hyperphenylalaninemia and thus cannot be detected by the newborn screening for PKU. So far, three patients have been detected and in all of them initial investigations of CSF pterins revealed high concentrations of dihydrobiopterin and biopterin (6). Using the new assay with cytokine-stimulated fibroblasts (7) and the DNA analysis (5), final diagnosis was established. It has been suggested that in patients with SR deficiency, peripheral BH₄ biosynthesis proceeds via the route catalyzed by carbonyl, aldose, and dihydrofolate reductases (5) and that in the brain 7,8-dihydrobiopterin cannot be converted to BH₄ due to the low dihydrofolate reductase activity. To test the hypothesis that in patients with SR deficiency brain 7,8-dihydrobiopterin originates from sepiapterin we investigated their CSF using a new HPLC system for yellow fluorescing pterins. This was confirmed by increased levels of sepiapterin, but not xanthopterin and 3'-hydroxysepiapterin in both patients. Sepiapterin was not detectable in CSF from patients with dihydropteridine reductase deficiency, another form of BH₄ deficiency presenting with high 7,8-dihydrobiopterin in the brain (12). In contrast to the brain enzyme, peripheral dihydrofolate reductase may efficiently reduce 7,8-dihydrobiopterin to BH₄ and thus maintain hydroxylation of phenylalanine in the liver. The amount of BH₄ produced in peripheral tissue is sufficient to prevent hyperphenylalaninemia. We were also not able to detect any sepiapterin in urine of patients with SR deficiency.

The HPLC method we described is simple and fast and can detect as little as 1 nmol/L of sepiapterin. Measurement of sepiapterin in CSF may not only be important for the rapid diagnosis of SR deficiency but may also contribute to the elucidation of the

negative effect of sepiapterin and positive effect of BH₄ on the neuronal NOS (13). Recently, it has been shown that not only 7,8-dihydrobiopterin but also sepiapterin can uncouple the NOS reaction by releasing the prebound BH₄ (14) and that under BH₄-deficient conditions consumption of NADPH can become uncoupled from NO synthesis, resulting in the production of superoxide anions and peroxyntirite (15). We proposed recently that in patients with SR and dihydropteridine reductase deficiency uncoupling of the NOS reaction may lead to neuronal cell dysfunction (6,11). This event may potentate neurological abnormalities caused by dopamine and serotonin depletion in these patients.

ACKNOWLEDGMENTS

The authors thank M. Killen for editorial work. This work was supported by the Swiss National Science Foundation, Grant 31-54183.98.

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Received October 26, 2001, and in revised form November 19, 2001

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