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Enzymatic diagnosis of homocystinuria by determination of cystathionine-ß-synthase activity in plasma using LC-MS/MS

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ABSTRACT

Background: Cystathionine β-synthase (CBS) is released into plasma from organs expressing this enzyme. Decreased plasma CBS activity has been demonstrated in CBS-deficient patients with 16 different genotypes. The aim of this study was to determine plasma CBS activity in patients carrying 11 additional genotypes using two LC-MS/MS methods.

Patients and methods

CBS activity was measured in EDTA or heparin plasma using either a previously described or a newly developed LC-MS/MS method optimized for analysis of the reaction product, 3,3-$^{2}H_{2}$-cystathionine, as its butyl ester derivative. We analyzed plasma samples from 26 CBS-deficient patients with known genotypes and 57 controls.

Results: We developed a new LC-MS/MS method for simple and sensitive determination of CBS activity. Plasma CBS activity was low (i.e., 0.001–0.036 of the multiples of median control values, MoM) in patients homozygous for the prevalent Hispanic mutation c.572C > T (p.T191M) but was highly elevated (2.95 MoM) in a single patient homozygous for the c.1330G > A (p.D444N) mutation. Patients with the remaining nine genotypes exhibited decreased activities (0.00–0.22 MoM), which did not overlap with the controls (0.29–2.10 MoM).

Conclusions: The determination of CBS activity in plasma is a rapid and non-invasive procedure for detecting a subgroup of CBS-deficient patients with distinct genotypes.

1. Introduction

Cystathionine β-synthase (CBS, L-serine hydrolyase, EC 4.2.1.22) is the first enzyme in the transsulfuration pathway, in which the potentially toxic amino acid homocysteine is converted to cysteine. CBS deficiency (MIM *236200) is a well-known genetic disease and represents the most common cause of homocystinuria. Diagnostic hallmarks of this disease are grossly elevated concentrations of plasma total homocysteine, combined with decreased plasma concentrations of cysteine and cystathionine and variable elevation of plasma methionine [1]. CBS deficiency is inherited as an autosomal recessive trait, and common clinical features include developmental delay/intellectual disability, dislocation of the optic lenses and/or severe myopia, skeletal abnormalities and/or osteoporosis and vascular diseases, such as thromboembolism [2]. Expressivity is variable for all of the clinical signs, and it has been suggested that an unknown subset of patients with CBS deficiency may suffer from a mild vascular form of the disease or may even be asymptomatic [3,4]. Two phenotypic variants are recognized: pyridoxine responsive and non-responsive homocystinuria, with the former usually, but not always, being milder than the latter [5].

Thus far, more than 160 different mutations in the *CBS* gene have been identified worldwide (http://cbs.lf1.cuni.cz/search_res.php), approximately 87% of which are missense mutations. The pyridoxine responsive mutation p.I278T affects 45% of the alleles found in Europe, while the pyridoxine non-responsive variation p.T191M is the most common in Spain, Portugal and American Hispanics [6,7]. These mutations are in turn highly population-specific and determine biochemical behavior. DNA analysis may be used to confirm the diagnosis only when mutations with known pathogenicity are found at both patient *CBS* alleles, and its utility is limited in other situations.

The demonstration of decreased enzyme activity is a common diagnostic approach in patients with inborn errors of metabolism (IEMs), including CBS deficiency. Because the majority of enzymes that are relevant to IEMs are located within cells, the determination of enzymatic activity requires sampling of patient tissue in most cases, ranging from simple venipuncture for the isolation of blood cells to biopsies of skin or even of organs (e.g., liver and muscle). In addition, laborious,

Abbreviations: CBS, Cystathionine ß-synthase; EDTA, Ethylenediaminetetracetic acid; DTT, Dithiothreitol; IEM, Inborn errors of metabolism; IS, Internal standard; LC-MS/MS, Liquid chromatography-tandem spectrometry; MoM, Multiples of median; PLP, Pyridoxal 5′-phosphate; SAM, S-adenosyl-L-methionine.

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time-consuming and technically demanding culture of tissues, such as skin, may be required to produce sufficient amounts of cells expressing the enzyme of interest. Intracellular enzymes can be released into the plasma in small amounts, and the amounts of these non-plasma-specific enzymes in body fluids may increase if cell integrity is impaired. Clinical chemistry laboratories utilize this phenomenon to routinely analyze the activities of liver, pancreas, muscle, myocardium, prostate and other organ-specific enzymes as biomarkers of diseases [8]. The determination of enzyme activities in extracellular fluids is only rarely used for routine diagnostic purposes in IEMs, with the exception of some lysosomal storage diseases or biotinidase deficiency.

Recently, we have demonstrated that CBS is released into the plasma from organs expressing this enzyme and that its activity can be determined using a sensitive LC-MS/MS assay employing deuterium-labeled substrates. Moreover, we confirmed that the activity of this enzyme is decreased in a substantial proportion of plasma samples from CBS-deficient patients [9].

In the present work, we report studies from two different laboratories addressing plasma CBS activity in homocystinuric patients measured by LC-MS/MS, extending the number and spectrum of previously published genotypes. Laboratory A used the previously described method [9], while in Laboratory B, we developed a modified method described in detail in this paper.

2. Materials and methods

2.1. Plasma samples

In Laboratory A, EDTA plasma samples were obtained from ten genotyped Czech CBS-deficient patients (five females and five males, age range from 1 to 73 years). Eight patients were pyridoxine responsive. The most common mutation in the group was the pyridoxine responsive mutation c.833 T > C (p.I278T), found either in homozygosity ($n = 3$) or in compound heterozygosity ($n = 2$). Two patients (CZ-1 and CZ-2) were homozygous for the pyridoxine non-responsive mutation c.210-1G > C (p.A69fs*94), while CZ-8 and CZ-9 were compound heterozygotes for c.146C > T/c.1007G > A (p.P49L/p.R336H), and CZ-10 was a compound heterozygote for c.1224-2A > C/c.1330G > A (p.W409_G453del/p.D444N). Twenty-three EDTA plasma control samples were included in this study (twelve females, eleven males, age range from 1 to 54 years). The EDTA plasma samples were stored at −20 °C and CBS activity was determined less than 3 months since collection.

In Laboratory B, the heparin plasma samples were obtained from twenty-one patients (20 Spanish and one of North African origin), consisting of ten females and eleven males, ranging in age from days to 46 years, who were referred to our laboratory for diagnosis or monitoring of treatment. The samples corresponded to two groups: a) subjects with a known genotype ($n = 16$) and b) patients with classical clinical and biochemical features of CBS deficiency (hyperhomocysteinemia >150 μmol/L, hypermethioninemia >500 μmol/L, normal excretion of organic acids), but without genetic confirmation ($n = 5$).

Most of the genotyped patients carried the pyridoxine non-responsive mutation c.572C > T (p.T191M) in either homozygosity ($n = 11$) or compound heterozygosity ($n = 2$) (c.572C > T/c.253delG) and (c.572C > T/c.209 + 1G > T). Case E-13 was homozygous for the variation c.1330G > A (p.D444N), and E-14 and E-15 were compound heterozygotes for c.333C > G/c.1330G > A (p.P111L/p.D444N) and c.209 + 1G > A/c.19dupC (delEx1or153del57/p.Q7fs).

Anonymous heparin plasma control samples were obtained from samples remaining after routine analysis in the laboratory. This collection included thirty-four individuals (twenty-two males, twelve females) divided into four age groups: <2 years, 2–9 years, 10–15 years and adults. All of the analyzed samples were kept frozen at −20 °C until analysis.

2.2. Sample suitability

In Laboratory A, only EDTA plasma samples stored at −20 °C for less than 3 months since collection with a maximum of 3 freeze–thaw cycles were used without further testing. In Laboratory B archived samples were used and biotinidase activity was determined in the plasma samples as an internal control of sample integrity [10]. Normal activity (4.5–12.2 nmol/min/mL) was used as an inclusion criterion in this study, and undetectable biotinidase activity, usually found in plasmas that had been kept frozen longer than four years, was applied as an exclusion criterion. Plasma samples from control individuals with elevated transaminases or/and severe hepatic damage clearly showed extremely elevated CBS activity (614–5783 nmol/L/h) and are not presented in this paper.

2.3. Chemical reagents and apparatus

Laboratory A used the reagents and equipment described previously [9]. Laboratory B used the following reagents: (2-amino-2-carboxyethyl) homocysteine-3,3,4,4-d_4 ($^2H_4$-cystathionine), as an internal standard (IS), purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada); 2,3,3-$^2H_3$-L-serine, as a reaction substrate, from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA); and L-homocysteine-thiolactone hydrochloride, L-cystathionine, dithiothreitol (DTT), pyridoxal 5′-phosphate (PLP) and S-adenosyl-L-methionine (SAM) from Sigma-Aldrich (St. Louis, Missouri, USA). Hydrogen chloride in 1-butanol was obtained from Fluka (St. Louis, Missouri, USA), methanol and water (LC-MS grade) were obtained from Merck (Darmstadt, Germany), and ammonium acetate was obtained from BDH Prolabo (Barcelona, Spain). The LC-MS/MS system consisted of an 1100 Agilent Series HPLC (Santa Clara, California, USA) coupled to an Applied Biosystems API 2000 QTrap (Carlsbad, California, USA).

2.4. CBS assay

The CBS assay was performed as described in [9] in Laboratory A and in a slightly modified manner in Laboratory B. Briefly, 80 μL of plasma was added to 25 μL of the reaction solution (200 mmol/L Tris–HCl (pH 8.6), 0.5 mmol/L SAM, 1 mmol/L PLP and 40 mmol/L 2,3,3-$^2H_3$-L-serine). The assay was initiated by the addition of 5 μL of starting solution (final concentrations are shown) prepared by incubating 280 mmol/L homocysteine thiolactone in 1.225 mol/L NaOH at 37 °C for 5 min, followed by the addition of 100 mmol/L Tris–HCl (pH 8.6), 10 mmol/L DTT and 1 mol/L HCl.

The final assay mixture (110 μL) was incubated at 37 °C in a water bath for 4 h or was stopped immediately if a blank was being processed. The reaction was then stopped by adding 100 μL of 30% TCA containing 1 μmol/L of the IS ($^2H_4$-cystathionine) and then kept at 4 °C until LC-MS/MS analysis. Finally, the suspension was centrifuged to remove precipitated proteins, and the supernatant was dried under a nitrogen stream.

2.5. LC-MS/MS analysis

The sample preparation and LC-MS/MS analysis conditions used in Laboratory A can be found in [9].

Laboratory B employed a newly developed LC-MS/MS method for the quantification of the enzyme reaction product 3,3-$^2H_2$-cystathionine. A dried sample was derivatized by adding 50 μL of hydrogen chloride (3 mol/L) in 1-butanol for 20 min at 65 °C, then dried again and reconstituted in 200 μL of mobile phase. A 10 μL aliquot of each sample was then injected into a Symmetry C18 column (100 mm × 2.1 mm, particle size 3.5 μm, Waters). The mobile phase consisted of 10 mmol/L ammonium acetate in a mixture of H_2O:MeOH (40:60). The flow rate was 150 μL/min, and the run time was 13 min. Butylated cystathionines were analyzed using positive multiple reaction monitoring (MRM) mode. The MRM transitions were 335 → 190 for L-cystathionine,