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Simultaneous determination of cystathionine, total homocysteine, and methionine in dried blood spots by liquid chromatography/tandem mass spectrometry and its utility for the management of patients with homocystinuria

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ABSTRACT

Background: Disorders of homocysteine and B-vitamin metabolism represent a significant problem in clinical practice. Establishing the diagnosis requires specialized tests with demanding preanalytical requirements. To advance the detection of patients with these disorders, we developed a method for the simultaneous determination of cystathionine (Cysta), methionine (Met) and total homocysteine (tHcy) in dried blood spots (DBSs).
Methods: A punch from a DBS sample was mixed with a solution of isotopically labeled internal standards, and analytes were extracted using methanol/0.1% formic acid/0.5 mol/L dithiothreitol. The extract was injected into an LC–MS/MS system operating in MRM mode.
Results: The analytical performance of the method employing DBS is adequate for its purpose and the type of sample. Compared with Cysta, tHcy and Met plasma levels, our method exhibited a negative bias between −3.8% and −42.2% due to the lower concentrations of these analytes in erythrocytes. The tHcy level and the Met/Cysta ratio in DBS enabled the clear detection of 12 patients with disorders of transsulfuration and with genetic and nutritional remethylation defects.
Conclusions: The ease of collecting and transporting DBS samples may advance diagnostic procedures in patients with neuropsychiatric disorders and thromboembolism. Consequently, this approach may facilitate detection and simplify the monitoring of patients with homocystinuria.
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1. Introduction

Homocystinuria represents a group of rare genetic disorders characterized by markedly elevated plasma levels of total homocysteine (tHcy) and its increased excretion in the urine. Currently, 8 distinct enzyme deficiencies caused by mutations in the respective genes are recognized as causes of homocystinuria. In homocystinuria, the plasma tHcy concentrations typically range between 50 and 400 μmol/L compared to physiological levels of 5–15 μmol/L. Two types of homocystinuria can be distinguished based on the location of the enzyme defect and the resulting changes in methionine (Met) and cystathionine (Cysta) concentrations. Defects in the remethylation (RM) pathway, such as methylene tetrahydrofolate reductase deficiency, and intracellular methylcobalamin synthesis defects of complementation groups cblC, cblD (including cblD variants), cblE, cblF, cblG and cblJ are characterized by low-normal to decreased plasma Met levels and high-normal to elevated Cysta levels. In contrast, the hallmark of cystathionine beta-synthase (CBS) deficiency, which affects the transsulfuration pathway of homocysteine (Hcy), is plasma with elevated Met level and low-normal to decreased Cysta levels. Thus, the ratio of Met/Cysta may be a suitable marker for differentiating these two types of homocystinuria [1,2]. Homocysteine metabolism depends not only on the intact function of the genes described above but also on the nutritional supply of B vitamins (i.e., folates, riboflavin, cobalamin and pyridoxine) and their transport and intracellular metabolism. Thus, markedly elevated plasma total homocysteine concentration between 30 and 100 or greater than 100 μmol/L (i.e., moderate or severe hyperhomocysteinemia, respectively) is a general sign of genetic or severe nutritional disturbances in the transsulfuration and RM pathways.

Most patients with disorders of Hcy and B-vitamin metabolism are ascertained in pediatric, hematological and neurological practices after presenting symptoms. Newborns with homocystinurias are also

Abbreviations: Hcy, homocysteine; Cysta, cystathionine; Met, methionine; tHcy, total homocysteine; DBSs, dried blood spots; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; RM, remethylation; CBS, cystathionine beta-synthase; Hcy-Hcy, homocystine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CV, coefficient of variation; TCEP, tris-(2-carboxyethyl)phosphine; LOD, limit of detection; LLOQ, lower limit of quantification.

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diagnosed in the presymptomatic phase in some neonatal screening programs worldwide. The prevalence of all homocystinurias as a group remains unknown; published CBS deficiency studies revealed prevalence rates between 1:1800 in Qatar and 1:900,000 in Japan [3]. Homocystinurias are clinically heterogeneous, although they share a high risk of thromboembolism, which is attributed to grossly elevated levels of tHcy [4]. Some patients with CBS deficiency may also suffer from connective tissue abnormalities and/or developmental delay, cognitive impairment and epilepsy. Patients with RM defects do not exhibit connective tissue abnormalities, but their neurological and psychiatric complications are usually more severe, exhibiting, e.g., white matter abnormalities and occasionally impairment in hematopoiesis, leading to megaloblastic anemia [5]. The diagnostic work-up for all these conditions requires plasma tHcy determination with specific preanalytical conditions (time limit for centrifugation, cooling of samples after collection and during transportation) and the availability of special assays for serum/plasma B-vitamins, Met and Cysta.

It has been shown that early detection and timely dietary and/or pharmacological treatment can prevent many clinical complications associated with homocystinuria and can substantially improve the quality of life of patients; as a result, these diseases have become a widely recognized target for routine neonatal screening [6,7]. The primary screening markers assessed in routine newborn screening programs for homocystinuria are the Met level and the Met/phenylalanine ratio; however, these markers suffer from low sensitivity and specificity [8, 9]. The use of tHcy as a primary marker in dried blood spots (DBSs) substantially increases the specificity and sensitivity of homocystinuria screening, although this method is used only in Qatar due to its high cost and lengthy analysis [10]. Consequently, a number of screening programs for homocystinuria have combined the measurement of Met as a primary marker with a second-tier analysis of tHcy in DBS samples that exhibit abnormal Met concentrations [11].

DBSs have been used for decades to screen newborns for a predefined set of disorders of amino acid metabolism, such as phenylketonuria, as well as to monitor dietary treatment. More recently, the DBS method has become instrumental in screening high-risk populations for inborn errors of metabolism, such as fatty acid oxidation defects, biotinidase deficiency and lysosomal storage disorders (e.g., considerably increasing the detection of Fabry disease [12]). Considering the simplicity of collecting and transporting DBS samples, LC–MS/MS analysis may potentially also be used as the first step in screening clinically symptomatic high-risk populations for the two types of homocystinuria and severe B-vitamin deficiencies.

A number of methods for measuring tHcy and/or Met in DBS have been reported [13–17], although no analysis of Cysta in DBS has yet been published. Thus, we aimed to develop a simple LC–MS/MS method for the simultaneous measurement of Cysta, tHcy and Met. This method was optimized to detect not only marked elevations of tHcy in DBS but also to provisionally and simultaneously differentiate transsulfuration and remethylation defects. The presented method may be utilized a/as a second-tier method in neonatal screening, b/for screening high-risk patients with putative genetic defects or severe B-vitamin deficiencies, and c/for monitoring therapy in patients with homocystinuria.

2. Materials and methods

2.1. Chemicals and reagents

Cysta, Hcy, homocystine (Hcy-Hcy), Met, methionine-d_3 (d_3-Met) and dithiothreitol (DTT) were purchased from Sigma-Aldrich. Cystathionine-d_4 (d_4-Cysta) was obtained from CDN isotopes and homocystine-d_8 (d_8-Hcy-Hcy) from Cambridge Isotope Laboratories. All the solutions and solvents were of the highest available purity and were suitable for LC–MS analysis. All stock solutions were stored at −20 °C.

2.2. Calibrators and samples

To prepare DBS calibrators, blood samples from healthy anonymous donors without any hematological diseases were collected in EDTA tubes and enriched with 2.5, 5, 25, 50, 100, 150 and 200 μmol/L of Hcy-Hcy; 5, 10, 50, 100, 200, 300, 400, 500 and 600 μmol/L of Met; and 0.50, 1.00, 5.00 and 10.00 μmol/L of Cysta. These spiked blood samples were spotted onto filter cards (Whatman 903, GE Healthcare Life Sciences, USA), dried overnight at room temperature and stored at −20 °C.

To determine the reference ranges for the comparative study and to assess the recovery and stability of the analytes, we used anonymous control plasma samples and/or DBS. To determine the reference ranges in newborns, we used random DBS samples from routine newborn screenings; for the remainder of the studies, we employed aliquots of EDTA plasma and/or DBS samples remaining after negative routine biochemical genetic screening for inborn errors of metabolism. Samples were collected from patients with homocystinuria after informed consent was obtained; we had access to twelve archived DBS samples collected at the time of diagnosis and sixty EDTA plasma and DBS samples collected from 21 patients during routine clinical visits (hematocrit values were within appropriate age-related reference ranges). All DBS samples were stored at room temperature prior to analysis, and the plasma samples were stored at −20 °C.

This study was approved by the Ethics Committee of the General University Hospital in Prague, No. 30/10/IGA MZ ČR VFN.

2.3. DBS sample preparation

A single 3.2 mm (1/8 in.) disk was punched from each DBS and placed into a flat polypropylene 96-well microtiter plate (Chromsystems, Germany). A 20 μL mixture of isotopically labeled internal standards dissolved in water, which contained 2.5 μmol/L d_8-Hcy-Hcy, 5 μmol/L d_3-Met and 1.5 μmol/L d_4-Cysta, was added, followed by 20 μL of DTT (500 mmol/L in water) and 150 μL of methanol containing 0.1% formic acid. The extraction was performed by gentle laminar shaking at room temperature for 30 min. The extract was then transferred into polypropylene 96-well microtiter plates containing filter membranes (AcroPrep, 0.45 μm PTFE, 350 μL, Chromsystems) and filtered into another polypropylene 96-well microtiter plate by centrifugation (at 4000 U/min for 5 min). The plate was sealed and analyzed using LC–MS/MS.

2.4. LC–MS/MS instrumentation and conditions

The LC–MS/MS system consisted of an Agilent 1200 HPLC (Agilent Technologies, USA) and a triple–quadrupole MS/MS API 4000 system (AB SCIEX, Framingham, USA). LC separation was performed on a Waters C8 column (SunFire; 3.5 μm, 4.6 × 100 mm) by isocratic elution using a mobile phase consisting of 40% methanol and 0.1% formic acid in water. The flow rate was 350 μL/min, and the injection sample volume was 5 μL. Tandem mass spectrometry was performed using a turbo ion spray source operated in positive mode, and the multiple reaction monitoring (MRM) mode was used for the selected analytes and their internal standards. The mass calibration of both quadrupoles was optimized using a polypropylene glycol solution with an infusion pump. The following electrospray settings were used: voltage 5000 V, temperature 400 °C, nebulizer gas 40 (GS1), heater gas 30 (GS2) and curtain gas (CUR) 25 psi. The MRM transitions for all the compounds were optimized by the infusion of 10 μmol/L aqueous solution.

The LC–MS/MS parameters are shown in Table 1. Quantitative results were obtained by dividing the peak area of each analyte by the peak area of the corresponding isotopically labeled standard, and the ratios were converted to concentrations by applying factors derived from the calibration curve. The concentrations were calculated from the calibration curves using Analyst 1.5.2 software (AB SCIEX).

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