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Quantification of folate metabolites in serum using ultraperformance liquid chromatography tandem mass spectrometry

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ABSTRACT

Folate deficiency is considered a risk factor for many diseases such as cancer, congenital heart disease and neural tube defects (NTDs). There is a pressing need for more methods of detecting folate and its main metabolites in the human body. Here, we developed a simple, fast and sensitive ultraperformance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) method for the simultaneous quantifications of folate metabolites including folic acid, 5-methyltetrahydrofolate (5-MeTHF), 5-formyltetrahydrofolate (5-FoTHF), homocysteine (Hcy), S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). The method was validated by determining the linearity ($r^2 > 0.998$), sensitivity (limit of detection ranged from 0.05 to 0.200 ng/mL), intra- and inter-day precision (both CV < 6%) and recovery (each analyte was >90%). The total analysis time was 7 min. Serum samples of NTD-affected pregnancies and controls from a NTD high-risk area in China were analyzed by this method, the NTD serum samples showed lower concentrations of 5-MeTHF ($P < 0.05$) and 5-FoTHF ($P < 0.05$), and higher concentrations of Hcy ($P < 0.05$) and SAH ($P < 0.05$) compared with serum samples from controls, consistent with a previous study. These results showed that the method is sensitive and reliable for simultaneous determination of six metabolites, which might indicate potential risk factors for NTDs, aid early diagnosis and provide more insights into the pathogenesis of NTDs.

1. Introduction

Folate is converted to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR) once transported into the cell and functions as the carrier for one carbon unit involved in various biological processes particularly nucleotide metabolism, DNA damage repair and methylation action [1,2]. Reports have shown that folate deficiency is a risk factor for a variety of diseases such as megaloblastic anemia [3], cancer [4–6], congenital heart disease [7,8], and neural tube defects (NTDs) [9]. Therefore, it is of vital significance to develop a sensitive and fast method for detecting folate levels in the human body.

The analysis of folate provides a great challenge owing to its poor stability and extremely small amounts in biological matrix samples, and its compounds are complicated. There have been many methods developed for the detection of folate such as microbiological assay [10], radioimmunoassay [11,12], capillary electrophoresis [13] and chromatography [14,15]. The most useful methods are microbiological assay and radioimmunoassay in clinical practice. However, microbiological assays are time costly and lack repetition. Radioimmunoassay has the advantage of fast and simple for sample detection, but its results vary wildly with different kits. These methods detect the total folate, that is folate plus its derivatives, with low selectivity, and different results are found by different methods used for the same sample. When high performance liquid chromatography–tandem mass spectrometry (HPLC–MS) was used for folate analysis, it not only rapidly and effectively separated the folate compounds, but could meet the requirements of a low detection limit owing to the high sensitivity of mass spectrometer, and show great advantages in qualitative and quantitative analysis [16,17]. Therefore, HPLC–MS has great application potential for the folate compounds in serum. Liquid chromatography tandem mass spectrometry (LC/MS/MS) has been shown to provide a more efficient assessment of abnormal one-carbon metabolism [18,19]. In 2008, Zhang et al. established the LC/MS/MS

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method to detect the 10 compounds in folate- and homocysteine-mediated one-carbon metabolism [20]. Separating and detecting specific folate vitamers may be one of the greatest advantages of this work. With the development of high performance liquid chromatography (HPLC), the emergence of ultraperformance liquid chromatography (UPLC) prompted the development of chromatography. Some reports have shown that the ultraperformance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) has the advantage of being faster, more accurate, with higher sensitivity and specificity, compared with LC/MS/MS [21,22]. The use of UPLC is the emerging methodology of choice for folate determination in human health [23], the key folate forms including folic acid, tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MeTHF), 5-formyltetrahydrofolate (5-FoTHF), 5,10-methenylTHF [24,25] and S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) [26] were detected by use of the isotope dilution UPLC–MS/MS, which is demonstrated to have better accuracy (recovery) and precision. However, it has not been reported that UPLC–MS/MS were used to simultaneously quantify folic acid, 5-MeTHF, 5-FoTHF, homocysteine (Hcy), SAM and SAH in the same system. Here, we established simple, fast and sensitive UPLC/MS/MS method to simultaneously examine and compare the major components involved in the maternal folate–homocysteine (Hcy) metabolism. The potential of this method is to help investigate the underlying mechanism of diseases such as NTDs.

2. Materials and methods

2.1. Equipment

UPLC-quadrupole tandem mass spectrometer XEVO^{TM} TQ MS (Waters, Milford, MA, USA) was used for UPLC–MS/MS analysis. Separation was performed on a BEH C18 analytical column (2.1 mm × 50 mm I.D., 1.7 μm, Waters, USA) at 40 °C. Ultrapure water was prepared with a Milli-Q water purification system (Millipore, France).

2.2. Chemicals and reagents

The following compounds were obtained from Sigma–Aldrich (St. Louis, MO, USA): folic acid, 5-methyltetrahydrofolate (5-MeTHF), 5-formyltetrahydrofolate (5-FoTHF), homocysteine (Hcy), S-adenosyl-methionine (SAM), S-adenosyl-homocysteine (SAH), and dithiothreitol (DTT). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Waltham, MA, USA). Ascorbic acid and citric acid monohydrate were purchased from Beijing Chemical Company (Beijing, China).

2.3. UPLC–MS/MS

The mobile phases were set as follows: 0.1% (V/V) formic acid in water (eluent A), and acetonitrile (eluent B). The following linear elution gradient was used (flow rate, 400 μL/min): 0–1 min, 99.9%–99.9% A; 1–3 min, 99.9% A–82% A; 3–4 min, 82% A–10% A, 4–5 min, 10% A–10% A; 5–6 min, 10%–99.9%. The equilibration time was 1 min. The total analysis time was 7 min and the injection volume was 5 μL in each run.

The MS/MS operating parameters were obtained and optimized under positive-ion (ESI+) with multiple reactions monitoring (MRM). The capillary voltages of 3000 V and source temperature of 150 °C were adopted. The desolvation temperature was 400 °C. The collision gas flow was set at 0.13 mL/min. The cone gas and desolvation gas flow were 30 L/h and 900 L/h, respectively.

2.4. Validation of the method

2.4.1. Preparation of calibration standards and quality control sample

Stocked solutions for each standard were prepared at a concentration of 100 μg/mL in 50:50 (V/V) methanol/water (containing 100 μg/mL each of ascorbic acid, citric acid and DTT to inhibit oxidation) and stored at −80 °C. Calibrants were prepared by diluting the stocked solution with acetonitrile/water (1/9) (containing ascorbic acid, citric acid and DTT as above), resulting in concentrations of 0.5, 1, 2, 5, 10, 50 ng/mL for folic acid, 5-MeTHF, 5-FoTHF, and SAH; 2, 5, 10, 50, 100, 200 ng/mL for SAM; and 0.05, 0.1, 0.25, 0.5, 1, 2 μg/mL for Hcy. Quality control (QC) samples were spiked by adding low, medium, and high concentrations of standards into blank serum to obtain serum spiking solutions. All stocked solutions, working solutions and QC samples were stored at −80 °C and brought to room temperature before use.

2.4.2. Linearity

The calibration curves were obtained from plots of the peak-area versus the concentration of the standards. The concentrations of the metabolites in serum samples were determined by the equations of linear regression obtained from the calibration.

2.4.3. Intra-day and inter-day precision and recovery

Intra-day precision was evaluated by analysis of QC samples at different times on the same day. Inter-day precision were determined by repeated analysis of QC samples over five consecutive days. The calibration curves were calibrated every day to ensure the precision of the results. The extraction recoveries were determined by analysis of the blank serum spiked with standards. Three concentrations were studied: the center was the endogenous level, the low and high were 50% and 200% of the center, respectively.

2.4.4. Sensitivity and stability

The sensitivity of the assay was estimated using the limit of detection (LOD) and the limit of quantitation (LOQ) of the six compounds. The LOD and LOQ were defined as the minimum detectable values with signal-to-noise levels of 3 for LOD and 10 for LOQ. The compound stability for 0, 2, 4, 8, 16 and 24 h at −20 °C in serum was evaluated by repeated analysis at the medium concentration of QC samples. The precision and accuracy were determined by calculating the CVs (coefficient of variations).

2.5. Sample selection and pretreatment

The serum samples were collected from the NTD-affected pregnant women, and the details have been described previously [20]. 50 μL dithiothreitol (DTT) (10 mg/mL) was added to 100 μL serum, vortexed for 1 min, and then treated with 500 mL of methanol containing 100 μg/mL each of ascorbic acid and citric acid. The mixture was vortexed for 2 min and then centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was transferred to a 1.5 mL Eppendorf tube and dried under nitrogen at room temperature. The residue was dissolved in 60 μL aliquots of acetonitrile:water (1:9) (containing 100 μg/mL of ascorbic acid, citric acid, and 10 mg/mL DTT), and stored at −80 °C before analysis.

2.6. Statistical analysis

Linear regression analysis was used to verify the linearity of the calibration curves. Folic acid, 5-MeTHF, 5-FoTHF, Hcy, SAM, and SAH concentrations were expressed as mean ± standard deviation (s.d.) and examined by Student's *t*-test. All statistical analyses were performed with SPSS16.0 (SPSS Inc., Chicago, IL, USA).