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ScienceDirect
Journal of Chromatography B, 863 (2008) 94–100
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous quantification of 11 pivotal metabolites in neural tube defects by HPLC–electrospray tandem mass spectrometry

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Received 30 July 2007; accepted 4 January 2008
Available online 16 January 2008

Abstract

One-carbon metabolism that involves folate metabolism and homocysteine metabolism plays a powerful role in embryonic development. Any impairment to this metabolism during the neurulation process would trigger the occurrence of neural tube defects (NTDs). The great importance of one-carbon metabolism necessitates the establishment of methodology to determine the relative compounds involved in the metabolic cycles. We have developed a sensitive method for measurement of 11 pivotal compounds by using high-performance liquid chromatography coupled to mass spectrometry (HPLC–MS/MS) in sera of pregnant women. Use of an aqueous chromatography column increased retention time and separation of the polar compounds in the system, resulting in fewer co-elution and interference from the other compounds that can lead to ion suppression. Calibration curves suitable for the analysis of maternal serum were linear ($r^2 > 0.997$) with limits of detection from 0.05 to 1 ng/mL. Intra-day coefficients of variation (CVs) and inter-day CVs were both lower than 11%. With the developed method, 96 serum samples including 46 cases and 50 controls were analyzed. The established method provided a reliable method for quantifying most of the compounds involved in the one-carbon metabolism simultaneously, thus made it possible to elucidate NTDs with multiple factors instead of one single and provided a solid foundation for the diagnosis and prevention of NTDs as well as some other one-carbon metabolism related diseases.

Keywords: Neural tube defects; One-carbon metabolism; Tandem mass spectrometry; Folate; Homocysteine

Abbreviations: NTDs, neural tube defects; HPLC–ESI–MS/MS, high-performance liquid chromatography–electrospray tandem mass spectrometry; FA, folic acid; THF, tetrahydrofolate; 5-FT, 5-formyltetrahydrofolate; 5-MT, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; Met, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Hcy, homocysteine; Cysta, cystathionine; His, histidine; Ser, serine; MTHFD, methylenetetrahydrofolate-dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; MAT, methionine adenosyl transferase; MT, methyl transferase; SAHH, S-adenosyl homocysteine hydrolase; CBS, cystathionine beta synthase; DTT, dithiothreitol; SPE, solid-phase extraction; QC, quality control; MRM, multiple reaction monitoring.

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1570-0232/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2008.01.010

1. Introduction

Neural tube defects (NTDs) are considered to be one of the most common congenital defects produced when the open neural tube of human embryo fails to close during the early stage of pregnancy, which will lead to infant morbidity and mortality. NTDs are considered to be multifactorial diseases and both genetic and environmental factors contribute to the malformations. The prevalence of NTDs is 1 per 1000 births in the United States [1]. In China, it is 5–6 per 1000 births in northern areas, 1 per 1000 births in southern areas [2], and 16–18 per 1000 births in the area of our study. The lives lost and the treatment costs have led to tremendous expense to society.

It is well known that one-carbon metabolism, in which folate acts as a cofactor for enzymes involved in DNA and RNA biosynthesis, is tightly associated with NTDs [3]. Many researchers have demonstrated that folic acid [2,4,5] and homocysteine [6,7] play important roles in the occurrence of NTDs.

Pathways involved in folate and homocysteine related one-carbon metabolism are depicted in Fig. 1, and the compounds investigated in our study are underlined in the figure. Mechanism elucidation of NTDs must be based on the quantification of as many correlative compounds as possible, which is considered to be a promising and challenging strategy in the field of metabolite analysis [8]. Many methods, such as microbiological assays [9], HPLC with UV, fluorescence, or electrochemical detection [10], GC/MS [11], LC/MS [12], and LC/MS/MS [13], have been published to detect and quantify folates, amino acids, and other aforementioned metabolites [14] in biological samples.

It is a great challenge to quantify the 11 pivotal metabolites simultaneously because of the following complications: (a) the instabilities of the analytes. Folates are susceptible to light, heat, and other oxygenous conditions, while homocysteine exists as a multiplicity of several forms (free homocysteine monomer, homocysteine–homocysteine disulfide, homocysteine–cysteine disulfide, etc.) [15] that makes the accurate determination of the compounds much more difficult. (b) The wide concentration ranges of the analytes. Folates concentrations in body fluids are much lower than some amino acids, such as Hcy and Met. (c) The strong polarities of amino acids increase the difficulty of separation on the HPLC column. To the best of our knowledge, no method for the simultaneous quantification of metabolites that cover the two metabolic cycles has been reported.

Here, we present a selective and sensitive high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) method for simultaneous quantification of 11 relevant metabolites involved in one-carbon metabolism in human serum. In addition, an improvement in sample extraction and stabilization was performed, which accelerated serum pretreatment and minimized sample degradation. 96 serum samples including 46 NTDs affected women and 50 healthy women were analyzed with the established method. After data analysis with the Student's *t*-test, several potential risk factors were screened out.

2. Experimental procedures

2.1. Chemicals and reagents

Folic acid (FA), tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MT), 5-formyltetrahydrofolate (5-FT), homocysteine (Hcy), methionine (Met), S-adenosylhomocysteine (SAH), S-adenosylmethionine (SAM), cystathionine (Cysta), serine (Ser), histidine (His), and dithiothreitol (DTT) standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium formate, formic acid, ascorbic acid, citric acid, and 2-mercaptoethanol of AR grade were purchased from Beijing Chemical Reagents Company (Beijing, China). Acetonitrile and methanol of HPLC grade were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Ultrapure water (18.2 MΩ) was prepared with a Milli-Q water purification system (Millipore, France).

2.2. Samples

Serum samples of 46 NTDs affected pregnant women in Lvliang of Shanxi Province, China were collected as cases and 50 serum samples of healthy pregnant women in the same area and Huairou of Beijing, China as controls. All blood samples were centrifuged to obtain serum in the hospital and sent to our laboratory, where they were stored at −80 °C until sample preparation. All study participants gave informed consent.

2.3. Preparation of samples

Before analysis, 100 μL of aqueous DTT (15 mg/mL) were added to 250 μL aliquots of serum, vortexed for 1 min, and then treated with 1 mL of methanol containing ascorbic acid and citric acid (both 100 μg/mL, final pH 4.5–5.0). The mixture was vortexed for 2 min and then centrifuged at 6000 rpm for 15 min at 4 °C. The clear supernatant was transferred to a 1.5 mL polypropylene tube, and dried under a gentle stream of nitrogen at room temperature. The residue was reconstituted with 100 μL of a mixture of methanol–water (3:1, v/v) containing 10 μg/mL of ascorbic acid, citric acid, and DTT, and stored at −20 °C before analysis.

2.4. HPLC–MS/MS methods

Quantification of FA, THF, 5-MT, 5-FT, Hcy, Met, SAH, SAM, Cysta, Ser, and His were performed using reverse-phase HPLC–MS/MS. The separation of the samples was performed on an HP1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) using an Ultimate™ AQ-C18 column (250 mm × 4.6 mm i.d., 5 μm particle size, Welch Materials, MD, USA) with an attached Alltech guard column (7.5 mm × 4.6 mm i.d., 5 μm particle size) at a flow rate of 0.5 mL/min. Mobile phase A was water containing 5 mM ammonium formate and 0.15% (v/v) formic acid, and mobile phase B was acetonitrile containing 0.15% (v/v) formic acid. The column was held at 25 ± 1 °C throughout the assay. The injection of samples (20 μL) was followed by a linear gradient starting at

Table 1
Compound-dependent MS parameters for each analyte

Compound	MRM transition	OR (V)	RNG (V)	RO2 (V)	RO3 (V)	ST3 (V)
FA	442.3 → 295.1	46	170	−32	−34	−48
THF	446.2 → 299.2	66	260	−38	−40	−54
5-MT	460.2 → 313.2	56	220	−38	−40	−56
5-FT	474.3 → 327.1	86	330	−38	−40	−56
Hcy	136.0 → 90.1	33	140	−24	−26	−40
Met	150.1 → 104.2	36	140	−24	−26	−42
SAH	385.2 → 134.1	44	210	−36	−38	−60
SAM	398.9 → 250.2	41	180	−32	−34	−72
Cysta	223.2 → 134.1	36	140	−28	−30	−52
His	156.2 → 110.0	39	160	−30	−32	−50
Ser	106.1 → 60.2	101	360	−28	−30	−38

100% mobile phase A to 80% mobile phase B over 25 min. Subsequently, the column was equilibrated with the initial mixture for 5 min. The effluent was split 4:1 using a valved three-way split, so that approximately 100 μL/min was introduced into the source of the mass spectrometer. Mass spectrometric analyses were performed on a Sciex API 3000 triple-quadrupole mass spectrometer (PerkinElmer Sciex, Canada) in the positive-ion mode using multiple reaction monitoring (MRM), and monitoring the transitions *m/z* 442.3 → 295.1 for FA; 446.2 → 299.2 for THF; 460.2 → 313.2 for 5-MT; 474.3 → 327.1 for 5-FT; 136.0 → 90.1 for Hcy; 150.1 → 104.2 for Met; 385.2 → 134.1 for SAH; 398.9 → 250.2 for SAM; 223.2 → 134.1 for Cysta; 156.2 → 110.0 for His; 106.1 → 60.2 for Ser.

The temperature of the turbo ion electrospray was set at 350 °C. The collision gas (nitrogen) was set at 6 mTorr, and nebulizer gas (nitrogen), curtain gas (nitrogen), and assistant drying gas (air) were used at a flow rate of 8, 2, and 4 L/min, respectively. The ion spray voltage was 5300 V. The MS/MS operation parameters for all analytes were obtained and optimized via positive ion mode electrospray ionization. Multiple reaction monitoring transitions for each analyte were individually optimized for protonated analyte molecules [M + H$^+$] and stable, protonated fragments in continuous flow mode by connecting an infusion pump directly to the TurboIonSpray probe, and the flow rate was set at 15 μL/min. Specific compound-dependent MS/MS parameters for each analyte are given in Table 1.

2.5. Calibration procedure

2.5.1. Stock solutions, working solutions, and quality control samples

Stock solutions for each standard were prepared at a concentration of 1 mg/mL in methanol–water (50:50, v/v) and stored at −20 °C. For folates, the standard solutions contained 100 μg/mL of ascorbic acid, citric acid, and DTT to inhibit oxidation. Working solutions were prepared by diluting the stock solutions with acetonitrile (containing antioxidants as above), resulting in con-

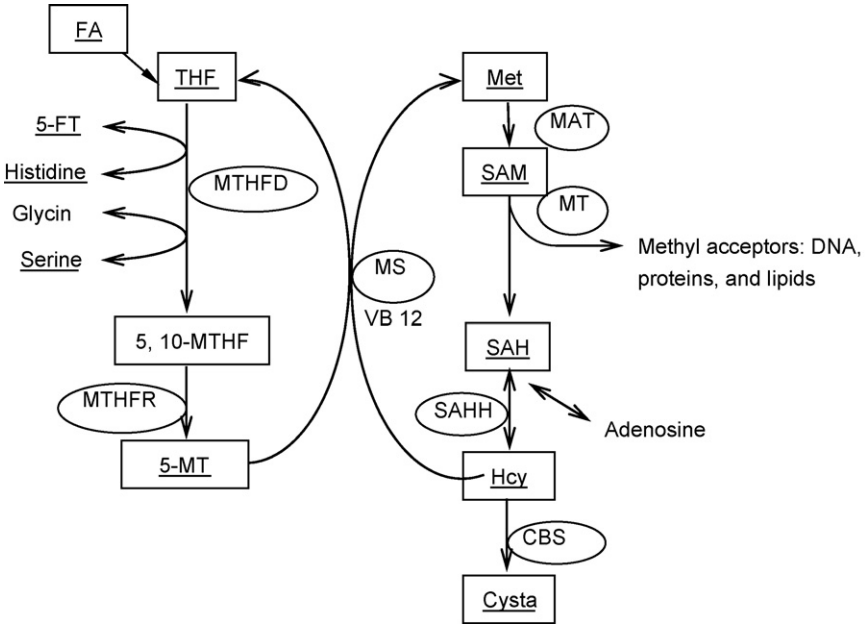

Fig. 1. The simplified folate and homocysteine related one-carbon metabolisms.

Table 2
The regression equations and limits of detection of 11 compounds

Compound	Regression equation	Linear range (ng/mL)	r	LOD (ng/mL)
FA	$y = 197.7x + 125.89$	0.5–40	0.9990	0.25
THF	$y = 21.811x - 48.507$	5–200	0.9997	1
5-MT	$y = 201.67x - 98.415$	0.5–40	0.9988	0.1
5-FT	$y = 169.11x + 0.0885$	0.5–40	0.9995	0.1
Hcy	$y = 325.26x + 510.58$	50–2000	0.9999	1
Cysta	$y = 244.24x + 261.91$	0.5–40	0.9998	0.25
Ser	$y = 94.348x + 29.655$	10–500	0.9986	0.25
SAM	$y = 74.44x - 220.06$	10–100	0.9985	1
SAH	$y = 219.77x + 39.038$	0.5–40	0.9998	0.25
Met	$y = 100.17x + 1160.3$	100–8000	0.9994	0.05
His	$y = 207.48x + 26961$	500–10,000	0.9995	0.1

centrations of 0.5, 1, 2, 5, 10, 50, 100, 200, 400, and 500 ng/mL for FA, THF, 5-MT, 5-FT, Cysta, and SAH (working solution I), 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL for Hcy, Ser, and SAM (working solution II), and 100, 200, 500, 1000, 2000, 5000, 10,000, and 20,000 ng/mL for Met and His (working solution III).

Quality control (QC) samples were prepared by spiking 250 μL aliquots of blank serum with low, medium, and high concentrations of standards, respectively. QC samples were prepared as described in Section 2.3 and stored at −20 °C until needed.

2.5.2. Calibration curves

External calibration method was used for the quantitative analysis. Calibration curves were obtained by the plots of the peak-area versus the concentration of the standards. The concentrations of the metabolites in serum samples were determined by using the equations of linear regression obtained from the calibration curves.

2.6. Method validation

Intra-day precision (each $n = 5$) were evaluated by analysis of QC samples at different times on the same day. Inter-day precision ($n = 6$) were determined by repeated analysis of QC samples spiked with standards of medium concentration twice per day over three consecutive days. The calibration curves were calibrated everyday by analyzing two working solution samples before analyzing the serum samples to ensure the precision of the results. Precision was determined by the coefficients of variation (CVs). Signals three times higher than the peak noise height were regarded as the limits of detection (LOD) for the analytes.

The extraction recovery was determined by comparing the peak areas obtained from QC samples with the un-extracted standard working solutions at the same concentration in the same solvent.

The compound stability for 0, 4, 8, 16, and 24 h at −20 °C in serum was evaluated by repeated analysis at the medium concentration of QC samples.

2.7. Statistical analysis

The mass spectrometry data were processed using Macquan software (PE Sciex). Linear regression analysis (Excel) was used to verify the linearity of the calibration curves. Comparisons between case studies and controls were done using the Student's t-test. $p < 0.05$ was considered statistically significant.

Table 3
Precisions and recoveries

Compound	Intra-day ($n = 5$)						Inter-day ($n = 6$)	Spiked concentration		
	Serum-l[a]		Serum-m[a]		Serum-h[a]		Serum-medium[a] CV (%)	C_l (ng/mL)	C_m (ng/mL)	C_h (ng/mL)
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)				
FA	80.7	5.5	84.7	3.7	106.6	5.9	6.9	1	2	4
THF	87.1	10.3	93.8	5.5	105.7	4.5	7.2	10	20	40
5-MT	112.8	3.7	92.5	3.3	101.7	5.7	6.5	5	10	20
5-FT	83.0	7.5	111.4	9.5	94.8	2.0	10.1	1	2	4
Hcy	114.9	4.7	80.0	5.7	82.5	3.5	8.8	500	1000	2000
Cysta	76.2	7.7	86.6	5.5	87.4	5.6	10.2	1	2	4
Ser	110.3	1.9	105.5	6.3	94.5	5.3	6.4	50	100	200
SAM	85.2	4.7	93.1	7.4	106.4	3.0	7.0	25	50	100
SAH	119.9	8.6	108.8	6.3	92.0	6.1	7.0	2.5	5	10
Met	111.9	3.0	116.3	0.9	97.4	4.0	1.9	500	1000	2000
His	84.5	4.3	114.8	4.1	94.7	3.3	3.9	1000	2000	4000

[a] Serum samples spiked with low, medium, or high concentrations.

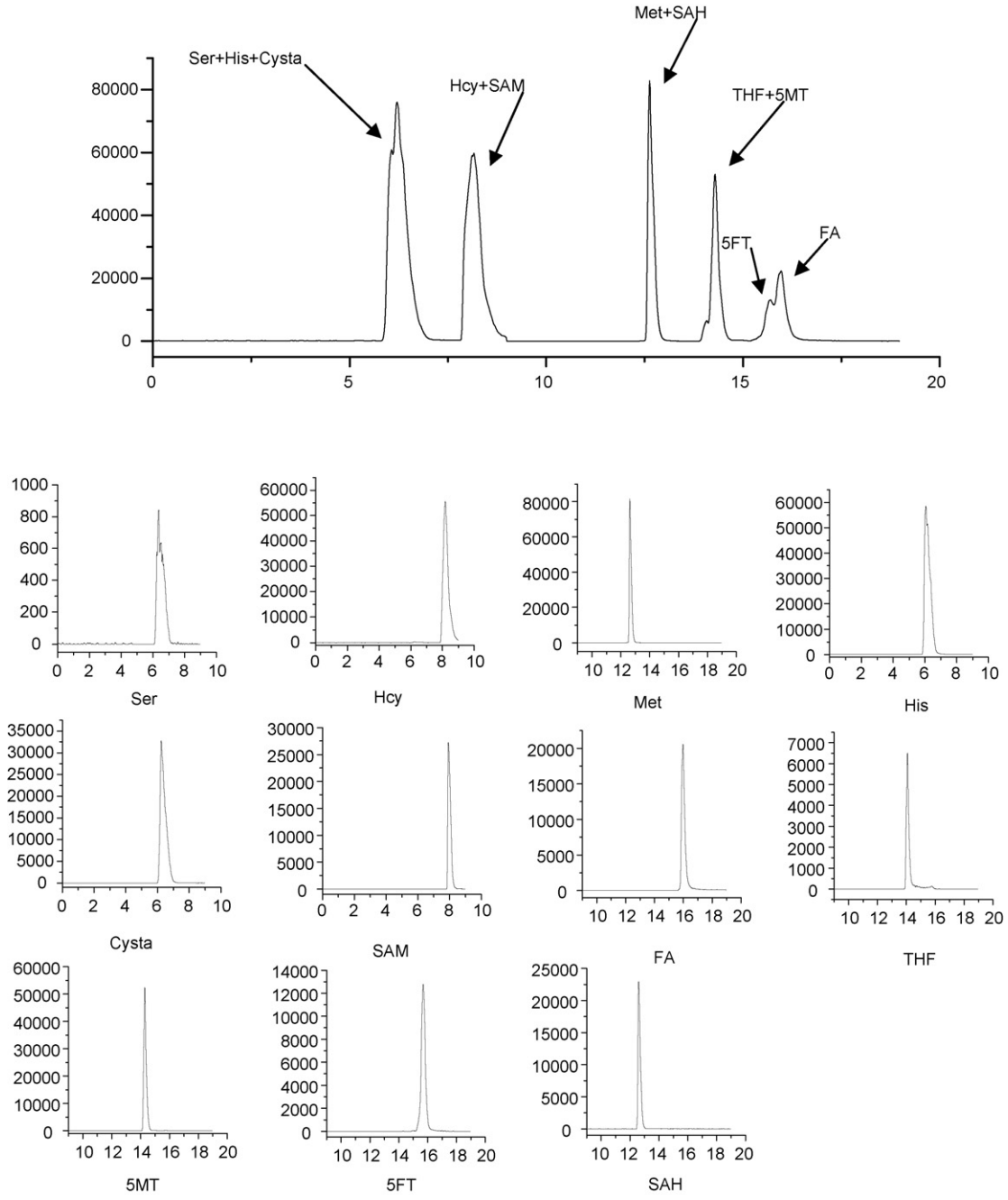

Fig. 2. Total ion LC/MS/MS chromatogram and extracted ion chromatograms (EICs) of each analyte in control sample fortified with stock solution.

3. Results and discussion

3.1. Optimization of sample preparation

Due to the complexity of the system, i.e., the instabilities of folates and homocysteine, effective and reproducible stabilization and extraction of the analytes should be top priority. Due to the instabilities of folates, it was necessary to add antioxidants to the system to prevent degradation. Based on literature review, it was found that ascorbic acid and 2-mercaptoethanol were the most frequently used antioxidants, while citric acid was a good synergist of them [13,16–19]. Homocysteine presents the problem of disulfide bond formation. It has been reported that DTT is effective at reducing homocysteine disulfides to homocysteine monomers and stabilizing the monomers once they are formed [20–21].

Different combinations of ascorbic acid, citric acid, 2-mercaptoethanol, and DTT of varying concentrations were evaluated for protective and reductive effects on the system. Optimal concentrations of antioxidants and reducer were determined as described in Section 2.3.

Although mass spectrometry can provide high specificity because of its ability to monitor selected mass ions, it still has problems related to interference [22]. One of the most important

factors that can affect the quantitative performance of the mass detector is ion suppression, which is caused by the presence of material other than the target compound during the ionization process and can lead to erroneous results [23].

To reduce the ion suppression from sample matrix, a specimen cleanup procedure was needed. Specimen cleanup was carried out by protein precipitation with acids (including metaphosphoric acid, trichloroacetic acid, and perchloric acid), methanol, acetonitrile, or combinations thereof [24–27], solid-phase extraction (SPE) [14,28,29], and solvent extraction. To optimize sample extraction, we experimented with protein precipitation, SPE, and ultrafiltration. Due to the complexity of the system and the thermal instability of the analytes, the analytes were eluted in different periods of SPE and degraded during the time-consuming procedure of ultrafiltration. Then, protein precipitation methods were evaluated. Serum samples were deproteinized with methanol and acetonitrile with or without formic acid, respectively. Due to poor solubilities in acetonitrile, some of the analytes were separated from the supernatant and resulted in low recoveries. The method for sample extraction was eventually determined as described in Section 2.3.

3.2. Optimization of chromatography and mass spectrometry conditions

Extensive chromatographic separation is not required for HPLC–MS/MS, since it has high selectivity. However, in order to minimize ion suppression from both matrix effects and interferences from co-eluted compounds in the system, it was essential to increase chromatographic separation. Derivatization methods are conventionally used for hydrophilic amino acid separation and determination, but these methods were not suitable for this thermally unstable system. In this study, we evaluated eight chromatography columns and several combinations of four mobile phases. The HPLC columns tested included Venusil MP-C18 (5 μm, 2.1 mm × 100 mm, Agela Technologies Inc.), Venusil MP-C18 (5 μm, 2.1 mm × 150 mm, Agela Technologies Inc.), Venusil XBP-C18 (5 μm, 2.1 mm × 150 mm, Agela Technologies Inc.), Venusil XBP-C18 (5 μm, 2.1 mm × 150 mm, Agela Technologies Inc.), Agilent XDB-C18 (5 μm, 4.6 mm × 250 mm, Agilent Technologies), Waters Xterra-C18 (5 μm, 3.9 mm × 150 mm, Waters), Waters Symmetry-C18 (3.5 μm, 2.1 mm × 100 mm, Waters), Ultimate AQ-C18 (5 μm, 4.6 mm × 250 mm, Welch Materials Inc.), and the volatile buffers were ammonium formate, ammonium acetate, formic acid, and acetic acid. Retention times, separations, intensities of the analytes, as well as analysis time for each sample were the factors for evaluation. The optimal chromatographic conditions were determined as described in Section 2.4.

To obtain the highest selectivity and lowest limit of quantification, tandem mass spectrometry with MRM mode was used. Parameters such as collision energy, capillary voltage, cone voltage, and nitrogen pressure in the collision cell were optimized in both positive and negative ion mode. Most analytes provided better results in positive ion mode. Detailed MS conditions are described in Section 2.4.

Table 4
Quantification and statistical results of serum samples

Compound	Concentration		p^a
	Control ($n=50$)	Case ($n=46$)	
FA (nmol/L)	1.39 (2.93)	1.56 (3.15)	0.799
5-MT (nmol/L)	14.58 (9.06)	9.32 (4.52)	<0.01
5-FT (nmol/L)	4.35 (2.60)	2.64 (1.59)	<0.01
Hcy (μmol/L)	6.92 (3.72)	8.14 (4.38)	0.160
Met (μmol/L)	13.80 (12.44)	10.34 (9.00)	0.327
SAH (nmol/L)	9.25 (4.13)	12.60 (7.58)	<0.05
SAM (nmol/L)	49.41 (19.43)	47.76 (24.70)	0.348
Cysta (nmol/L)	7.20 (6.94)	8.60 (6.46)	0.193
His (μmol/L)	19.55 (7.48)	19.55 (8.27)	0.953
Ser (μmol/L)	1.03 (1.05)	1.39 (1.42)	0.366

[a] p-Value from Student's t-test (natural log transformed).

Shown in Fig. 2 are typical chromatograms of a control serum fortified with stock solution and prepared and subjected to LC–ESI–MS/MS analysis as described in Section 2.

3.3. Calibration curves and limits of detection

The regression equation of calibration curves and their correlation coefficients (r) were calculated as shown in Table 2. All the calibration curves and limits of detection were suitable for the analysis of maternal serum except that of THF for its low concentration in serum.

3.4. Precision, extraction recovery, and stability

The data from QC samples were calculated to estimate the intra-day precision, inter-day precision, extraction recoveries and stabilities. The results showed good stability of the analytes during processing and storage. The detailed results are listed in Table 3.

3.5. Case–control study

The present method was applied to the analysis of serum samples of cases and controls. The analyte THF was not observed in any of the serum samples at the detection limit of this assay. All of the other ten compounds were observed. Student's t-test was carried out after natural log transformation of the data and the results are shown in Table 4.

Previous folate intervention studies [2,4] have suggested that the reduction in risk of neural tube defects was associated with folic acid supplementation. The conclusion that folic acid has a protective effect on neural tube defects was concluded based on the reduced recurrence or occurrence after the supplement of folic acid. However, who should be ultimately responsible for the reduction of the risk is still unknown. Our findings that the serum levels of 5-MT and 5-FT in healthy pregnant women were much higher than that of NTDs affected pregnant women suggests that 5-MT and 5-FT may play important roles in keeping the metabolic pathway normal and we suppose that the periconceptional supplemented folic acid prevent neural tube defects by normalizing the disordered states of one-carbon metabolism.

Therefore, it could also be presumed that any factors that reduce the level of disturb the metabolism, such as a deficiency of 5,10-methylenetetrahydrofolate reductase (MTHFR) [30] and an abnormality in methionine synthase [7] may be risk factors for neural tube defects.

Furthermore, we found significantly higher concentrations of SAH ($p < 0.05$) in case subjects than in controls. The increased SAH is a potent product inhibitor of most methyltransferase, which might lead to dysregulation of gene expression, protein function, and lipid and neurotransmitter metabolism [31–33]. Thus, we concluded that SAH is another risk factor for NTDs.

4. Conclusion

Triple-quadrupole tandem mass spectrometry with multiple reaction monitoring brings high selectivity and high sensitivity. The use of this technique makes it possible to quantify trace substances in complex biological samples simultaneously. Although MRM may lead to a loss of information of substances other than the targets, it can provide accurate quantitative information of pivotal substances in the system. Therefore, MRM is still an optimal choice for trace analysis of systems in which the targets are relatively clear.

Conventional methods such as chemiluminescent immunoassays and fluorescence polarization immunoassays can only determine a total concentration of folates or homocysteine. However, NTDs are multifactorial diseases that are caused by complex and interactive effects of environmental conditions, gene aberrations, and metabolic abnormalities. So, there may be many potential causes or risk factors of NTDs, and it is hard to explain the occurrence of NTDs with one single risk factor, i.e., it requires quantification of as many related compounds as possible to clarify the mechanism of NTDs, which is an impossible mission for conventional methods. And based on literature review [19,20,34,35], it was found that all of the methods targeted a small portion of compounds in the one-carbon metabolism; hence comprehensive information regarding the metabolic cycles cannot be obtained. However, in order to acquaint ourselves with the full view of folate and homocysteine related one-carbon metabolism, as many compounds as possible involved in the metabolism should be investigated.

The presented method provided a reliable method for quantifying most of the compounds involved in the one-carbon metabolism simultaneously, thus established the foundation of research for mechanism elucidation of NTDs. So that it is now possible to identify those with multiple risk factors of NTDs instead of one single risk factor. In conclusion, the presented method and findings will provide a solid foundation for prenatal diagnosis and prevention of NTDs, as well as some other one-carbon metabolism related diseases such as coronary heart disease.

Acknowledgement

This work was supported by a grant from the Major State Basic Research Development Program of China (973 Program) (No. 2001CB510305 and 2007CB511903).

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