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원 저

혈장 중 황함유 화합물과 메틸말론산의 신속 간편한 분석법 개발; GC-MS-SIM을 이용한 호모시스테인혈증의 진단

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A Rapid, Simple Determination of Sulfur-containing Compounds and Methylmalonic Acid on Plasma using GC-MS-SIM for the Diagnosis of Homocysteinemia

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Purpose: If early diagnosis is not made, patients with metabolic disorders as homocystinemia rapidly progress to physical defect or mental retardation resulted in storage of the toxic material into the brain. Therefore, it is necessary to develop an analytical method for a rapid screening and/or correct confirmation diagnosis.

Methods: The standard solution of sulfur amino acids spiked plasma was subjected to protein precipitation with methanol, and then consecutively derivatized with trimethylsilyl (TMS) and trifluoroacyl (TFA) and determined by GC-MS. The formation of TMS derivative of the hydroxyl and TFA derivative of amino functional group was performed by BSTFA and MBTFA, respectively. Selective ion monitoring (SIM) mode was used for quantification with selected specific ions.

Results: A calibration curve on standard spiked pooled plasma showed a linear relationship with correlation coefficient of 0.9936-0.9992 for all compounds over the range of 0.1-300 ng. The precision and accuracy were within S.D. of 1-15% and RSD of 1-15% for intra-day assay at 2 ng/mL, 15 ng/mL and 30 ng/mL. LOD and LOQ was 0.4 ng/mL and 4 ng/mL respectively.

Conclusion: A rapid analytical method was developed to quantify sulfur amino acids and methyl malonic acid, after two-step derivatization procedure with good sensitivity and specificity on human plasma. Advantages of a new method are simplicity and rapidity. The method could be useful for routine analysis, diagnosis of homocysteinemia.

Key words: GC-MS-SIM, Sulfur compounds, Trimethylsilylation, Trifluoro-acylation, Homocystinemia

Introduction

Sulfur containing amino acids such as methionine and homocysteine serve numerous vital func-

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tions in cellular biology, biochemistry and pharmacology. Their clinical roles are very critical for sustaining life, as methionine is required for protein synthesis[1]. Methioninesulfoxide or methionine sulfone were derived from the oxidation of sulfur of methionine[2]. Excess amount of methionine leads to hypermethioninemia and lack of methionine causes lipid peroxidation. Analysis of the methylmalonic acid is a valuable tool for the diagnosis of cobalmin deficiency, one of the homocyteinemia[3, 4]. Elevation of the plasma concentration of homocysteine and its metabolites are risks factor for cardiovascular diseases[5, 6]. Homocysteinuria and homocystinuria are two common metabolic defects related to homocysteine[7, 8].

Currently available analytical methods include capillary electrophoresis (CE)[9–11] and high–performance liquid chromatography (HPLC) using UV–Vis detector[12, 13], HPLC coupled with coulometric electrochemical detection[14, 15], capillary electrophoresis with reverse pulse ampherometric detector[16], ion exchange chromatography[17], gas chromatography mass spectrometry (GC–MS)[18–23], HPLC–FTMS[24], liquid chromatography–tandem mass spectrometry (LC–MS/MS)[25]. Each of these methods has some basic limitations such as sample preparation with complexity, sample processing time, run times or validation parameters.

Trimethylsilyl derivatives are the most commonly employed silylation derivatization procedures for GC–MS analysis[26]. Until now, reports on derivatization techniques with sulfur amino acids have included N–trifluoroacetyl (TFA)–*O*–isopropyl derivatives[18], tert–butyldimethylsilyl (TBDMS)[23], alkyl chloroformate derivatization[20], and methylchloroformate and toluene derivatives [27]. The present study demonstrated a more effective quantitative determination using two step derivatives, first silylation of hydroxyl group using BSTFA (Bis–trimethylsilyltrifluoroacetamide) and then acylation of amino group using MBTFA (N–methyl–bis–(trifluoroacetamide)) and then analyses by GC–MS.

In the view of their biological and clinical importance, there has been an increasing demand for analytical methods to detect sulfur amino acids for a number of applications. Therefore, we developed a new simultaneous quantitative analytical method with homocysteinemia related sulfur compounds and methylmalonic acid using GC–MS–SIM.

Materials and methods

1. Materials

Methionine (2–amino–4–(methylthio) butanoic acid), homocysteine (2–amino–4–sulfanylbutanoic acid), homocystine (L–4,4'–dithiobis (2–aminobutanoic acid)), methionine sulfoxide (2–amino–4–(methylsulfinyl) butanoic acid), methionine sulfone (2–amino–4–(methylsulfonyl) butanoic acid), methyl malonic acid (2–methylpropanedioic acid) were purchased from TCI Co

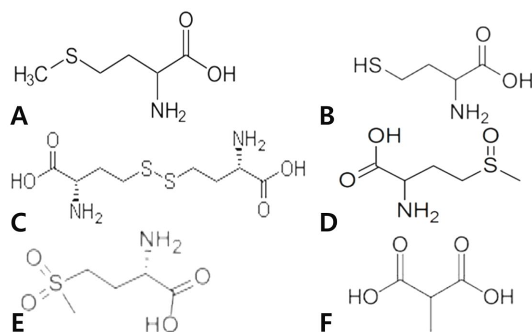

Fig. 1. Structure of (A) Methionine. (B) Homocysteine. (C) Homocystine. (D) Methionine sulfoxide. (E) Methionine sulfone. (F) Methylmalonic acid.

(Osaka, Japan (Fig. 1). All chemicals and organic solvents including acetonitrile and methanol including S-aminoethyl cysteine (IS, internal standard) were of analytical-reagent grade and purchased from J. T. Baker or YAKURI pure chemical co. LTD (Osaka, Japan). N-Methyl- N-trifluoroacetamide (MSTFA), N-Methyl-bis (trifluoroacetamide) (MBTFA) as derivatisation reagent was purchased from Sigma-Aldrich (MA, USA). Distilled water was prepared by Millipore-Milli QTM Thermo Vap (TAITEC model DTU-2C, Japan) was used for evaporation and derivatization. A shaker (TAITEC, Tokyo, Japan) and a centrifuge (Eppendorf model 5424) (Hamburg Germany) were used for mixing and centrifuging the specimens in different steps.

2. Sample preparation

A 50 μL of a plasma specimen were deproteinized with 500 μL of 10% methanol solution. Fifty μL of IS solution was then added. The mixture was centrifuged at 3,000 rpm at room temperature for 3 min. Hundreds μL of supernatant were dried under a gentle nitrogen stream at 80℃ for 10 min.

The TMS derivative of a carboxylic functional group was performed by adding 50 μL of BSTFA. As an indicator reagent 10 μL methyl orange was then added. The mixture was subjected to gentle vortex-mixing for 1 min until the color of the indicator changed from red to yellow. The mixture was then reacted at 80℃ for 10 min. For trifluoroacyl derivative (TFA) of the amino functional group, 20 μL of MBTFA was added and reacted at 60℃ for 30 min. The completed TMS-TFA derivatives of the carboxylic and amino acid functional group were cooled for 5 min (Fig. 2). The residue was transferred to an autosampler vial containing a low volume insert for analysis by GC-MS.

3. Gas chromatography - mass spectrometry

Analyses were carried out on a Hewlett-Packard- 6890 Series II GC-MS system (PA, USA) consisting of a Model 6890N gas chromatograph, a Model 5973N mass-selective detector, a HP Hewlett-Packard 3365 MSD Chemstation, and a Model 6890 series injector. A HP-5 MS (30 m×0.25 mm I.D., 0.25 ㎛) fused-silica capillary column was used for the separation of sulfur amino acids. Helium as a carrier gas was set to a flow rate of 1 mL/min with column head pressure of 130 kPa. The split ratio was 1:10. The GC oven temperature was programmed from 100℃ (3 min hold) to 180℃ (1 min hold) at a rate of 30℃/min then to 240℃ (0.5 min hold) at a rate of 20℃ and finally to 300℃ (1 min hold) at a rate

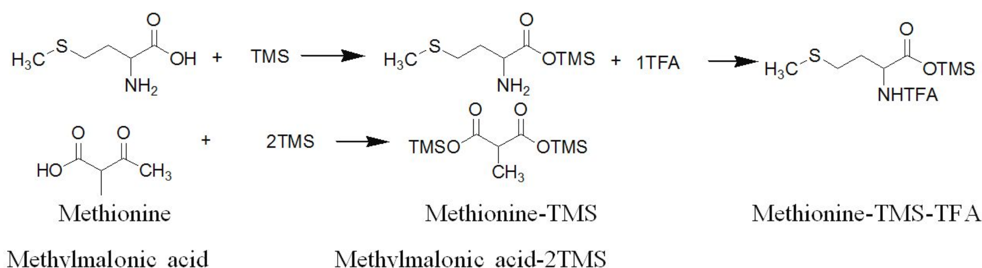

Fig. 2. Reaction scheme for TMS-TFA derivative for methionine and methylmalonic acid.

of 50℃. The temperature of the interface and inlet were 300℃ and 280℃, respectively.

The MS was executed in selected ion monitoring (SIM) mode with dwell time of 100 ms. One μL aliquot of the final derivative was injected into the GC-MS with the split ratio of 10:1.Mass spectrum and chromatogram of the TMS-TFA derivatives were obtained in full scan mode with a scan range from m/z 50 to 550 (Fig. 3, 4). Table I shows the ions used for quantification

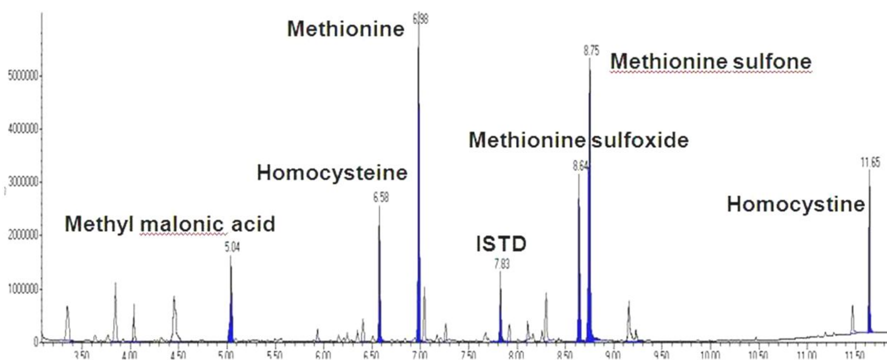

Fig. 3. Mass spectra of TMS-TFA derivative for sulfur compounds and methymalonic acid.

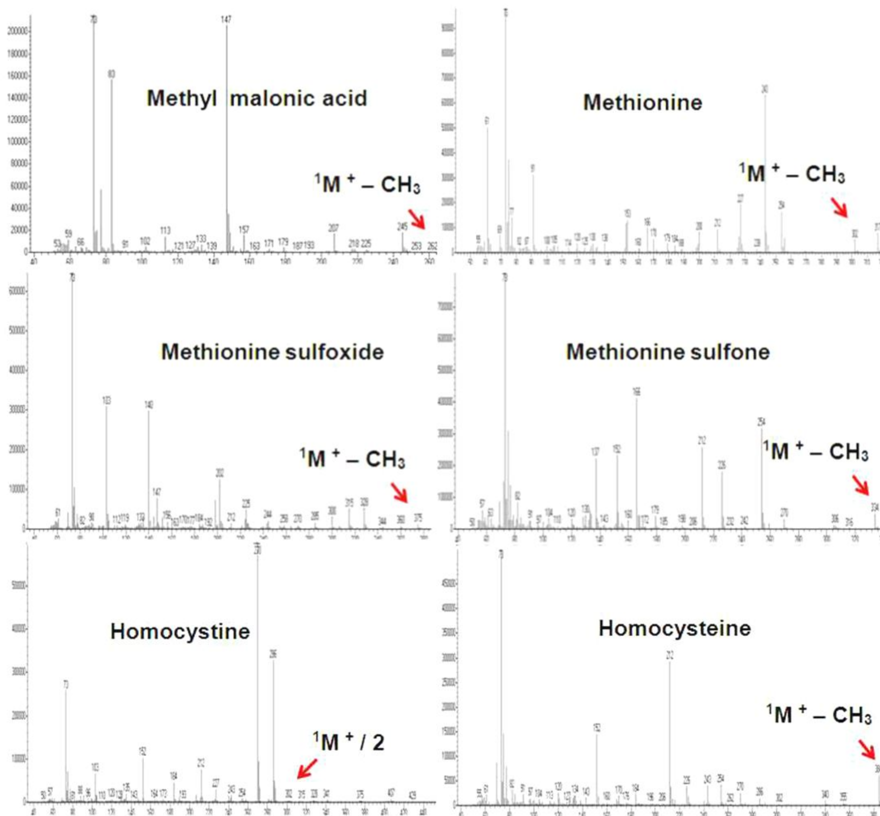

Fig. 4. Chromatograms of mixtures of sulfur compounds and methymalonic acid.

(QI) and confirmation ion (CI).

4. Specimen collection and preparation of standard solution

For control plasmas, specimens were collected from thirty healthy volunteers (18–30 years of age) in polyethylene tubes. The plasma specimens were immediately stored at −20℃ until analysis. All control subjects were subjected to the same diagnostic procedure at the same facility.

Stock solutions of each sulfur amino acids and internal standard were dissolved in methanol (1,000 μg/mL) and stored in a refrigerator until the analysis. Each stock solution was further diluted to different concentrations with methanol as working solution. Methyl orange (200 ppm) was prepared by dissolving in a mixture of acetonitrile and trifluoroacetic acid (6:4) and used as indicator.

5. Calibration

The calibration was performed by spiking pooled plasma specimen with 0.5, 1, 2, 10, 20 and 35 ng/mL of standard solutions and internal standard solution (10 ng/mL) of methionine, homocysteine, homocystine, methionine sulfoxide, methionine sulfone and methyl malonic acid.

6. Validation

The method was validated according to the US Food and Drug Administration (FDA) bioanalytical method validation guidelines[27].

The analyte concentration where the signal to noise ratio was greater than 3 was chosen as the limit of detection (LOD) and that greater than 10 was chosen for limit of quantitation (LOQ)[27]. Accuracy for intra-day assay was measured by the following equation; [measured concentration−apparent concentration]/[apparent concentration]×100%[27]. Precision for intra-day assay was calculated using standard spiked pooled plasma and was expressed as coefficient of variation (CV). Recovery tests were performed by comparing quantitative result of extracted and non-extracted spiked plasmas after fortified with low (2 ng/mL), medium (15 ng/mL), and high (30 ng/mL) concentration of sulfur compounds and methylmalonic acid.

Results

TMS derivatization of the hydroxyl group and TFA derivatization of the amine group were quantitatively completed by the addition of BSTFA and MBTFA (Fig. 2).

The mass spectrum was obtained at electron impact ionization mode in full scan with a scan range from 50 to 550 m/z. Molecular ion and other fragmentation ion of all compounds investigated were confirmed concurrent with library matching (Fig. 3). Molecular ion was chosen as a confirmation ion and a product ion with highest intensity was selected as a quantification ion (Table 1). A representative GC-MS chromatogram of the

Table 1. Parent and Product Ion for the Sulfur Compounds

Sulfur amino acid	F.W	M+	QI, CI
Methylmalonic acid	118	262	147,218
Methionine	149	317	227,243
Methionine sulfoxide	165	390	140,202
Methionine sulfone	181	349	166,254
Homocystine	268	604	270,286
Homocysteine	135	399	152,212

standard mix solution of sulfur compounds and methylmalonic acids shown in Fig. 4.

Linearity of calibration curve ranged from 0.5–5.0 ng/mL with a correlation coefficient between 0.993–0.999 for all compounds investigated showing good linearity (Fig. 5). Limit of detection and limit of quantitation was determined as 0.4 ng/mL and 4 ng/mL, respectively. In order to observe carry–over effect six blank plasmas were run after running the highest concentration of standard solutions and demonstrated no visible interference peak (data not shown).

Precision and accuracy of the method were evaluated on standard spiked plasma at 3 different concentration for intra–day assay. The precision was within 1 to 15% of RSD. The recoveries of the analytes from plasma at low, medium and high concentration ranged from 86% to 122%. Overall, the method was suitable enough for screening and diagnostic purposes (Table 2).

Discussion

Rapid and sensitive analytical method with two step derivatization was developed and validated under a simple sample preparation within 12 min of chromatographic separation.

Homocysteinemia is a metabolic disorder that effects eyes, central nervous system, skeletal and vascular system[3]. The disorder is characterised by several biochemical alterations resulting in increased levels of sulfur amino compounds and methylmalonic acid. As a result plasma sulfur compounds and methylmalonic acid concentration monitoring has emerged as a useful tool for the diagnosis and monitoring the presence of metabolic disorders[3–5].

We developed a new method using two step derivatization, for carboxylic group with silylation and amino group with acylation. This two–step derivatizaton technique results in high sensitivity and resolution for the quantification of both sulfur

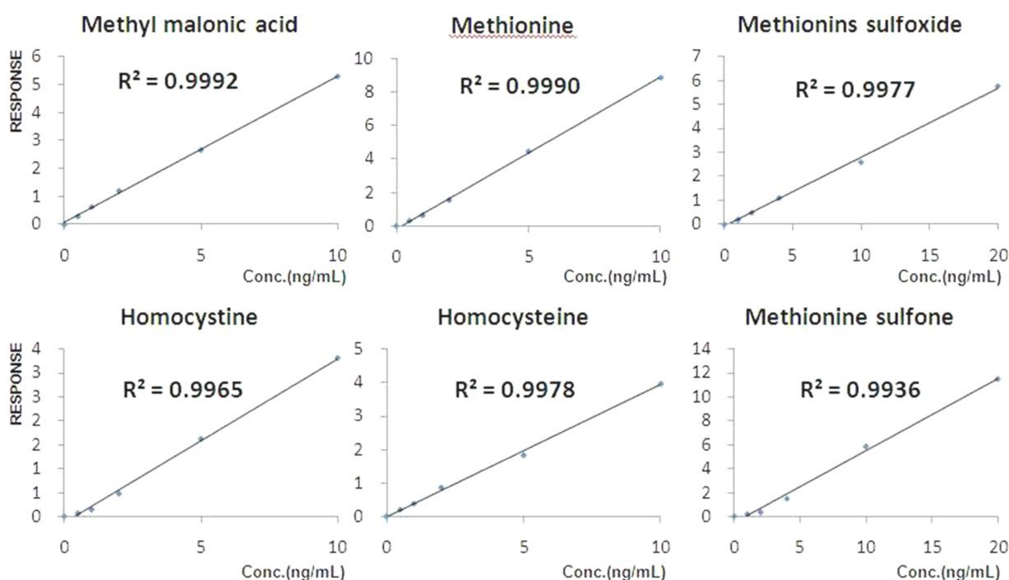

Fig. 5. Calibration curves of a mixture of sulfur compounds and methymalonic acid for GC–MS– SIM analyses.

Table 2. Precision and Recovery of the Intra-day Assay

Compounds	Conc. (ng/mL)	Conc. Calculated	Precision RSD (%)	Recovery (%)
Methionine	2	2.57	0.01	128.5
	15	15.36	0.83	102.4
	30	29.2	1.19	97.3
Homocysteine	2	2.45	0.04	122.5
	15	15.87	0.88	105.8
	30	31.4	0.13	104.6
Methionine sulfoxide	2	1.97	0.13	98.5
	15	14.25	1.75	95
	30	26.01	0.08	86.7
Methionine sulfone	2	1.76	0.04	88
	15	14.88	0.76	99.2
	30	27.1	0.58	90.3
Methyl Malonic acid	2	1.99	0.05	99.5
	15	14.88	0.7	99.2
	30	21.07	0.43	70.2

Abbreviations: Conc., concentration; RSD, relative standard deviation.

Table 3. Analytical Method Comparison for Sulfur Compounds by GC-MS

	Sample Preparation	Calibration range (μM)	LOD (μM)
Windel berg et al., 2005[21]	methylchloroformate and toluene derivatization	MMA: 0.03-100, homocysteine: 0.1-100, methionine: 1-1,000	MMA: 0.03, homocysteine: 0.1, ethionine: 1 L
Valerio et al., 2005[22]	bis-tert-butyldimethylsilyl (TBDMS)	methionine sulfoxide: 0-153, methionine: 0-169	methionine sulfoxide: 1 pmol, methionine: 0.1 pmol
Mashima e tal., 2003[23]	bis-tert-butyldimethylsilyl (TBDMS)	homocysteine: 1-400, methionine: 0.02-8	homocysteine: 1, methionine: 0.02
Švagera et al., 2012[20]	methyl, ethyl and propyl chloroformate derivatization	Homocysteine and methionine: 0.5-7 μg/mL	methionine: 0.27 μg/mL, homocysteine: 0.25 μg/mL

compounds and methylmalonic acid.

Svagera et al[20], Windelberg et al[21], and Mashima et al[23] reported the analysis of homocysteine and methionine with GC-MS. Methionine sulfoxide was analysed by Valerio et al[22]. Table 3 is summarized the reported analytical method for methionine by other researcher where the LOD is similar or inferior to our study.

To our limited knowledge this study showed for the first time two-step derivatization targeted sulfur related compounds and methylmalonic acid by GC-MS-SIM. Since homocysteinemia excreted higher amount of sulfur amino acid and methylmalonic acid in plasma, LOD and LOQ shown in this study is sensitive enough for the diagnosis or following monitoring of homocysteinemia.

Conclusion

The main advantage of this method is the simple two step derivatization following protein precipitation without expensive and time consuming SPE or LLE. Thus, this method could provide useful analytical tool for the diagnosis and monitoring of treated patients with homocysteinemia.

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