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Simultaneous determination of methionine and total homocysteine in human plasma by gas chromatography–mass spectrometry

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Abstract

A gas chromatographic–mass spectrometric method for the simultaneous determination of methionine and total homocysteine in human plasma is described. DL-[$^2H_4$]Methionine and DL-[$^2H_8$]homocystine were used as internal standards. The method involved reduction of the disulfide bond with dithiothreitol, purification by cation-exchange chromatography using a BondElut SCX cartridge and derivatization with isobutyl chlorocarbonate in water–ethanol–pyridine. Quantitation was performed by selected-ion monitoring of the quasi-molecular ions of *N*(*O*,*S*)-isobutyloxycarbonyl ethyl ester (IBC-OEt) derivatives for methionine and [$^2H_4$]methionine, respectively, and the fragment ions ([M+H–COOisoBu–COOEt]$^+$) for IBC-OEt derivatives for homocysteine and [$^2H_4$]homocysteine, respectively. The sensitivity, specificity, accuracy and precision of the method were demonstrated to be satisfactory for measuring concentrations of methionine and total homocysteine in human plasma.

Keywords: Methionine; Homocysteine

1. Introduction

Homocysteine is a thiol-containing amino acid formed from methionine through the intermediates *S*-adenosylmethionine and *S*-adenosylhomocysteine. Homocysteine is then metabolized by remethylation or transsulfuration [1,2]. In the remethylation pathway, homocysteine acquired a methyl group from 5-methyltetrahydrofolate (MTHF) or from betaine to form methionine. The reaction with MTHF is vitamin B_{12}-dependent, whereas the reaction with betaine is vitamin B_{12}-independent. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5′-phosphate (PLP)-containing enzyme, cystathionine β-synthase. Cystathionine is hydrolyzed by a second PLP-containing enzyme, γ-cystathionase, to form cysteine and α-ketobutyrate.

The plasma concentration of homocysteine is markedly increased in patients with genetic disorder called homocystinuria [3]. Furthermore, an elevated plasma homocysteine (hyperhomocysteinemia) is currently considered as an independent risk factor for cardiovascular disease [4–6] and can result from genetic or nutrient-related disturbances in homocysteine metabolism [7]. To understand the mechanisms of plasma homocysteine elevation, it is necessary to quantitatively assess methionine transmethylation,

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and homocysteine remethylation and transsulfuration in hyperhomocysteinemic patients.

A methionine loading test has been used as the most common methods for detecting mild hyperhomocysteinemia in patients with premature cardiovascular disease [8–11]. The test is based on measurement of the plasma homocysteine before and 2, 4 or 6 h after a high dose of methionine (0.1 g/kg). An inherent problem is that this method does not permit a distinction between endogenous homocysteine and homocysteine derived from administered methionine. Furthermore, the method cannot clarify which pathway is impaired in the methionine–homocysteine metabolism.

The use of gas chromatography–mass spectrometry (GC–MS) and isotopically labeled compounds as tracers has enjoyed broad application in metabolic studies [12–15]. One of the major advantages of this technique is that endogenous and exogenous compounds having the same basic structure can be differentiated easily by employing stable isotopically labeled compounds. We have initiated studies to assess the methionine–homocysteine metabolism after administration of stable isotope-labeled methionine. In the present work, we have developed a isotope dilution GC–MS method for the determination of methionine and total homocysteine in human plasma.

2. Experimental

2.1. Chemical and reagents

DL-[3,3,4,4-$^2H_4$]Methionine ([$^2H_4$]methionine; >99% atom ^2H) and DL-[3,3,3′,3′,4,4,4′,4′-$^2H_8$]-homocystine ([$^2H_8$]homocystine; 97.9% atom ^2H) were purchased from CDN Isotopes (Quebec, Canada). Methionine, isobutyl chlorocarbonate and dithiothreitol were obtained from Wako (Osaka, Japan). Homocystine was obtained from Nacalai Tesque (Kyoto, Japan). A cation-exchange BondElut SCX cartridge was purchased from Varian (Harbor City, CA, USA). All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Stock solutions

The stock standard solutions of methionine (1.52 mg/100 ml), [$^2H_4$]methionine (14.89 mg/100 ml), homocystine (2.75 mg/100 ml) and [$^2H_8$]homocystine (2.79 mg/100 ml) were prepared in 100 m*M* pyridine. Storage of these solutions at 4°C did not result in any detectable decomposition for more than 3 months. All analyses were performed by diluting the stock solutions with 100 m*M* pyridine.

2.3. Gas chromatography–mass spectrometry with selected-ion monitoring (GC–MS-SIM)

GC–MS-SIM measurements were made with a Shimadzu (Kyoto, Japan) QP-1000EX quadrupole gas chromatograph–mass spectrometer equipped with a data processing system. A methylsilicone bonded-phase fused-silica capillary column SPB-1 (10 m×0.25 mm I.D.) with 0.25 μm film thickness (Supelco, Bellefonte, PA, USA) was connected directly to the ion source. Helium was used as the carrier gas at a column head pressure of 0.8 kg/cm^2. A split–splitless injection system Shimadzu SPL-G9 operating in the splitless mode was used with a septum purge flow-rate of 1.0 ml/min and split vent flow-rate of 30 ml/min. The purge activation time was 2 min after injection. The initial column temperature was set at 120°C. After the sample injection, it was maintained for 2 min and was increased at 40°C/min to 280°C and maintained there for 1 min. The temperature of the injector was 280°C. The mass spectrometer was operated in chemical ionization (CI) mode with isobutane as the reagent gas at pressure of $2\cdot10^{-5}$–$5\cdot10^{-5}$ Torr (1 Torr=133.322 Pa). The ionization voltage and ionization current were 200 eV and 150 mA, respectively. The ion source temperature was 280°C. SIM was performed on the quasi-molecular ions at *m/z* 278 and 282 for the *N*(*O*,*S*)-isobutyloxycarbonyl ethyl ester (IBC-OEt) derivatives of methionine and [$^2H_4$]methionine, respectively, and on the fragment ions ([M+H–COOisoBu–COOEt]$^+$) at *m/z* 190 and 194 for the IBC-OEt derivatives of homocysteine and [$^2H_4$]homocysteine, respectively.

2.4. Sample preparation

Frozen plasma samples were thawed at room temperature. To a PTFE-lined screw-cap culture tube (100×16 mm I.D.) were added 0.1 ml of plasma, 0.1 ml of water, and 10 nmol of [$^2H_4$]methionine and 2 nmol of [$^2H_8$]homocystine as the internal standards dissolved in 0.1 ml of 100 m*M* pyridine. Following addition of 1% dithiothreitol in acetonitrile (0.05 ml), the resulting solutions were stood at room temperature for 30 min. The samples were deproteinized with 10% trichloroacetic acid (0.2 ml) on a vortex mixer for 10 s. After centrifugation at 3000 rpm for 5 min, the supernatant was applied to a BondElut SCX cartridge, which was pre-washed and activated with 3 ml of methanol, 3 ml of a mixture of methanol–0.1 *M* hydrochloric acid (1:1, v/v) and 3 ml of 0.1 *M* hydrochloric acid. The cartridge was washed with 1 ml of water and 1 ml of a mixture of water–ethanol (2:1, v/v), and then eluted with 0.5 ml of a mixture of water–ethanol–pyridine (30:16:4, v/v) into a PTFE-lined screw-cap conical centrifuge tube (100×16 mm I.D.). To the eluent was added isobutyl chlorocarbonate (0.05 ml) and the solution was mixed on a vortex mixer for 10 s. The sample was extracted with 1 ml of chloroform. After evaporating to dryness under a stream of nitrogen, the residue was dissolved in 20 μl of ethyl acetate. A 1–2 μl volume of the solution was subjected to GC–MS-SIM.

2.5. Calibration curves and quantitation

To each of a series of standards containing known amounts of methionine (0.2–10 nmol) and homocystine (0.2–10 nmol) dissolved in 0.2 ml of 100 m*M* pyridine, 10 nmol of [$^2H_4$]methionine and 2 nmol of [$^2H_8$]homocystine dissolved in 0.1 ml of 100 m*M* pyridine were added as the internal standards. Each sample was prepared in triplicate. The samples were derivatized and analyzed as described above. The peak-area ratios (*m*/*z* 278 versus *m*/*z* 282 for methionine and *m*/*z* 190 versus *m*/*z* 194 for homocysteine) were determined in triplicate. The calibration graphs were obtained by an unweighted least-squares linear fitting of the peak-area ratios versus the mixed molar ratios on each sample. Plasma concentrations were calculated by comparing the peak-area ratios obtained from the unknown samples with those obtained from the standard mixtures.

2.6. Accuracy and precision

Accuracy and precision were determined by assaying five preparations of 0.1-ml portions of pooled human plasma spiked with several concentrations of methionine (0.2, 0.5, 1, 2 and 5 nmol) and homocystine (0.2, 0.5, 1, 2 and 5 nmol). After preparation of the sample for GC–MS-SIM as described above, the peak-area ratios were measured.

2.7. Human plasma sample

Seventeen healthy volunteers, 11 men and six women aged 21–49 years (mean 26.4±8.5 years), participated in the study. Informed consent was obtained from all subjects. After an overnight fast, venous blood samples were taken into a EDTA-containing tube Veneject II (Terumo, Tokyo, Japan) between 09:00 and 10:00 and was immediately centrifuged at 1000 *g* for 15 min at room temperature. The plasma sample was immediately frozen and stored at −20°C.

3. Results and discussion

Homocysteine in human plasma is predominantly bound with itself, cysteine and protein sulfhydryl groups to form homocystine, homocysteine–cysteine and protein-bound homocysteine, respectively. Total homocysteine refers to all the forms of homocysteine. Determination of total homocysteine in plasma requires the reduction of the disulfide bond between homocysteine and other thiols. The following three reductants were examined; dithioerythritol, dithiothreitol and tri-*n*-butylphosphine. The use of dithioerythritol and tri-*n*-butylphosphine gave the interfering peaks in the vicinity of the peak of homocysteine on GC. Dithiothreitol was selected for this assay.

GC–MS-SIM is widely accepted as the most

accurate and specific method for the determination of small amounts of substances in biological materials. The use of stable isotopically labeled analogues as internal standards for the GC–MS analysis offers major advantages that they behave in almost identical manner to the analyte through all steps in the extraction, derivatization, and chromatographic procedures. Commercially available DL-[3,3,4,4-$^2H_4$]-methionine and DL-[3,3,4,4,3′,3′,4′,4′-$^2H_8$]homocystine were chosen for the analytical internal standards.

Several derivatization methods have been used to measure sulfur amino acids. Stabler and co-workers have reported the use of *tert*-butyldimethylsilyl derivatives [16,17]. The high natural abundance of ^{29}Si and ^{30}Si present in the derivatives results in a higher background which may limit the analytical precision and accuracy of SIM measurements. A new approach to amino acid derivatization by using alkyl carbonate reagents in a medium of water–alcohol–pyridine was described by Hušek and co-workers [18–21]. A single-step procedure allowed the simultaneous acylation of the amine and thiol groups, and esterification of the carboxylic group. A mechanism was proposed based on the formation of an intermediate mixed carboxylic–carbonic acid anhydride followed by the exchange with an alcohol [22]. Various combination of alkyl carbonate reagents and alcohols were used to generate a variety of *N*(*O*,*S*)-alkoxycarbonyl alkyl ester derivatives for determination of homocysteine by GC–MS [23,24]. Pietzsch and co-workers have reported the determination of homocysteine by using *N*(*O*,*S*)-ethyloxycarbonyl ethyl ester derivatives [25]. However, the *N*(*O*,*S*)-ethyloxycarbonyl ethyl ester derivatives of methionine gave an ion $[M+H-4]^+$ in the chemical ionization mode with isobutane as the reagent gas. Although the ion was a very low intensity, the analytical accuracy and precision may be limited when the SIM was performed on the quasi-molecular ions using the analogue incorporated four deuterium atoms as internal standard. Propyloxy- and isopropyloxy-derivatives of methionine also gave the ion $[M+H-4]^+$, the corresponding ions were not observed in butyloxy- and isobutyloxy-derivatives. The isobutyloxycarbonyl ethyl ester derivatives have shorter retention times than the butyloxy derivatives and were selected for the assay. The retention times for methionine and homocysteine were about 4.6 and 5.85 min, respectively.

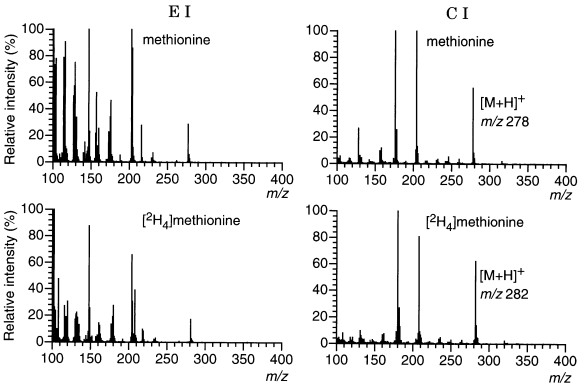

Fig. 1. Electron ionization (EI) and chemical ionization (CI) mass spectra of the *N*(*O*)-isobutyloxycarbonyl ethyl ester derivatives of methionine and [$^2H_4$]methionine.

Fig. 1 shows the electron ionization (EI) and the CI mass spectra of the *N*(*O*)-isobutyloxycarbonyl ethyl ester derivatives of methionine and [$^2H_4$]methionine. The quasi-molecular ions $[M+H]^+$ at m/z 278 and 282 in the CI method were chosen for quantitation by the SIM method. Fig. 2 shows the EI and CI mass spectra of the *N*(*O*,*S*)-isobutyloxycarbonyl ethyl ester derivatives of homocysteine and

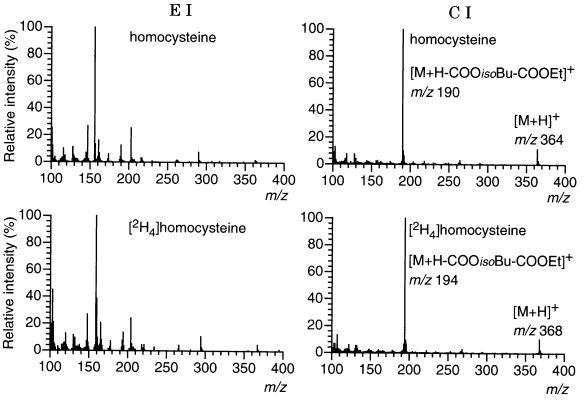

Fig. 2. Electron ionization (EI) and chemical ionization (CI) mass spectra of the *N*(*O*,*S*)-isobutyloxycarbonyl ethyl ester derivatives of homocysteine and [$^2H_4$]homocysteine.

[$^2H_4$]homocysteine. Quasi-molecular ions of low abundance are present. Intense fragment ions corresponding to the neutral loss of molecules of COOisoBu and COOEt were found at m/z 190 and 194 in the mass spectra of unlabeled and labeled homocysteine, respectively. Exhibiting no loss of label, these ions were chosen for quantitation by the SIM method. Calibration curves were prepared from a series of samples containing various amounts of methionine in the range of 0.2–10 nmol or homocystine in the range of 0.2–10 nmol. Each sample was assayed as the *N*(*O*,*S*)-isobutyloxycarbonyl ethyl ester derivative by monitoring the ion intensities at m/z 278 and 282, and m/z 190 and 194. When the peak-area ratios were plotted against the mix molar ratios, a good correlation was found, $y=1.0084x+0.0015$ ($r=1.0000$) for methionine and $y=1.0814x+0.0059$ ($r=1.0000$) for homocysteine.

Ion-exchange columns such as a Dowex 50W X8 have been used as a simple method for extracting amino acids from biological fluids prior to GC–MS analysis [15,25]. However, evaporation of the eluent solvent was laborious and time-consuming. In the present method we used a mixture of water–ethanol–pyridine (30:16:4, v/v) as the eluent solvent from BondElut SCX cartridge. The solvent was directly used as a reaction medium for the derivatization of amino acids with isobutyl chlorocarbonate. When the mixture of water–ethanol–pyridine (30:16:4, v/v) was used as the eluent solvent, the total recovery from human plasma was found to be high yield (70±1% for methionine and 69±2% for homocysteine, $n=3$).

The accuracy and precision of the assay was determined by spiking 0.1-ml aliquots of human plasma with multiple standard solution of methionine and homocystine. Representative SIM profiles of plasma sample are shown in Fig. 3. There was no interference in the vicinity of the peaks of analyses in the SIM. The estimated amounts were in good agreement with the actual amounts added (Tables 1 and 2). The intra- and inter-day precision of the assay were less than 10% for each amino acids at all concentrations. The lower limit of quantitation (LOQ) for the present method was determined by spiking 0.1-ml aliquots human plasma with [$^2H_4$]methionine and [$^2H_8$]homocystine. The LOQ

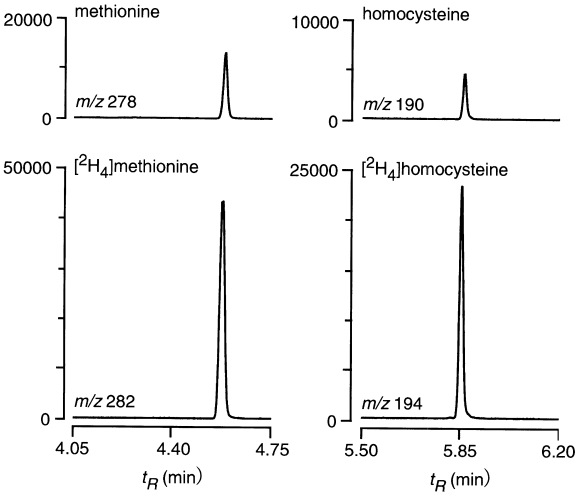

Fig. 3. Selected-ion monitoring profiles of extracts from human plasma.

was around 50 pmol/ml plasma for [$^2H_4$]methionine and around 200 pmol/ml plasma for [$^2H_4$]homocysteine.

The validated method was applied to determine the concentrations of methionine and total homocysteine in human plasma. The mean±SD concentrations of methionine and total homocysteine in 17 adults were 30.4±5.63 nmol/ml (range 21.3–47.2) and 9.3±2.17 nmol/ml (range 5.3–12.8), respectively. The data are in agreement with previously results in healthy subjects [16,26].

The present method provides a sensitive and reliable technique for the simultaneous determination of methionine and total homocysteine in plasma with good accuracy and precision. The method can be applied to pharmacokinetic and metabolic studies of methionine with a particular interest in evaluating the transmethylation and remethylation.

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Table 1
Intra-day accuracy and precision for determination of methionine and homocysteine in plasma

Methionine					Homocysteine				
Added (nmol)	Expected (nmol/ml)	Found (nmol/ml)[a] (mean±SD)	RSD (%)	R.E. (%)	Added (nmol)	Expected (nmol/ml)	Found (nmol/ml)[a] (mean±SD)	RSD (%)	R.E. (%)
–	–	16.84±0.81	4.78	–	–	–	7.31±0.39	5.31	–
0.20	18.88	19.31±0.67	3.47	2.25	0.41	11.41	10.80±0.40	3.69	−5.29
0.51	21.95	22.22±1.98	8.90	1.24	1.03	17.56	16.69±1.30	7.77	−4.94
1.02	27.05	27.03±0.92	3.40	−0.08	2.05	27.81	26.86±0.07	0.24	−3.40
2.04	37.26	37.18±0.60	1.62	−0.21	4.10	48.31	50.80±1.80	3.54	5.16
5.11	67.89	67.87±2.85	4.20	−0.02	10.25	109.81	109.02±6.33	5.81	−0.71

[a] $n=3$.

Table 2
Inter-day accuracy and precision for determination of methionine and homocysteine in plasma

Methionine					Homocysteine				
Added (nmol)	Expected (nmol/ml)	Found (nmol/ml)[a] (mean±SD)	RSD (%)	R.E. (%)	Added (nmol)	Expected (nmol/ml)	Found (nmol/ml)[a] (mean±SD)	RSD (%)	R.E. (%)
–	–	15.78±0.24	1.52	–	–	–	7.90±0.55	6.99	–
0.20	17.82	17.53±0.79	4.53	−1.64	0.41	12.00	12.68±1.15	9.06	5.72
0.51	20.88	20.78±0.29	1.39	−0.50	1.03	18.15	17.22±1.07	6.21	−5.11
1.02	25.99	25.50±1.08	4.23	−1.89	2.05	28.40	26.94±0.88	3.27	−5.14
2.04	36.20	36.09±0.41	1.14	−0.29	4.10	48.90	49.18±1.81	3.68	0.58
5.11	66.83	66.94±0.22	0.33	0.17	10.25	110.40	110.54±1.28	1.16	0.13

[a] $n=3$.

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