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Synthesis of L-[3,3,4,4,*S*-Methyl-$^2H_7$]Methionine for Use as a Substrate for the Methionine Loading Test

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SUMMARY

Optically pure L-[3,3,4,4,*S*-methyl-$^2H_7$]methionine (L-[$^2H_7$]-methionine) for use as a substrate for the methionine loading test has been prepared. The racemic [$^2H_7$]methionine was prepared from DL-[3,3,4,4-$^2H_4$]methionine (DL-[$^2H_4$]methionine) by conversion of the *S*-CH_3 group to a C^2H_3 group. The racemate was resolved by stereospecific hydrolysis of the *N*-acetylated derivative with acylase. After an intravenous administration of L-[$^2H_7$]methionine in a rat, the concentration of L-[$^2H_7$]methionine and the metabolites in plasma were determined by GC-MS-SIM. L-[$^2H_4$]Homocysteine and L-[$^2H_4$]methionine were detected in plasma at 30 min after dosing. These results show that L-[$^2H_7$]methionine was converted by de-methylation and subsequent re-methylation to form L-[$^2H_4$]methionine.

Key words: methionine, homocysteine, hyperhomocysteinemia, deuterium, GC-MS, loading test

INTRODUCTION

Homocysteine is a sulfur-containing amino acid produced by demethylation of methionine through the intermediates *S*-adenosylmethionine and *S*-adenosylhomocysteine. Homocysteine is then either re-methylated by accepting a methyl group from either betaine or

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5-methyltetrahydrofolate to form methionine or catabolized irreversibly to form cystathionine. Moderate hyperhomocysteinemia is an independent risk factor for arteriosclerotic vascular disease (1-3) and can result from genetic or nutrient-related disturbances in homocysteine metabolism (4). To understand the pathophysiology of hyperhomocysteinemia, it is necessary to quantitatively assess the importance of methionine transmethylation, homocysteine remethylation and transsulfuration in patients. An L-methionine loading test has been used as one of the most common methods for detecting moderate hyperhomocysteinemia in patients with premature cardiovascular disease (5-7). An inherent problem of this method is that it does not permit a distinction between endogenous and exogenous L-methionine in biological fluids after dosing. The method also requires a large dose of L-methionine (50-100 mg/kg body weight) to obtain high concentrations beyond the endogenous level of L-methionine in the patients.

We have initiated studies to assess methionine-homocysteine metabolism after administration of stable isotope-labelled L-methionine. L-[3,3,4,4,*S*-Methyl-$^{2}H_7$]methionine (L-[$^{2}H_7$]methionine) was chosen as a tracer. The *S*-$C^{2}H_3$ label of L-[$^{2}H_7$]methionine is removed during transmethylation to form L-[3,3,4,4-$^{2}H_4$]homocysteine (L-[$^{2}H_4$]-homocysteine), and then L-[$^{2}H_4$]homocysteine is re-methylated to form L-[3,3,4,4-$^{2}H_4$]methionine (L-[$^{2}H_4$]methionine). Since non-labeled L-methionine, L-[$^{2}H_4$]methionine and L-[$^{2}H_7$]methionine are distinguishable from each other by GC-MS, it has become feasible to investigate the extent of homocysteine re-methylation after L-[$^{2}H_7$]methionine dosing. The present paper describes the preparation of optically pure L-[$^{2}H_7$]methionine for use as a substrate for the methionine loading test from commercially available DL-[$^{2}H_4$]methionine.

EXPERIMENTAL

DL-[3,3,4,4-$^{2}H_4$]Methionine (DL-[$^{2}H_4$]methionine; >99% atom ^{2}H) and DL-[3,3,3',3',4,4,4',4'-$^{2}H_8$]homocystine (DL-[$^{2}H_8$]homocystine; 97.9% atom ^{2}H) were purchased from CDN isotopes (Quebec, Canada). [$^{2}H_3$]Methyl iodide (>99.5% atom ^{2}H) was purchased from E. Merck (Darmstadt, Germany). A cation-exchange BondElut SCX cartridge column was

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purchased from Varian (Harbor City, OH, USA). ^1H and ^{13}C NMR spectra were recorded on a Bruker (Rheinstetten, Germany) DRX500 spectrometer. The samples for ^1H and ^{13}C NMR were dissolved in [$^2H_4$]methanol or 0.1M sodium deuteroxide in deuterium oxide (0.5 ml) containing [$^2H_4$]methanol as a reference for ^{13}C NMR. Chemical shifts were expressed in δ (ppm) downfield from methanol (3.35 ppm) and H^2HO (4.8 ppm) for ^1H NMR and [$^2H_4$]methanol (49.0 ppm) for ^{13}C NMR, respectively. The GC-MS-SIM analysis was conducted on a Shimadzu (Kyoto, Japan) QP1000EX gas chromatograph-mass spectrometer equipped with a data processing system and were performed under the conditions described previously (8).

DL-[$^2H_7$]Methionine.

To a solution of DL-[$^2H_4$]methionine (1.0 g, 6.5 mmol) in liquid ammonia (ca. 100 ml) was added small pieces of metallic lithium (200 mg) at -78°C. The resulting blue solution was stirred at -78°C with progress of the reaction being monitored by ^1H NMR as follows. Aliquots of the solution (ca. 1 ml) were removed, evaporated and re-dissolved in 0.1M sodium deuteroxide in deuterium oxide, and the disappearance of the *S*-CH_3 proton signal (δ 2.01 ppm) of unreacted DL-[$^2H_4$]methionine monitored. Additional amounts of lithium were added in two portions (30 mg each) at 2 h intervals. After being stirred for 6 h, ammonium chloride (0.5 mg) was added to the reaction mixture and the color of the solution was discharged. To the solution was added excess of [$^2H_3$]methyl iodide (1 ml, 15 mmol). The solution was stirred for 2 h at -78°C and then allowed to stand at room temperature to remove ammonia. The remaining off-white residue was dissolved in water (20 ml) and evaporated under reduced pressure. Again the residue was dissolved in water (10 ml) and the pH was adjusted to 4 with 1M hydrochloric acid. The solution was applied to a cation-exchange Dowex 50W X8 column (130 x 20 mm I.D., H^+ form), washed with water (200 ml) and eluted with 1M ammonia (150 ml). After evaporating the solvent under reduced pressure, the residue was dissolved in water (20 ml) and the pH was adjusted to 5 with 1M hydrochloric acid. The solvent was evaporated under reduced pressure to yield a colorless solid, which was recrystallized with water-ethanol to yield DL-[$^2H_7$]methionine (0.75 g, 72 %) as a

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colorless crystalline solid. m.p. 261-263°C (dec.). Anal. Calcd. for $C_5H_4{}^2H_7NO_2S$, C, 38.44; H(^{2}H), 7.10; N, 8.96; O, 24.98; S, 20.52. Found C, 38.24; H(^{2}H), 6.94; N, 8.83. ^{1}H NMR (0.1M NaO^{2}H) δ 3.35 (1H, s, 2-H). ^{13}C NMR (0.1M NaO^{2}H) δ 13.89-14.57 (S-C), 29.37-30.05 (4-C), 33.88-34.50 (3-C), 56.04 (2-C), 183.81 (1-C).

Resolution of DL-[$^2H_7$]methionine.

A solution of DL-[$^2H_7$]methionine (600 mg, 3.8 mmol) in acetic acid (15 ml) was refluxed for 5 min, and acetic anhydride (2 ml, 21 mmol) was added thereto. The reaction mixture was refluxed for 5 min, and then stirred at room temperature for 2 h. After removal of the solvent under reduced pressure, the residual syrup was reconstituted with water (5 ml x 3) followed each time by evaporation to yield *N*-acetyl-DL-[$^2H_7$]methionine (751 mg, 99%) as a colorless solid. m.p. 98-100°C. Anal. Calcd. for $C_7H_6{}^2H_7NO_2S$, C, 42.40; H(^{2}H), 6.61; N, 7.06; O, 27.76; S, 16.17. Found C, 42.26; H(^{2}H), 6.73; N, 6.88. ^{1}H NMR ([$^2H_4$]methanol) δ 1.98 (3H, s, MeCO), 4.51(1H, s, 2-H). ^{13}C NMR ([$^2H_4$]methanol) δ 14.02-14.69 (S-C), 22.35 (<u>Me</u>CO), 29.91-30.57 (4-C), 31.05-31.68 (3-C), 52.68 (2-C), 173.45 (CONH), 175.17 (1-C).

The *N*-acetyl derivative (718 mg, 3.6 mmol) was dissolved in water (30 ml), and the pH was adjusted to 7.8 with 1M ammonia. Porcine kidney acylase (10 mg) was added and incubated at 37°C for 48 h. The enzymic hydrolysis was terminated by acidification with acetic acid to pH 4. After the enzyme was filtered off with the aid of charcoal, the filtrate was concentrated to ca. 5 ml under reduced pressure. The solution was applied to a Dowex 50W X8 column (H^+ form, 150 x 10 mm I.D.). The column was washed with water (250 ml) and then eluted with 1M ammonia (200 ml). The solvent was evaporated under reduced pressure. The residue was dissolved in water (10 ml) and the pH was adjusted to 5 with acetic acid. The solvent was evaporated under reduced pressure and the residue was recrystallized with water-ethanol to yield L-[$^2H_7$]methionine (181 mg, 64%) as a colorless solid. Anal. Calcd. for $C_5H_4{}^2H_7NO_2S$, C, 38.44; H(^{2}H), 7.10; N, 8.96; O, 24.98; S, 20.52. Found C, 38.15; H(^{2}H), 6.98; N, 8.81. The enantiomeric purity was analyzed by HPLC with a chiral stationary phase Crownpak CR column (150 x 4 mm ID, Daicel Chemical, Tokyo, Japan) using the following conditions: mobile

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phase, 0.07% $HClO_4$; flow rate, 0.5 ml/min; detection, UV 200 nm. The amino acid was eluted at t_R 6.2 min as a single peak and the enantiomeric purity was estimated to be >99.8% e.e..

The water washings described above were evaporated under reduced pressure. The residue was dissolved in acetone (5 ml) and the solution was stirred for 10 min, followed by evaporation to yield *N*-acetyl-D-[$^2H_7$]methionine (354 mg) as a colorless solid. The compound (350 mg, 1.7 mmol) was dissolved in 2M hydrochloric acid (15 ml) and the solution was refluxed for 3 h. The solution was concentrated to ca. 5 ml under reduced pressure and applied to a Dowex 50W X8 column (H^+ form, 150 x 10 mm I.D.). The column was washed with water (250 ml) and then eluted with 1M ammonia (200 ml). The solvent was evaporated under reduced pressure. The residue was dissolved in water (10 ml) and the pH was adjusted to 5 with acetic acid. The solvent was evaporated under reduced pressure and the residue was recrystallized with water-ethanol to yield D-[$^2H_7$]methionine (188 mg, 68%) as a colorless solid. Anal. Calcd. for $C_5H_4{}^2H_7NO_2S$, C, 38.44; H(^{2}H), 7.10; N, 8.96; O, 24.98; S, 20.52. Found C, 38.25; H(^{2}H), 7.02; N, 8.75. The enantiomeric purity was analyzed by HPLC under the conditions described above. The amino acid was eluted at t_R 4.1 min as a single peak and the enantiomeric purity was estimated to be >99.8% e.e..

Dose experiment.

After an overnight fast, a Sprague-Dawley male rat weighing 240 g was administered an intravenous bolus dose of L-[$^2H_7$]methionine (32 μmol/kg) in saline (0.1 ml) via a femoral vein under anesthesia with pentobarbital. At 30 min after dosing, the rat was sacrificed and blood (5 ml) was collected. Plasma was separated and stored at -20°C until analysis.

The double isotope dilution method was employed to measure L-[$^2H_7$]methionine, L-[$^2H_4$]methionine, endogenous L-methionine, L-[$^2H_4$]homocysteine and endogenous L-homocysteine in plasma simultaneously. The plasma sample was divided into two sets (0.1 ml each). To one aliquot of each sample was added DL-[$^2H_4$]methionine (10 nmol) and DL-[$^2H_8$]homocystine (2 nmol) as analytical standards. Two aliquots of plasma were subjected to GC-MS-SIM analysis as described

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previously (8). SIM was performed on the quasi-molecular ions at m/z 278, 282 and 285 for the $N(O,S)$-isobutyloxycarbonyl ethyl ester (IBC-OEt) derivatives of L-methionine, DL-[$^2H_4$]methionine and L-[$^2H_7$]methionine, respectively, and at m/z 364 and 368 for the IBC-OEt derivatives of L-homocysteine and DL-[$^2H_4$]homocysteine, respectively.

Calculation.

The following calculations were used to determine L-methionine, L-[$^2H_4$]methionine and L-[$^2H_7$]methionine levels, as assayed by the double isotope dilution method. The peak area ratio values for m/z 278 : 282 and m/z 285 : 282 were determined by GC-MS-SIM of the sample free of the internal standard and designated a and b, respectively. The corresponding ratio values for the sample containing the internal standard are designated c and d, respectively. The amounts of L-methionine (x), L-[$^2H_4$]methionine (y) and L-[$^2H_7$]methionine (z) in the original sample were then calculated from the following equations:

$$a = k_1 \frac{x}{y} + t_1 \qquad b = k_2 \frac{z}{y} + t_2$$

$$c = k_1 \frac{x}{y+p} + t_1 \qquad d = k_2 \frac{z}{y+p} + t_2$$

where p denotes the amount of internal standard, and k_n and t_n (n = 1, 2) denote the values for the slope and intercept of the calibration curve, respectively. Therefore,

$$x = \frac{(a-t_1)(c-t_1)}{k_1(a-c)} p \qquad y = \frac{(c-t_1)}{(a-c)} p = \frac{(d-t_2)}{(b-d)} p \qquad z = \frac{(b-t_2)(d-t_2)}{k_2(b-d)} p$$

The concentration values for L-[$^2H_4$]homocysteine and endogenous L-homocysteine were calculated using a similar method.

RESULTS AND DISCUSSION

Synthesis of L-[$^2H_7$]methionine.

The synthesis of S-methyl labelled methionine had been achieved by treating homocysteine (9), S-benzylhomocysteine (10,11) or methionine

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(12) with sodium in liquid ammonia to give the thiolate anion of homocysteine and subsequent methylation with a labelled methyl iodide. The drawback to these methods was that it was difficult to separate the methionine from sodium chloride, which was a by-product, by recrystallization with water-ethanol. The use of lithium in place of sodium enabled a high recovery of pure labelled methionine to be achieved by recrystallization because lithium chloride was soluble in ethanol (13).

The synthetic route to L-[$^2H_7$]methionine from commercially available DL-[$^2H_4$]methionine is illustrated in Figure 1. Conversion of DL-[$^2H_4$]methionine to DL-[$^2H_7$]methionine was performed by the method of Billington and co-workers (13) with minor modification. Reduction of DL-[$^2H_4$]methionine with lithium in liquid ammonia and subsequent methylation with [$^2H_3$]methyl iodide proceeded smoothly to give DL-[$^2H_7$]methionine in 72% yield. The ^1H NMR spectrum of DL-[$^2H_7$]methionine showed no proton signals for 3-H, 4-H and *S*-CH_3. Deuterium incorporation can also be monitored indirectly from isotope-induced shifts in ^{13}C NMR spectroscopy (14, 15). In the proton-decoupled ^{13}C NMR spectrum, the signal for the carbon directly attached to the deuterium shifts its centre of resonance by 0.3-0.6 ppm and spin-spin coupling produces a characteristic multiplet, hence the signals for C^2H, C^2H_2 and C^2H_3 would give a triplet, a quintet and a septet, respectively. The proton-decoupled ^{13}C NMR spectrum of the DL-[$^2H_7$]methionine gave complex signals at δ 13.89 - 14.57, 29.37 - 30.50 and 33.88 - 34.50 ppm for *S*-C, 4-C and 3-C, respectively (Figure 2). The chemical ionization mass spectra showed that the quasi molecular ion at m/z 285 for the *N*-isobutyloxycarbonyl ethyl ester (IBC-OEt) derivative of DL-[$^2H_7$]-methionine was three mass units higher than that of DL-[$^2H_4$]methionine (Figure 3). The isotopic purity of DL-[$^2H_7$]methionine was estimated to be 99.3 atom % ^2H, based on the ion intensities in the region of the molecular ion measured by GC-MS.

Following *N*-acetylation of DL-[$^2H_7$]methionine with acetic anhydride in acetic acid, stereospecific hydrolysis of the *N*-acetyl derivative was performed with porcine kidney acylase to yield L-[$^2H_7$]methionine. The remaining *N*-acetyl-D-[$^2H_7$]methionine was hydrolyzed with hydrochloric acid to yield D-[$^2H_7$]methionine. The enantiomeric purity of L- and D-[$^2H_7$]methionine were determined by HPLC with a chiral stationary

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column (Crownpak CR) and were found to be >99.8 % (e.e.), respectively. A problem is incomplete hydrolysis of the L-enantiomer, which causes enantiomeric contamination of the L-isomer to the D-product. The high optical purity of D-[$^2H_7$]methionine showed that the hydrolysis of the *N*-acetyl-DL-[$^2H_7$]methionine with acylase was almost complete.

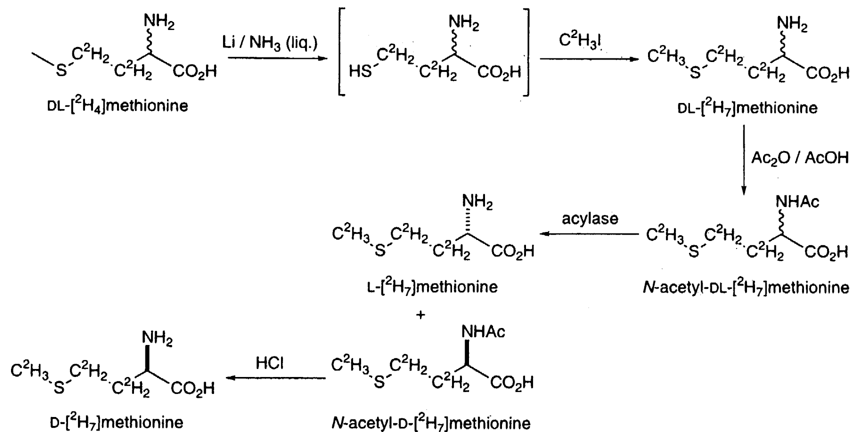

Figure 1 Synthesis of D- and L-[$^2H_7$]methionine

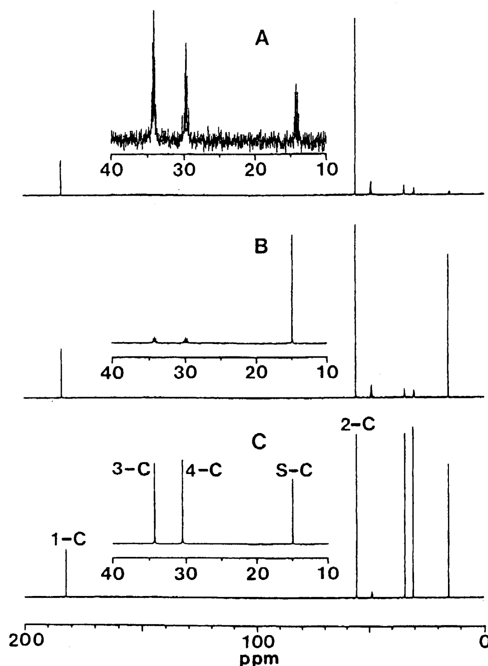

Figure 2 Proton decoupled ^{13}C NMR spectra for (A) [$^2H_7$]methionine, (B) [$^2H_4$]methionine and (C) methionine in 0.1M sodium deuteroxide containing [$^2H_4$]methanol as reference.

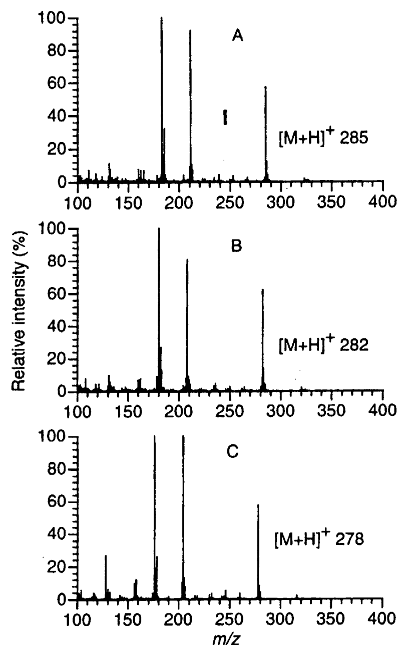

Figure 3 Chemical ionization mass spectra for *N*-isobutyloxylcarbonyl ethyl ester derivatives of (A) [$^2H_7$]-methionine, (B) [$^2H_4$]methionine and (C) methionine.

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Table 1 Accuracy and precision for GC-MS-SIM determination of L-[$^2H_7$]methionine in plasma

Added (nmol/ml)	Found (nmol/ml)						R.S.D.	Relative error
	Individual values				Mean	± S.D.	(%)	(%)
0.52	0.56	0.55	0.53	0.59	0.56	± 0.03	4.59	8.71
1.03	0.95	1.03	0.91	0.96	0.96	± 0.05	5.07	-6.65
10.30	10.33	9.59	9.83	10.12	9.97	± 0.33	3.26	-3.24
103.03	102.08	104.16	102.57	103.44	103.06	± 0.92	0.89	0.03

Determination of L-[$^2H_7$]methionine in plasma by GC-MS-SIM.

The accuracy and precision for the assay of L-[$^2H_7$]methionine by GC-MS-SIM was determined by spiking 0.1 ml aliquots of blank human plasma with multiple standard solutions of L-[$^2H_7$]methionine in the range of 0.05 - 10.3 nmol and DL-[$^2H_4$]methionine (0.1 nmol) as analytical standard. There were no interferences from endogenous compounds in the vicinity of the peaks of the analytes in the mass fragmentograms. The results are presented in Table 1. Good agreement between theory and experiment was observed, the relative error being less than 9 %. The inter-assay relative standard deviation (R.S.D.) was less than 6%.

Following a bolus intravenous administration of L-[$^2H_7$]methionine (32 µmol/kg) in a rat, the plasma concentration of L-methionines and L-homocysteines were determined. The concentrations of L-[$^2H_7$]-methionine, L-[$^2H_4$]methionine and endogenous L-methionine in plasma at 30 min after administration were 5.19, 3.65 and 50.16 nmol/ml, respectively, whereas those of L-[$^2H_4$]homocysteine and endogenous L-homocysteine were 0.93 and 4.81 nmol/ml. These results show that re-methylation from L-[$^2H_4$]homocysteine to L-[$^2H_4$]methionine could be observed by using L-[$^2H_7$]methionine as the substrate. A pharmacokinetic study of L-[$^2H_7$]methionine including quantitative assessment of transmethylation and re-methylation is now in progress and will be described in detail elsewhere.

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The stable isotope methodology presented here coupled with mass spectrometry could provide a useful tool for the pharmacokinetic and metabolic studies of methionine.

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