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Journal of Chromatography B, 823 (2005) 203–208
JOURNAL OF CHROMATOGRAPHY B
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Short communication

Determination of D- and L-enantiomers of methionine and [$^2H_3$]methionine in plasma by gas chromatography–mass spectrometry

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Received 26 March 2005; accepted 3 June 2005
Available online 11 July 2005

Abstract

A method for the stereoselective determination of D- and L-enantiomers of both methionine and [$^2H_3$]methionine in rat plasma was developed using gas chromatography–mass spectrometry with selected-ion monitoring (GC–MS-SIM). DL-[$^2H_7$]Methionine was used as analytical internal standard to account for losses associated with the extraction, derivatization and chromatography. The amino acids were purified by cation-exchange chromatography using BondElut SCX cartridge and derivatized with HCl in methanol to form methyl ester followed by subsequent *N*-acylation with optically active (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride to form diastereomeric amide. Quantification was performed by SIM of the molecular-related ions of the diastereomers on the chemical ionization mode. Endogenous L-methionine concentrations in 50 μl of rat plasma were measured with relative intra- and inter-day precision of 4.0 and 6.3%, respectively. The intra- and inter-day reproducibility in the amounts of D- and L-[$^2H_3$]methionine determined were in good agreement with actual amount added.

Keywords: D-Methionine; GC–MS; Stable isotope; Chiral inversion

1. Introduction

D-Amino acids are now recognized to be widely present in a variety of mammals [1–8]. In addition, significant amounts of D-amino acids are present in foods we usually ingest [9–11]. Some D-amino acids are metabolized by D-amino-acid oxidase to the corresponding α-keto acids, which may be converted into the L-enantiomers. The utilization of exogenous D-amino acid depends on whether it can be efficiently transformed to the L-isomer. In the nutritional studies, a widely accepted method involves a comparison of growth rate of animals fed a control diet with that of animals fed a diet containing the D-amino acid in place of the corresponding L-enantiomer. The efficiency of D-amino acid was indirectly estimated from the dosage of the D-amino acid required to achieve the same growth rate in control animal. The finding that D-methionine was well utilized for growth in rat suggested that D-methionine was almost converted into the L-enantiomer [12]. However, little information is available on the extent that D-methionine is converted into the L-enantiomer in vivo because L-methionine formed is indistinguishable from endogenous L-methionine.

The use of gas chromatography–mass spectrometry (GC–MS) and stable-isotope labeled compounds as tracers has enjoyed broad application in metabolic studies for endogenous compounds [13,14]. One of the major advantages of this technique is that an endogenous compound and its exogenously administered labeled analog are separately measurable by using GC–MS.

We have initiated studies to characterize the kinetic behavior of methionine enantiomers and to estimate the fraction of D-methionine that is converted into the L-enantiomer after administration of stable isotope-labeled D-methionine.

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doi:10.1016/j.jchromb.2005.06.010

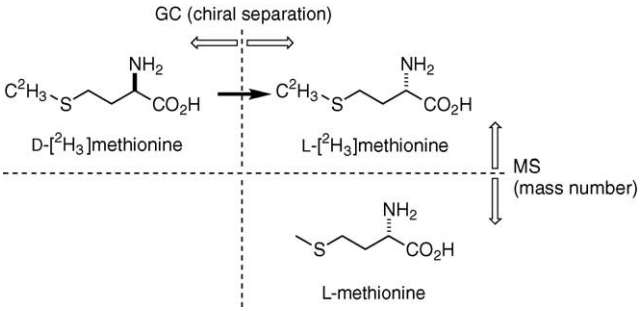

Fig. 1. Strategy for determining chiral inversion of D-methionine in vivo.

Fig. 1 shows the strategy for determining the chiral inversion of D-methionine. When an exogenously administered stable-isotope labeled D-methionine is converted into the L-enantiomer, the formed labeled L-methionine and non-labeled endogenous L-methionine are distinguishable from each other by GC–MS. Successful application of this methodology is dependent on separation of methionine enantiomers on chromatographic procedure. The present paper describes a procedure for the simultaneous determination of non-labeled endogenous and labeled methionine enantiomes in rat plasma.

2. Experimental

2.1. Materials

D- and L-methionine were purchased from Wako Pure Chemicals (Osaka, Japan). DL-[*S*-Methyl-$^2H_3$]methionine (DL-[$^2H_3$]methionine, 99.2 atom ^2H %) and DL-[3,3,4,4-$^2H_4$]methionine (>99 atom ^2H %) were purchased from CDN isotopes (Que., Canada). D- and L-[$^2H_3$]methionine were prepared from DL-[$^2H_3$]methionine in our laboratory by the method of Greenstein and Winitz [15] with minor modification. DL-[3,3,4,4,*S*-Methyl-$^2H_7$]methionine (DL-[$^2H_7$]methionine) was prepared from DL-[3,3,4,4-$^2H_4$]methionine (CDN isotopes) in our laboratory as described previously [16]. (*S*)-(+)-α-Methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl) and 10% HCl in methanol were purchased from Tokyo Kasei (Tokyo, Japan). A strong cation-exchange solid-phase extraction column BondElut SCX (H^+ form, size 1 ml/100 mg) was purchased from Varian (Harbor City, CA, USA). Chloroform stabilized with amylene was purchased from Cica-Merck (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade and used without further purification.

2.2. Stock solutions

Stock solutions of L-methionine (100 nmol/ml), DL-[$^2H_3$]methionine (200 nmol/ml) and DL-[$^2H_7$]methionine (100 nmol/ml) were prepared in methanol. The stock solutions were further diluted serially to prepare the standard solutions for calibration curve and quality control (QC) samples. The standard solution of DL-[$^2H_7$]methionine as internal standard was prepared at 10 nmol/ml in methanol. Storage of these solutions at 4 °C did not result in any detectable decomposition for more than 6 months.

2.3. Sample preparation

To a 50 μl of rat plasma was added DL-[$^2H_7$]methionine (1.0 nmol) as an analytical internal standard. The plasma sample was deproteinized and extracted with ethanol (1 ml) on a vortex mixing for 0.5 min. After centrifugation at 3000 rpm for 10 min, the supernatant was dried with a gentle stream of nitrogen at 40 °C. The residue was dissolved in 1 ml of 40 mM HCl and then applied to an activated BondElut SCX cartridge. The cartridge was pre-washed and activated with 3 ml of methanol, 3 ml of a mixture of methanol and 0.1 M HCl (1:1, v/v) and 3 ml of 0.1 M HCl. The cartridge was washed with 1 ml of water and 1 ml of methanol, and methionine species were then eluted with 0.5 ml of 10% HCl in methanol. The eluate was directly heated at 60 °C for 1 h. After removal of the solvent under a stream of nitrogen at 40 °C, the residue was reconstituted in 100 μl of 2% (+)-MTPA-Cl in chloroform, shaken for 0.5 min on a vortex mixer and left at room temperature for 1 h. After washing the reaction mixture with water (1 ml × 2), the solvent was evaporated at room temperature under a stream of nitrogen. The residue was dissolved in 20 μl of ethyl acetate and a 1–2 μl of the solution was subject to GC–MS-SIM.

2.4. GC–MS-SIM

GC–MS-SIM analyses were performed on a Shimadzu (Kyoto, Japan) QP1000EX quadrupole gas chromatograph–mass spectrometer equipped with a data processing system. A methylsilicone bonded-phase fused-silica capillary column SPB-1 (15 m × 0.25 mm i.d.) with a 0.25 μm film thickness (Supelco, Bellefonte, PA, USA) was connected directly into 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 a 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, increased at 15 °C/min to 250 °C and hold at 250 °C for 1 min. The temperature of the injector was 280 °C. The mass spectrometer was operated in chemical ionization mode with isobutane as the reactant gas at a pressure of 2×10^{-5} Torr to 5×10^{-5} Torr. The ionization voltage and ionization current were 200 eV and 150 μA, respectively. The ion source temperature was 280 °C. SIM was performed on the molecular related ions at *m/z* 380, 383 and 387 for of the *N*-(+)-α-methoxy-α-trifluoromethylphenylacetyl methyl ester (MTPA-OMe) derivatives of methionine, [$^2H_3$]methionine and [$^2H_7$]methionine, respectively.

2.5. Calibration curves and quantification

To 50 μl portions of rat blank plasma were added each of standard solutions containing known amounts of DL-[$^2H_3$]methionine (0.05, 0.1, 0.2, 0.4, 1.0, 1.5, 2.0, 4, 10 and 15 nmol) or L-methionine (0, 0.25, 0.5, 1.0 and 2.0 nmol). To the samples were added DL-[$^2H_7$]methionine (1.0 nmol) as an internal standard. The samples were purified, derivatized and analyzed as described above.

The peak area values were determined at *m/z* 380 for MTPA-OMe derivatives of L-methionine, *m/z* 383 for those of D- and L-[$^2H_3$]methionine, and *m/z* 387 for those of D- and L-[$^2H_7$]methionine, and the peak area ratios (L-methionine/L-[$^2H_7$]methionine, D-[$^2H_3$]methionine/D-[$^2H_7$]methionine and L-[$^2H_3$]methionine/L-[$^2H_7$]methionine, respectively) were calculated. The curves were obtained by an unweighted least-squares linear fitting of the peak area ratios versus the amounts added 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

QC samples were prepared by spiking the QC solution for DL-[$^2H_3$]methionine into 50 μl aliquot of rat pooled plasma. Final concentrations were 1.0, 10 and 100 nmol/ml for each enantiomer. After preparation of the sample for GC–MS-SIM as described above, the peak area ratios were determined.

To evaluate the intra- and inter-day reproducibility, 50 μl aliquot of a rat pooled plasma were placed in individual tubes ($n = 16$), frozen and stored at −20 °C until analysis. Four of these samples were prepared and measured by GC–MS-SIM each day.

2.7. Dose experiment

Sprague–Dawley male rats weighing 260–350 g were used. After fasting for 12 h, D-[$^2H_3$]methionine (35 μmol/kg body weight) dissolved in saline (70 μmol/ml) was administered into the femoral vein under anesthesia with sodium pentobarbital (50 mg/kg body weight, i.p.). Blood samples (150 μl) were collected from the jugular vein using a heparinized syringe at 10 min before and 0.5, 1, 3, 5, 10, 15, 20, 30, 60, 90, 120, 180, 240, 300 and 360 min after the administration. Plasma was separated and stored at −20 °C until analysis.

3. Results and discussion

3.1. Selection of labeled compounds

Successful application of stable isotope methodology to the metabolic investigation is dependent upon availability of compounds labeled at predesigned position. We have chosen a deuterium-labeled D-methionine at *S*-methyl group (D-[$^2H_3$]methionine) as a tracer because the deuterium labels were placed at sufficient enough distance from amino group of methionine to avoid loss of the label under the de-amination and transamination process. We have also chosen another deuterium-labeled DL-methionine (DL-[$^2H_7$]methionine) as an analytical internal standard. The $^2H_7$-labeled internal standard mimics D- and L-[$^2H_3$]methionine and D- and L-methionine in plasma. Therefore, if the extraction and/or the derivatization are not 100% complete, the methionine species measurement will not be affected because the $^2H_7$-labeled internal standard will also undergo losses to the same extent as the D- and L-[$^2H_3$]methionine and D- and L-methionine.

3.2. GC–MS-SIM

The simultaneous determination of D- and L-enantiomers of both [$^2H_3$]methionine and methionine by GC–MS requires the GC separation of these compounds. Since methionine is not volatile and does not permit direct analysis on GC, it must be converted into suitable derivatives. Several derivatization methods have been reported to measure amino acids by GC–MS [17–21]. We have previously used MTPA-OMe derivative for separation of the leucine enantiomers [22]. One of the major advantages for using MTPA-Cl as a derivatizing reagent is that the reagent, lacking α-hydrogen, is highly resistant to racemization. Fig. 2 shows the reaction scheme for the derivatization of methionine. DL-Methionine is converted into the methyl ester by HCl in methanol followed by subsequent chiral *N*-acylation with MTPA-Cl, and the diastereomeric MTPA-OMe derivative was subject to GC–MS. Fig. 3 shows the GC separation of the MTPA-OMe derivative of D- and L-methionine. The derivatives of D- and L-enantiomer underwent baseline separation within 10 min and eluted in this order. Fig. 4 shows the CI mass spectra for the MTPA-OMe derivatives of methionine, [$^2H_3$]methionine and [$^2H_7$]methionine enantiomers. Because the respective MTPA-OMe derivatives produce strong molecular related ions $[M + H]^+$ at *m/z* 380, 383 and 387, we have chosen the ions as the selected-ion monitoring ions.

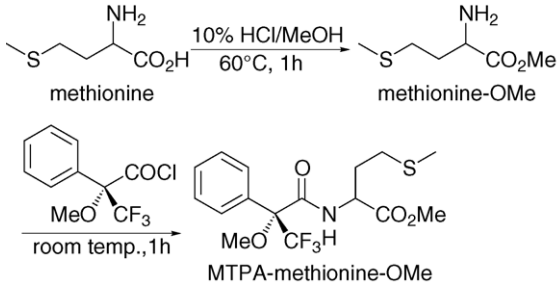

Fig. 2. Derivatization of methionine.

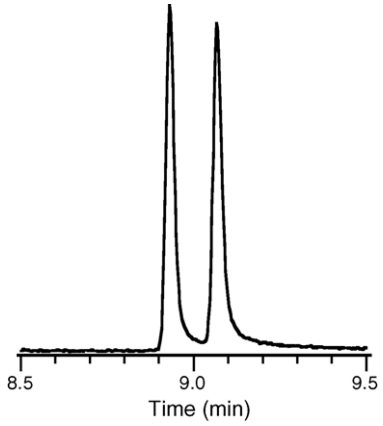

Fig. 3. Total ion chromatogram for MTPA-OMe derivative of D- and L-methionine. The retention times of the MTPA-OMe derivative of D- and L-methionine is 8.93 and 9.07 min, respectively.

3.3. Method validation

When a signal-to-noise (S/N) ratio of at least 3.0 was used as a criterion for a significant response, the limit of detection of the present GC–MS-SIM method was found to 500 fmol per injection for each D- and L-[$^2H_3$]methionine. The limit of quantification (LOQ) was determined by spiking 50 μl aliquots rat plasma with DL-[$^2H_3$]methionine and was found to be around 0.5 nmol/ml plasma for each enantiomer. Endogenous D-methionine was present in rat plasma and the level was around the LOQ.

Calibration curves for D-[$^2H_3$]methionine, L-[$^2H_3$]methionine and L-methionine were constructed by the addition of known amounts of DL-[$^2H_3$]methionine in the range of 0.05–15 nmol or L-methionine in the range of 0–2.0 nmol to rat plasma. When the peak-area ratio (y) were plotted against the amounts added (x), good correlations were found, $y = 2.0121x + 0.0053$ ($r = 0.9999$) for D-[$^2H_3$]methionine, $y = 1.9845x - 0.0065$ ($r = 0.9999$) for L-[$^2H_3$]methionine and $y = 2.0195x + 5.9092$ ($r = 0.9999$) for L-methionine.

The accuracy and precision of the method were determined by spiking 50 μl aliquots of blank rat plasma with the range of 0.05–5.0 nmol of D- and L-[$^2H_3$]methionine. Fig. 5 shows representative SIM profiles of a plasma sample. There was no interference from endogenous compounds in the vicinity of the peaks of analytes in the mass fragmentograms. The estimated amounts were in good agreement with the actual amounts spiked (Table 1). The intra- and inter-day precisions (R.S.D.) of the assay were less than 4% for D-[$^2H_3$]methionine and 3% for L-[$^2H_3$]methionine. The intra- and inter-day relative errors for each enantiomer were less than 7%. The reliability of the method was also determined by measuring endogenous L-methionine in 50 μl of rat plasma. As shown in Table 2, the relative intra- and inter-day precisions for L-methionine were less than 4 and 7%, respectively.

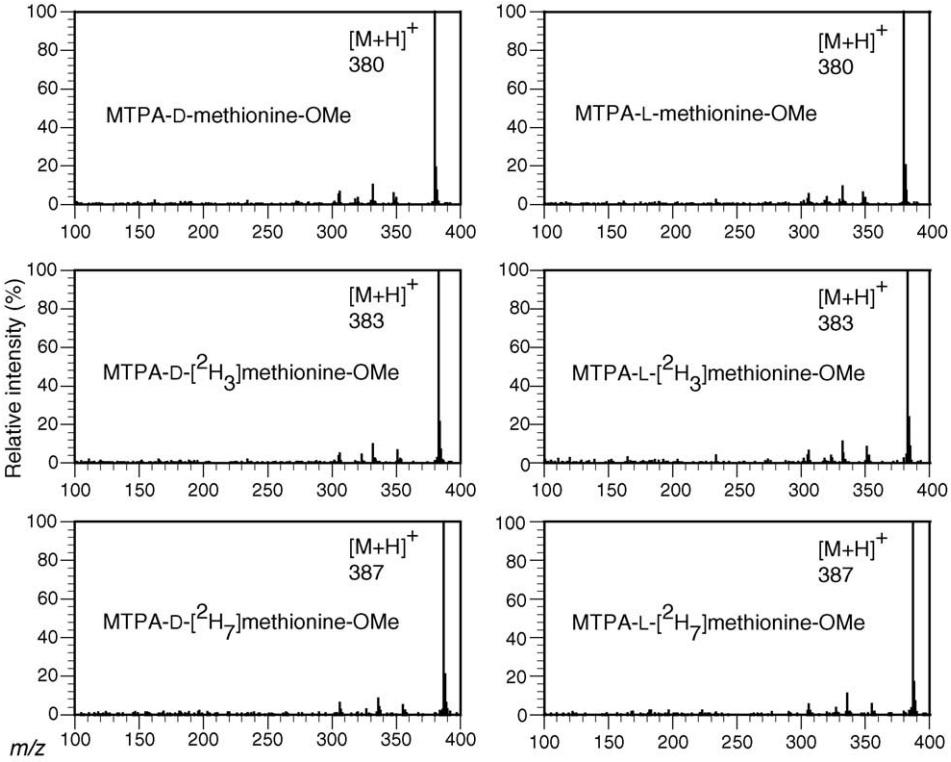

Fig. 4. Chemical-ionization mass spectra of MTPA-OMe derivatives of methionine, [$^2H_3$]methionine and [$^2H_7$]methionine enantiomers.

Table 1
Intra- and inter-day accuracy and precision for D-[$^2H_3$]methionine and L-[$^2H_3$]methionine spiked to rat plasma[a]

Spiked (nmol)	Expected (nmol/ml)	Intra-day (n = 4)			Inter-day (n = 4)		
		Found (nmol/ml)	R.S.D. (%)	RE (%)	Found (nmol/ml)	R.S.D. (%)	RE (%)
D-[$^2H_3$]Methionine							
0.05	1.00	1.04 ± 0.04	4.00	4.20	1.00 ± 0.03	2.62	0.45
0.5	9.98	9.90 ± 0.29	2.97	−0.75	10.05 ± 0.13	1.33	0.73
5.0	99.82	99.73 ± 1.67	1.67	−0.09	99.67 ± 1.01	1.01	−0.15
L-[$^2H_3$]Methionine							
0.05	1.00	1.06 ± 0.03	2.61	6.10	1.00 ± 0.04	3.94	0.20
0.5	9.98	9.92 ± 0.18	1.77	−0.61	10.04 ± 0.11	1.13	0.58
5.0	99.82	99.34 ± 1.65	1.66	−0.48	100.85 ± 1.72	1.70	1.03

[a] Results are expressed as mean ± S.D.

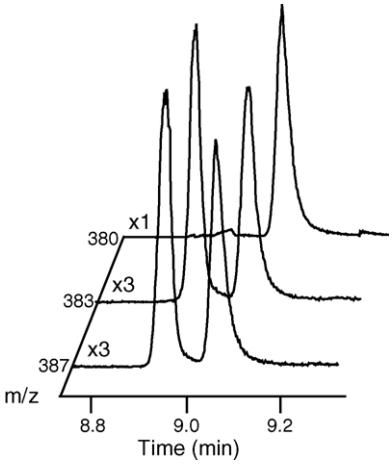

Fig. 5. Representative SIM profiles of rat plasma sample.

3.4. Application

The present stereoselective GC–MS-SIM method was applied for the quantification of plasma concentrations of D-[$^2H_3$]methionine, L-[$^2H_3$]methionine and endogenous L-methionine after a bolus intravenous administration of D-[$^2H_3$]methionine (35 μmol/kg body weight) to male rats. Fig. 6 shows representative plasma concentration–time profiles of D-[$^2H_3$]methionine, L-[$^2H_3$]methionine and endogenous L-methionine. After administration of D-[$^2H_3$]methionine, L-[$^2H_3$]methionine quickly appeared in the plasma. The kinetic study of D-[$^2H_3$]methionine including assessment of the chiral inversion of D-[$^2H_3$]methionine into the L-enantiomer will be described in details elsewhere.

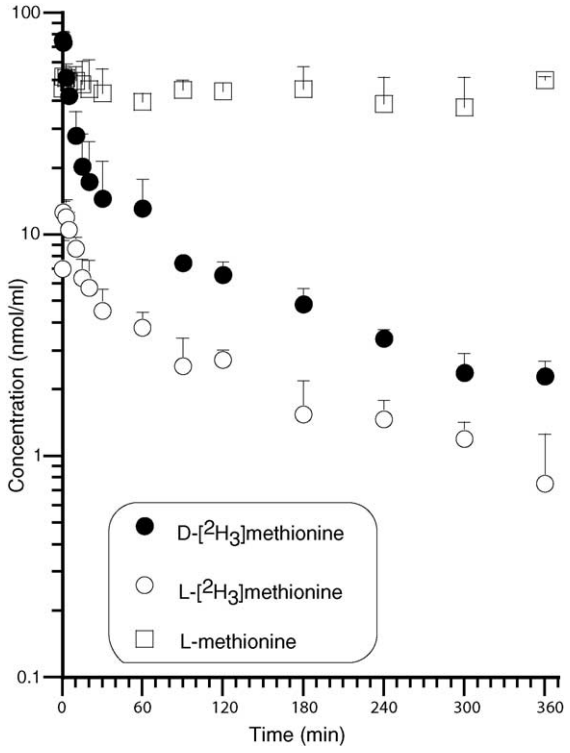

Fig. 6. Semi-logarithmic plots of plasma concentration vs. time curves of D-[$^2H_3$]methionine, L-[$^2H_3$]methionine and L-methionine in rats (n = 3) after an intravenous administration of D-[$^2H_3$]methionine (35 μmol/kg body weight).

Table 2
Intra- and inter-day variability of the measurement of L-methionine in rat plasma

Day	L-Methionine (nmol/ml)					
	Individual values				Intra-day	
					Mean ± S.D.	R.S.D. (%)
1	59.40	58.33	56.88	60.25	58.71 ± 1.45	2.47
2	58.47	60.34	57.11	55.58	57.87 ± 1.62	2.80
3	52.27	49.62	49.77	53.39	51.26 ± 1.87	3.64
4	58.53	59.31	54.48	59.22	57.88 ± 2.30	3.97
					Inter-day	
					Mean ± S.D.	R.S.D. (%)
					56.43 ± 3.55	6.30

4. Conclusions

The present method provides a sensitive and reliable technique for the simultaneous determination of D- and L-[$^2H_3$]methionine together with L-methionine in rat plasma. The method can be applied pharmacokinetic and metabolic

studies of methionine enantiomer with a particular interest in evaluating the extent of conversion of D-methionine into the L-enantiomer in vivo.

Acknowledgement

This work was supported by a grant provided by the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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