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Simultaneous determination of the enantiomers of leucine and [$^2H_7$]leucine in plasma by capillary gas chromatography–mass spectrometry

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Abstract

A method for the stereoselective assay of D- and L-enantiomers of both leucine and [$^2H_7$]leucine in rat plasma was developed using gas chromatography–mass spectrometry–selected-ion monitoring. DL-[$^2H_3$]leucine was used as an internal standard. The method involved purification by cation-exchange chromatography using BondElut SCX cartridge and derivatization with hydrochloric acid in methanol to form methyl ester followed by subsequent chiral derivatization with (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride to form diastereomeric amide. The derivatization made the separation of the leucine enantiomers possible with good gas chromatographic behavior. Quantitation was performed by selected-ion monitoring of the quasi-molecular ions of the diastereomers on the chemical ionization method. The sensitivity, specificity, accuracy and reproducibility of the method were demonstrated to be satisfactory for application to pharmacokinetic studies of leucine enantiomers.

Keywords: Enantiomer separation; Leucine; Amino acids

1. Introduction

Recent progress in chromatography on the separation of DL-amino acids reveals that significant amounts of D-amino acids are present in processed foods [1,2]. It has also ascertained that D-amino acids are present in a variety of mammals [3–13]. However, the physiological functions and the nutritional aspects of D-amino acids have not currently been elucidated. Some D-amino acids are metabolized by D-amino acid oxidase to the corresponding α-keto acids, which may be converted to L-isomers [14]. The utilization of exogenous D-amino acid depends on whether it can be efficiency transformed to the L-isomer. Utilization of D-amino acids for the purpose of growth or maintenance of nitrogen equilibrium was repeatedly confirmed by different species of animals [14]. The finding that D-leucine (D-Leu) was utilized for growth in rat indicated the inversion of D-Leu to L-Leu [15,16]. However, little information is currently available on the inversion because the L-Leu formed is indistinguishable from endogenous L-Leu.

The use of gas chromatography–mass spectrometry (GC–MS) and stable-isotopically labeled compounds as tracers has enjoyed broad application

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in pharmacokinetic studies [17–21]. 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. A radiotracer technique may also provide useful pharmacokinetic data but is less desirable because of the potential radiation hazard to humans.

We have initiated studies to characterize the pharmacokinetic properties of leucine enantiomers and to estimate the fraction of D-Leu that is inverted to L-lLu by stable isotope methodology. The present paper described a procedure for the simultaneous determination of leucine enantiomers in rat plasma after administration of the stable isotopically labeled leucine. The assay involved derivatization with an optically active reagent and GC–MS with selected-ion monitoring (SIM).

2. Experimental

2.1. Chemicals and reagents

L-Leu, D-Leu and DL-Leu were purchased from Peptide Institute (Osaka, Japan). DL-[2,3,3-$^2H_3$]leucine (DL-[$^2H_3$]Leu) and DL-[4,5,5,5,6,6,6-$^2H_7$]leucine (DL-[$^2H_7$]Leu) were purchased from Isotec (Miamisburg, OH, USA). (*S*)-(+)- and (*R*)-(−)-α-methoxy-α-trifluoromethylphenylacetyl chloride [(+)-MTPA-Cl and (−)-MTPA-Cl, respectively] and 10% hydrochloric acid 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 DL-Leu (10.05 μg/ml, 76.6 nmol/ml), DL-[$^2H_3$]Leu (13.75 μg/ml, 102.6 nmol/ml) and DL-[$^2H_7$]Leu (13.48 μg/ml, 97.5 nmol/ml) were prepared in methanol. Storage of these solutions at 4°C did not result in any detectable decomposition for more than six months. All analyses were performed by diluting the stock solutions with methanol.

2.3. Gas chromatography–mass spectrometry–selected-ion monitoring

Capillary GC–MS–SIM analysis was carried out 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 thin film (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 at 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 190°C and held at 190°C for 3 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}–5×10^{-5} Torr. The ionization voltage and ionization current were 200 eV and 150 μA, respectively. The ion source temperature was 280°C. Selected-ion monitoring was performed on the quasi-molecular ions of the (+)-MTPA derivative of leucine methyl ester ($^2H_0$, *m/z* 362; $^2H_3$, *m/z* 365; $^2H_7$, *m/z* 369).

2.4. Sample preparation for GC–MS–SIM

To 50 μl of rat plasma in a polypropylene microtube (1.5 ml) were added 137.5 ng of DL-[$^2H_3$]Leu dissolved in 100 μl of methanol as an analytical internal standard. The plasma sample was deproteinized and extracted with ethanol (0.5 ml×2) on a vortex mixer for ca. 0.5 min. After centrifugation at 3000 rpm for 10 min, the ethanol solution was transferred into another polypropylene microtube and evaporated at 40°C under a stream of nitrogen. The residue was dissolved in 0.5 ml of 40 m*M* hydrochloric acid and then applied to a BondElut SCX cartridge, which was pre-washed and activated with 3 ml of methanol, 3 ml of a mixture of methanol and

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 methanol, and then eluted with 0.5 ml of 10% hydrochloric acid in methanol into a PTFE-lined screw-cap conical centrifuge tube (100×16 mm I.D.). The eluent was directly heated at 60°C for 1 h. After removal of the solvent under a stream of nitrogen, the residue was reconstituted in 100 μl of 2% (+)- MTPA-Cl in chloroform, shaken for 30 s 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.

2.5. Calibration curves and quantitation

To each of a series of standards containing known amounts of DL-[$^2H_7$]Leu (2.7, 6.7, 13.5, 27.0, 67.4, 134.9, 674.2, 1348.5, 2696.9 and 6742.2 ng) or DL-Leu (5.0, 10.1, 20.1, 100.5, 502.6, 1005.2, 2010.5 and 5026.2 ng) dissolved in methanol, was added DL-[$^2H_3$]Leu (137.5 ng) dissolved in methanol as an analytical internal standard. Each sample was prepared in quadruplicate. After evaporation of the solvent at room temperature by a stream of nitrogen, the residue was dissolved in 0.5 ml of 10% hydrochloric acid in methanol and the sample was derivatized according to the procedure described above. The derivatized samples were analysed by GC–MS–SIM in triplicate. The peak area values of each analyte were monitored at *m/z* 362 for D- and L-Leu, *m/z* 365 for D- and L-[$^2H_3$]Leu, and *m/z* 369 for D- and L-[$^2H_7$]Leu. After correcting the peak-area values with the values of mutual contributions as shown in Table 1, the peak area ratios (D-Leu/D-[$^2H_3$]Leu, L-Leu/L-[$^2H_3$]Leu, D-[$^2H_7$]Leu/D-[$^2H_3$]Leu and L-[$^2H_7$]Leu/L-[$^2H_3$]Leu, respectively) were determined. The curves were obtained by an unweighted least-squares linear fitting of the peak-area ratios versus 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.

Table 1
Mutual contributions to ion intensity of various species in the channels monitored

Compound	*m/z* 362	*m/z* 365	*m/z* 369
D-Leu	100	0.332	0.051
D-[$^2H_3$]Leu	0.144	100	0.078
D-[$^2H_7$]Leu	0.049	0.219	100
L-Leu	100	0.332	0.058
L-[$^2H_3$]Leu	0.133	100	0.086
L-[$^2H_7$]Leu	0.091	0.296	100

2.6. Accuracy

Accuracy was determined by assaying four preparations of 50-μl portions of rat pooled plasma spiked with DL-[$^2H_7$]Leu (6.7, 27.0, 67.4, 134.9, and 674.2 ng), D-Leu (5.0, 25.13, 50.26, 251.3 and 502.6 ng) or L-Leu (251.4, 502.6, 1005.2 and 2513.1 ng). Following the addition of DL-[$^2H_3$]Leu (137.5 ng) dissolved in methanol as an internal standard, the samples were subjected to clean-up and derivatized according to the procedure described above. The samples were analysed by GC–MS–SIM and the peak-area ratios were determined.

2.7. Drug administration

Sprague-Dawley (S.D.) male rats weighing 250–350 g were used (n=6). After fasting for 12 h, each

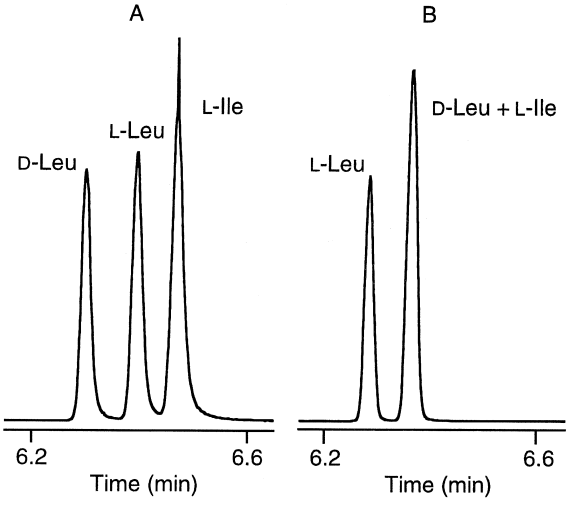
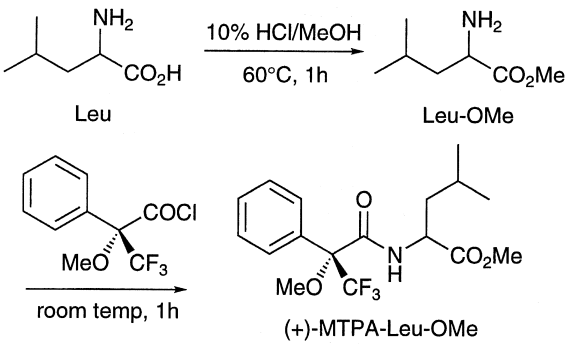


Fig. 1. GC separation of MTPA-OMe derivatives of D-Leu, L-Leu and L-Ile. (A) (+)-MTPA-OMe derivative, (B) (−)-MTPA-OMe derivative.

rat was administered intravenously DL-[$^2H_7$]Leu (2 mg/kg weight) in saline (0.5 ml of dosing solution/kg weight) via a femoral vein under anesthesia with ether. A blood sample (150 μl) was collected from the jugular vein using a heparinized syringe at 10 min before dosing and 0.5, 1, 3, 5, 10, 15, 20, 30, 60, 90, 120, 180, 240, 300 and 360 min after dosing. Plasma was separated and 50 μl of the plasma were used for the analysis mentioned above.



Scheme 1. Derivatization.

3. Results and discussion

Stable isotopically labeled D-Leu can be used to investigate the inversion of D-Leu, differentiating endogenous L-Leu from the labeled L-Leu formed from labeled D-Leu administered exogenously. It is necessary for the present study to use the compounds labeled at positions that are chemically and biologically inert. A commercially available [4,5,5,5,6,6,6-$^2H_7$]leucine ([$^2H_7$]Leu) was chosen for a biological internal standard, because the deuterium

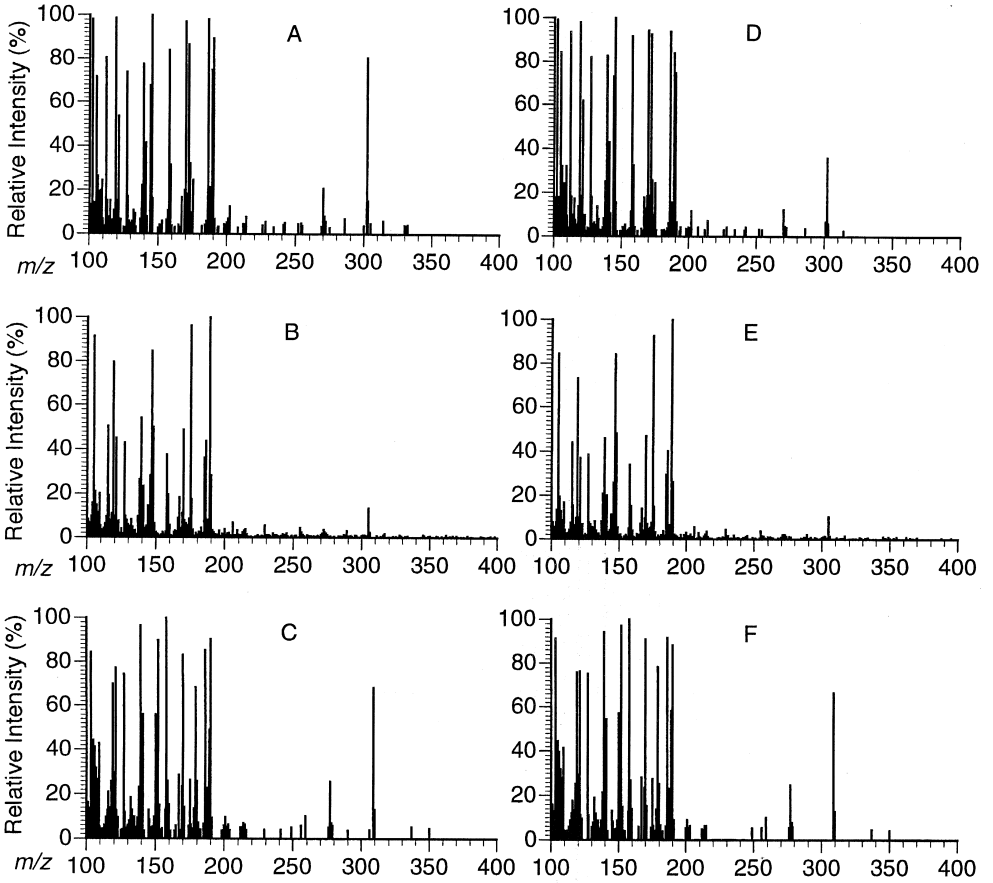


Fig. 2. Electron-ionization mass spectra of (+)-MTPA-OMe derivatives of D-Leu (A), D-[$^2H_3$]Leu (B), D-[$^2H_7$]Leu (C), L-Leu (D), L-[$^2H_3$]Leu (E) and L-[$^2H_7$]Leu (F).

labels were placed at sufficient enough distance from the amino group of leucine to avoid loss of the label under both the deamination and transamination process. [2,3,3-$^2H_3$]Leucine ([$^2H_3$]Leu) was used for an analytical internal standard.

Successful application of this methodology to the pharmacokinetic investigations is dependent on separation of leucine enantiomers on chromatographic procedure. Since amino acids are not volatile and do not permit direct analysis on GC, they must be converted into suitable derivatives. Separation of DL-amino acids on GC is achieved by the two methods. In one, DL-amino acids are derivatized with an achiral reagent followed by direct separation on a chiral column. In the second, DL-amino acids are derivatized with a chiral reagent followed by diastereomeric separation on an achiral column. The latter method requires that the chiral reagent has enough optical purity and no racemization of both amino acid and the chiral reagent occurs under derivatization. *N*-Trifluoroacetyl-L-prolyl chloride, (+)-2-butanol and optically active α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl) have been widely used as the chiral reagents in the diastereomeric method [22–28]. MTPA-Cl, lacking α-hydrogen, is highly resistant to racemization and is commercially available in its resolved form with high enantiomeric purity. There has been no reports on the use of this reagent for GC–MS analysis of DL-Leu and the determination of D-Leu in kinetic studies.

The simultaneous determination of D-Leu and L-Leu by GC–MS requires the GC separation of these compounds together with L-isoleucine (L-Ile) because of their identical mass numbers. D-Leu, L-Leu and L-Ile were converted to their methyl ester by

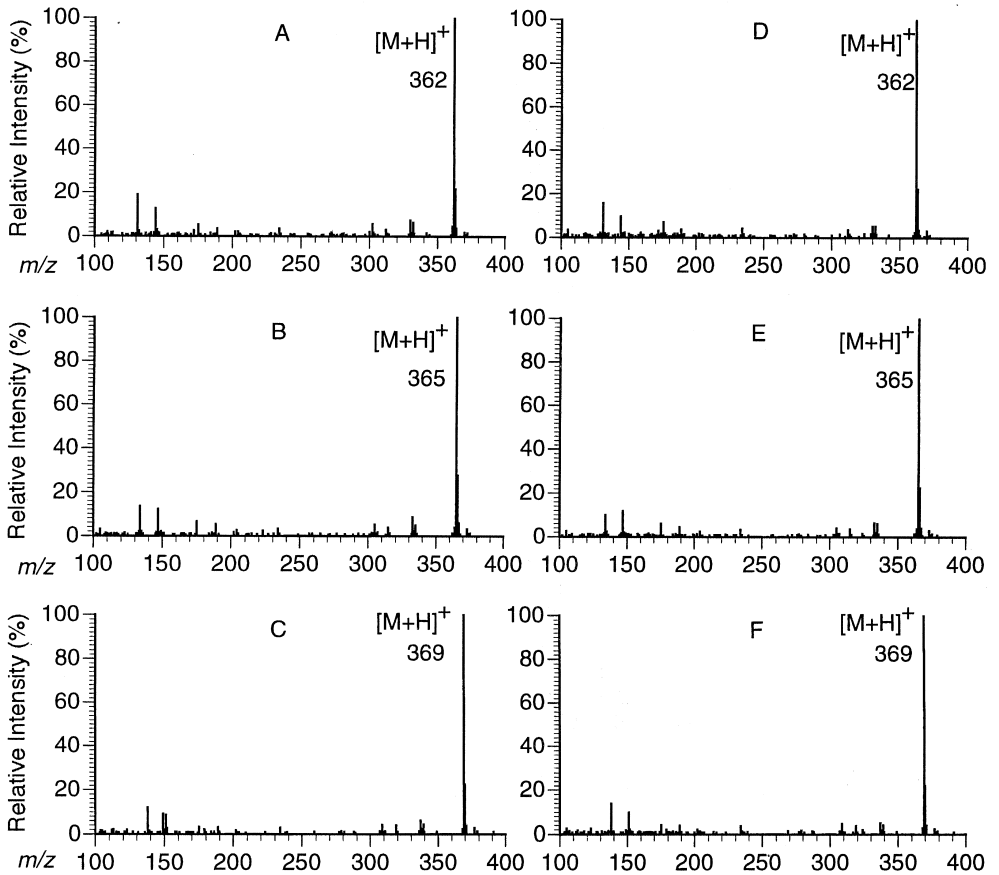


Fig. 3. Chemical-ionization mass spectra of (+)-MTPA-OMe derivatives of D-Leu (A), D-[$^2H_3$]Leu (B), D-[$^2H_7$]Leu (C), L-Leu (D), L-[$^2H_3$]Leu (E) and L-[$^2H_7$]Leu (F).

hydrochloric acid in methanol followed by *N*-acylation with (+)- or (−)-MTPA-Cl as shown in Scheme 1, and the products (MTPA-OMe derivatives) were subjected to GC–MS. Fig. 1 shows the GC separation of the MTPA-OMe derivatives of D-Leu, L-Leu and L-Ile. Using (+)-MTPA-Cl, the derivatives were well separated (Fig. 1A). The elution order was D-Leu, L-Leu and L-Ile. The R_s between the (+)-MTPA-OMe derivative of L-Leu and that of L-Leu and between L-Leu and L-Ile were 2.6 and 1.8, respectively. On the other hand, the (−)-MTPA-OMe derivative of L-Leu and D-Leu were well resolved, whereas D-Leu and L-Ile were not resolved on GC (Fig. 1B). Therefore, the (+)-form of MTPA-Cl was chosen as the chiral derivatization reagent.

Figs. 2 and 3 shows the electron ionization (EI) and the chemical ionization (CI) mass spectra of (+)-MTPA-OMe derivatives of D-Leu, D-[$^2H_3$]Leu, D-[$^2H_7$]Leu, L-Leu, L-[$^2H_3$]Leu and L-[$^2H_7$]Leu, respectively. In contrast to the strong quasi-molecular ion $[M+H]^+$ at *m/z* 362, 365 and 369 for the respective (+)-MTPA-OMe derivatives of non-labeled and labeled leucine in CI mass spectra, no molecular ion can be seen in the EI mass spectra. The quasi-molecular ions $[M+H]^+$ on the CI method were chosen for the selected-ion monitoring of (+)-MTPA-OMe derivatives. When a signal-to-noise (*S/N*) ratio of at least 3.0 was used as a criterion for a significant response, the detection limit of the present GC–MS(CI)–SIM method was found to 25 pg (200 fmol) per injection for each D- and L-[$^2H_7$]Leu (Fig. 4).

The present derivatization method may be suitable for the stereoselective analysis of leucine enantiomers because the (+)-MTPA-OMe derivative of DL-Leu can be prepared at high yield (>80%) and the subsequent separation of the diastereomers on capillary GC can be achieved within 7 min. Moreover, the high sensitivity of the GC–MS(CI)–SIM method is invaluable for analysis of leucine enantiomers present in small blood samples from laboratory animals. The method may be applicable for the stereoselective analysis of other DL-amino acids.

Because of the natural abundance of ^2H, ^{13}C and ^{18}O, a small peak at *m/z* 365 may appear in the mass spectrum of (+)-MTPA-OMe derivative of DL-Leu. In addition, there is also the possibility that the (+)-MTPA-OMe derivative of DL-[$^2H_3$]Leu could be

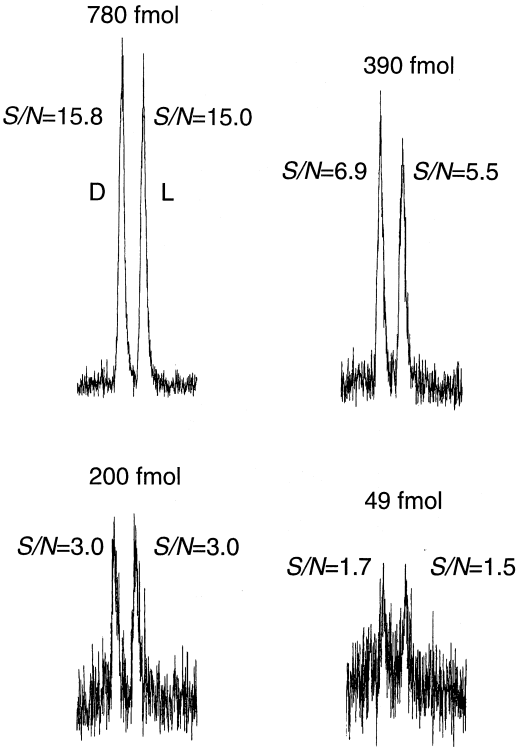


Fig. 4. Sensitivity of (+)-MTPA-OMe derivative of DL-[$^2H_7$]Leu by selected-ion monitoring.

contributed to the *m/z* 362 peak and the (+)-MTPA-OMe derivative of DL-[$^2H_7$]Leu to the *m/z* 362 and/or *m/z* 365 peaks. Precise analysis of the GC–MS–SIM data from (+)-MTPA-OMe derivatives of DL-Leu, DL-[$^2H_3$]Leu and DL-[$^2H_7$]Leu summarized in Table 1 indicated that no corrections for overlapping ions among the various isotopic compounds in question were necessary. The labeled compounds possessed sufficiently high isotopic purity that the contributions to the other ions were minor. However, there was the possibility that the concentration of endogenous L-Leu in the dose experiment was 10–1000 fold greater than that of L-[$^2H_7$]Leu. In such cases the contributions were significant, and corrections were made based on the values in Table 1.

Calibration curves were prepared from a series of samples containing various amounts of D-[$^2H_7$]Leu (1.34-3371.1 ng), L-[$^2H_7$]Leu (1.34–3371.1 ng), D-Leu (2.51–2512.1 ng), L-Leu (2.51–2512.1 ng) and a constant amount (137.5 ng) of DL-[$^2H_3$]Leu. Each sample was assayed as (+)-MTPA-OMe derivative

Table 2
Recovery of DL-[$^2H_7$]Leu

Eluent	Recovery (%)[a]
10% Ammonia in water	77.0±1.7
3% Ammonia in methanol	89.9±1.0
3% Hydrochloric acid in water	89.3±1.3
10% Hydrochloric acid in methanol	95.2±1.4

[a] Mean±S.D. (n=3).

in triplicate by monitoring the ion intensities at m/z 362, 365 and 369. When the peak-area ratios (y) were plotted against the mixed molar ratios (x), a good correlation was found between the observed peak-area ratios and the mixed molar ratios. Unweighted least-regression analysis gave the regression lines, $y=1.0647x-0.0243$ ($r=0.999$) for D-[$^2H_7$]Leu, $y=1.0218x+0.0253$ ($r=0.999$) for L-[$^2H_7$]Leu, $y=1.1019x-0.037$ ($r=0.999$) for D-Leu, $y=1.0825x-0.0335$ ($r=0.999$) for L-Leu.

Special attention was paid to extract DL-Leu from plasma samples. Physiological fluids requiring analysis for L-Leu are usually pre-treated with a denaturing reagent. Following removal of the precipitated protein by centrifugation, the amino acid is isolated by cation-exchange chromatography such as a Dowex 50W X8 column [29–31]. Rat plasma spiking DL-[$^2H_7$]Leu was deproteinized with ethanol and then applied to a Dowex 50W X8 column (50×5 mm, I.D.), followed by elution with 5 ml of 10% aqueous ammonia to recover of the analyte at high yield (70±15%, n=3). However, evaporation of the eluent was laborious and time-consuming. Recently, a solid-phase extraction column with strong cation-exchange property, BondElut SCX was utilized to extract catecholamines [32,33] and amino acids [34,35] from biological fluids. Amino acids spiked to urine were quantitatively recovered from BondElut SCX column with 0.9 ml of 1 *M* aqueous ammonia

Table 3
Accuracy of selected-ion monitoring of D-[$^2H_7$]Leu, L-[$^2H_7$]Leu, D-Leu and L-Leu in rat plasma

Added (μg/ml)	Expected (μg/ml)	Found (μg/ml)					R.S.D. (%)	Relative error (%)
		Individual values				Mean ±S.D.		
D-[$^2H_7$]Leu								
0.067		0.071	0.066	0.071	0.074	0.071±0.003	4.49	−4.81
0.27		0.25	0.26	0.27	0.25	0.26±0.006	2.23	3.46
0.67		0.65	0.70	0.70	0.67	0.68±0.025	3.75	−0.64
1.35		1.33	1.34	1.34	1.36	1.34±0.011	0.81	−0.37
6.74		6.70	6.70	6.70	6.73	6.71±0.013	0.19	0.50
L-[$^2H_7$]Leu								
0.067		0.060	0.073	0.063	0.065	0.065±0.006	8.64	3.13
0.27		0.27	0.28	0.26	0.26	0.27±0.009	3.53	1.50
0.67		0.69	0.70	0.68	0.68	0.69±0.009	1.25	−2.10
1.35		1.41	1.32	1.33	1.39	1.36±0.042	3.09	−0.93
6.74		6.44	6.71	6.50	6.57	6.56±0.120	1.83	2.76
D-Leu								
0.10		0.12	0.09	0.10	0.11	0.103±0.010	9.61	2.57
0.50		0.51	0.49	0.48	0.49	0.49±0.013	2.48	−2.19
1.01		1.00	1.02	1.05	0.98	1.01±0.030	2.93	0.50
5.03		5.14	5.02	5.00	5.06	5.05±0.063	1.25	0.57
10.05		10.41	10.12	10.42	9.47	10.11±0.445	4.41	0.54
L-Leu								
		18.95	18.86	17.26	18.32	18.35±0.777	4.23	
5.03	23.37	23.06	23.80	23.18	22.85	23.22±0.408	1.76	−0.64
10.05	28.40	26.78	26.24	26.60	26.03	26.41±0.340	1.28	−6.99
20.10	38.45	37.42	35.02	35.73	36.80	36.24±1.073	2.96	−5.75
50.26	68.61	69.57	65.17	73.07	65.24	68.26±3.809	5.57	−0.50

as an eluent [34]. Elution of *m*-tyrosine and *o*-tyrosine from BondElut SCX column was achieved with 0.5 *M* hydrochloric acid in water [35]. Addition of methanol to the eluent may improve recovery [36]. The extraction efficiency of DL-[$^2H_7$]Leu from rat plasma was tested with BondElut SCX using various composed eluents (Table 2). Replacement of water with methanol improved recovery of the analyte and the eluent was evaporated with ease. When 10% hydrochloric acid in methanol was used as the eluent, the recovery of DL-[$^2H_7$]Leu was closed to quantitatively and the eluent was directly heated at 60°C for 1 h to yield methyl ester of DL-[$^2H_7$]Leu.

The accuracy of the assay was determined for D-[$^2H_7$]Leu, L-[$^2H_7$]Leu, D-Leu, and L-Leu added to 50-μl aliquots of blank rat plasma. The plasma samples were spiked with multiple standard solutions of DL-[$^2H_7$]Leu in the concentration range 0.067–6.74 μg/ml, D-Leu in the concentration range 0.1–10.05 μg/ml and L-Leu in the concentration range 5.03–50.26 μg/ml, respectively. The results are presented in Table 3. The estimated amounts were in good agreement with the actual amounts added, the relative error being less than 4.9% for D-[$^2H_7$]Leu, 3.2% for L-[$^2H_7$]Leu, 2.6% for D-Leu and 7.0% for L-Leu. The inter-assay relative standard derivation (R.S.D.) for each enantiomers were less than 10% for all four leucines.

The present GC–MS–SIM method was applied for the quantitation of plasma concentrations of D-[$^2H_7$]Leu, L-[$^2H_7$]Leu and endogenous L-Leu after intravenous administration of DL-[$^2H_7$]Leu (2 mg/kg weight) to S.D. male rats ($n=6$). Plasma concentrations of D-[$^2H_7$]Leu and L-[$^2H_7$]Leu could be followed up to 5 h and 1 h, respectively, with no interference of endogenous L-Leu (Fig. 5). A pharmacokinetic study of D-Leu including assessment of the chiral inversion of D-Leu to L-Leu after administration of stable isotopically labeled D-Leu is now in progress and will be described in details elsewhere.

The present method provided a sensitive and reliable technique for determining the plasma levels of the labeled leucine enantiomers and endogenous L-Leu with good accuracy and precision. The method was confirmed to be applicable for assessing the pharmacokinetics of other D-amino acids as well as D-Leu. In addition, D-amino acid-labeled stable isotopes would be useful for studying chiral inversion of D-amino acids in humans as a substitute for radioactive compounds because of the absence of possible radiation hazards.

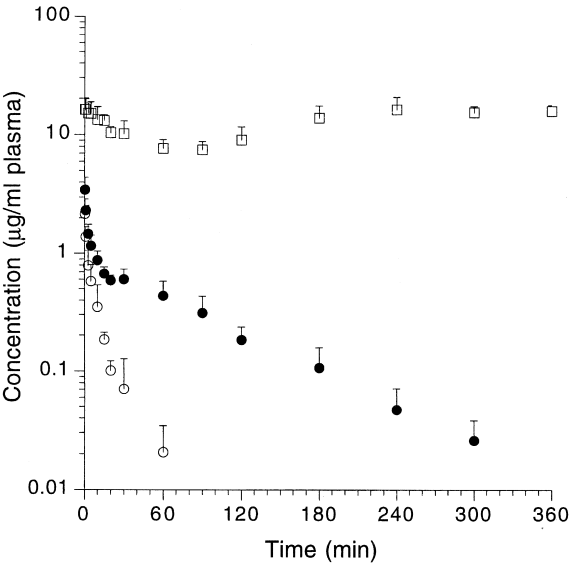


Fig. 5. Semi-logarithmic plots of plasma concentration versus time curves of D-[$^2H_7$]Leu (●), L-[$^2H_7$]Leu (○) and L-Leu (□) in Sprague-Dawley male rats ($n=6$) after an intravenous administration of DL-[$^2H_7$]Leu (2 mg/kg weight).

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