ASSESSMENT OF THE METABOLIC CHIRAL INVERSION OF d-LEUCINE IN RAT BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY COMBINED WITH A STABLE ISOTOPE DILUTION ANALYSIS

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ABSTRACT:

The stereoselective pharmacokinetics of leucine enantiomers in rats has been investigated to evaluate the inversion of d-leucine to l-enantiomer. After a bolus i.v. administration of d- or l-[2H7]leucine to rats, blood samples were obtained over 6 h after administration and analyzed by a stereoselective gas chromatography-mass spectrometry method. Racemic [2H7]leucine was used as an internal standard. The method involved methyl esterification and subsequent chiral derivatization with (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride to form the diastereomeric amide. The derivatization made possible the separation of leucine enantiomers with good gas chromatographic behavior. Plasma concentration of both d- and l-[2H7]leucine declined biexponentially, with elimination half-lives of 60 and 14 min, respectively. In contrast to the l-enantiomer, the d-enantiomer had a lower systemic clearance. When d-[2H7]leucine was administered, the l-enantiomer was found to rapidly appear in plasma. About 30% of an administered dose of the d-isomer was stereospecifically inverted to the l-enantiomer. There was no measurable inversion of the l- to d-enantiomer. This methodology has made it possible to evaluate the pharmacokinetics of each enantiomer of amino acids and estimate of chiral inversion after administration of d-amino acids.

The branched-chain amino acids (BCAA), l-leucine, l-isoleucine, and l-valine, can not be synthesized de novo in mammals (Meister, 1965). Diet and proteolysis of endogenous protein are the only sources of these amino acids for ongoing protein synthesis. The initial step in BCAA metabolism is a reversible transamination with α-ketoglutarate forming the respective branched-chain α-keto acids and glutamate (Taylor et al., 1970). Subsequently, the respective branched-chain α-keto acids may be decarboxylated, resulting in irreversible loss of BCAA. Therefore, during periods of limited protein intake, BCAA carbon could be conserved by decreasing the rate of irreversible loss or by altering the interconversion of BCAA and the corresponding α-keto acid.

Recent progress in chromatography on the separation of DL-amino acids reveals that significant amounts of D-amino acids are present in foods we usually ingest (Man and Bada, 1987; Brückner and Hausch, 1989). It has also ascertained that D-amino acids are present in a variety of mammals (Dunlop et al., 1986; Nagata et al., 1992; Hashimoto et al., 1993; Kera et al., 1995; Hamase et al., 1997). However, the physiological functions and the nutritional aspects of D-amino acids have not currently elucidated. Use of D-BCAA for the purpose of growth or maintenance of nitrogen equilibrium was repeatedly confirmed by different species of animals, and it was found that d-valine and d-leucine were used for growth in rat and human (Meister, 1965). The use of exogenous D-amino acids depends on whether it can be efficiently transformed to the L-isomers. However, little information is available on the extent that D-amino acid is inverted to L-amino acid because L-amino acid formed can be indistinguishable from endogenous L-amino acid.

The use of gas chromatography-mass spectrometry (GC-MS) and stable isotopically labeled compounds as tracers has enjoyed broad application in pharmacokinetic studies. One of the major advantages of this technique is that endogenous and exogenous compounds having the same basic structure can be differentiated easily by using stable isotopically labeled compounds (Baba et al., 1980; Shinohara et al., 1980; Matthews and Bier, 1983). A radiotracer technique may also provide useful pharmacokinetic data but is less desirable because of the potential radiation hazard to humans.

In an attempt to examine the pharmacokinetics of leucine enantiomers and to estimate the fraction that inverted from d-leucine to l-leucine, we have developed a GC-MS assay using the deuterium-labeled leucine (Hasegawa et al., 1999). We have reported a procedure for determining the enantiomers of the stable isotope-labeled and nonlabeled leucine in plasma with good accuracy and precision. This method is based on purification of leucine enantiomers by cation-exchange chromatography using a BondElut SCX column, derivatization with hydrochloric acid in methanol to form methyl ester followed by subsequent chiral derivatization with (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride [(+)-MTPA-Cl] to form the diastereomeric amides and quantitation by GC-MS with selected-ion
monitoring (GC-MS-SIM). The purpose of this study is to determine the fraction of the inversion of d-leucine to l-leucine in rat after i.v. administration of a stable isotope-labeled d-leucine.

Materials and Methods

Chemicals. d-Leucine was purchased from Peptide Institute (Osaka, Japan). dl-[2,3,3-2 H 7]-leucine (dl-[2 H 7]-leucine, >98 atom % 2 H) and dl-[4,5,5,5,5,5,6,6,6-2 H 7]-leucine (dl-[2 H 7]-leucine, >98 atom % 2 H) were purchased from Isotec (Miamisburg, OH). Porcine kidney acylase (E.C. 3.5.1.14), (S)-glyceraldehyde (99% e.e.), and 10% hydrochloric acid in methanol were purchased from Tokyo Kasei (Tokyo, Japan). BondElut SCX (H+ form, size 1 ml/100 mg) cation-exchange cartridge column was purchased from Varian (Harbor City, CA). Chloroform stabilized with anylene was purchased from Cica-Merck (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade and used without further purification.

1H NMR and 1H-decoupled 13C NMR spectra were determined on a Bruker (Tsukuba, Japan) DRX500 spectrometer. About 5 mg of the samples were dissolved in 0.5 ml of 0.15 M sodium deuterioxide in deuterium oxide containing 10 μl of 1H 7 methanol as a reference for 13C NMR. Chemical shifts were referenced to those of H 2 HO (δH 4.80) for 1H NMR and methanol (δC 49.0) for 13C NMR, respectively.

Resolution of dl-[2 H 7]-Leucine. A solution of dl-[2 H 7]-leucine (305 mg, 2.2 mmol) in 8 ml of acetic acid was refluxed for 5 min, and acetic anhydride (1 ml, 10.5 mmol) was added thereto. The reaction mixture was allowed to reflux 20 min, followed by the addition of 25% aqueous ammonia until the pH of the solution was adjusted to 5 with 1 M lithium hydroxide in water. The solution was evaporated under reduced pressure and the residue was reconstituted in 100 μl of methanol, and then eluted with 0.5 ml of methanol, and then eluted with 0.5 ml of 10% hydrochloric acid in methanol into a polytetrafluoroethylene-lined screw cap 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 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 1 to 2 μl of the solution was subject to GC-MS-SIM. The GC-MS-SIM analysis was conducted on a Shimadzu (Kyoto, Japan) QP1000EX gas chromatograph-mass spectrometer equipped with a data processing system. The operating conditions were the same as those described in a previous article (Hasegawa et al., 1999; Nakamichi et al., 1999). 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) was connected directly into the ion source. 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 the chemical ionization mode with isobutane as the reactant gas. The ionization voltage and ionization current were 200 eV and 1.2 μA, respectively. The ion source temperature was 280°C. SIM was performed on the quasi-molecular ions of the (+)-MTPA derivative of leucine methyl ester (m/z 362; 1H 3 m/z 365; 1H 3, m/z 369).

Data Analysis. Pharmacokinetic parameters were calculated by model-independent analysis using a macro-program MOMENT(EXCEL) (Tabata et al., 1999) running on Microsoft Excel. The half-life (t1/2) of the terminal elimination phase of the plasma concentration-time curve was estimated using a regression equation. The area under the plasma concentration-time curve (AUC) and the area under the first-moment time curve (AUMC) were calculated by the trapezoidal method, and were extrapolated to infinity using the last detectable plasma concentration and the terminal elimination rate constant. Mean residence time (MRT), total plasma clearance (CLtot), and apparent volume of distribution at steady state (Vdss) were calculated using the equations: MRT = AUC/CLtot, CLtot = Dose/AUC, and Vdss = CLtot/MRT, respectively. Peak plasma concentration (Cmax) and the time to achieve maximal plasma concentration (tmax) were determined directly from the observed data.

The fraction [F D 1] of d-[2 H 7]-leucine that was converted to l-[2 H 7]-leucine by the trypsin was calculated in the following equation:

\[
F_{D \rightarrow L} = \frac{AUC_{D \rightarrow L} \cdot Dose_{D}}{AUC_{L} \cdot Dose_{L}}
\]

where AUC_{D \rightarrow L} and AUC_{L} denoted the AUC of d-[2 H 7]-leucine after a dose of d-[2 H 7]-leucine and the AUC of l-[2 H 7]-leucine after a dose of l-[2 H 7]-leucine, respectively.

Results

Resolution of commercially available dl-[2 H 7]-leucine was carried out by the stereospecific hydrolysis of the N-acetyl derivative of

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\text{d-leucine (1 mg/kg b.wt. each), \( l-[2 H_7]\text{leucine (10 mg/kg b.wt.) or} \ l-[2 H_7]\text{leucine (1 mg/kg b.wt.) in saline (0.5 ml of dosing solution/kg b.wt.) at 10:00 AM under light ether anesthesia. Blood samples (150-μl) were collected from the jugular vein using a heparinized syringe at 10 min under ether anesthesia (just before sample collection) 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 stored at −20°C until analysis. Sample Preparation for GC-MS-SIM. To 50 μl of rat plasma in a 1.5-ml polypropylene microtube were added 137.5 μg of dl-[2 H 7]-leucine dissolved in 0.1 ml 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 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 mM hydrochloric acid and then applied to a BondElut SCX cartridge, which was prewashed 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 polytetrafluoroethylene-lined screw cap 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 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 1 to 2 μl of the solution was subject to GC-MS-SIM.}

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dl-[\textsuperscript{2}H\textsubscript{7}]leucine with porcine kidney acylase to give l-[\textsuperscript{2}H\textsubscript{7}]leucine (Fig. 1). The remaining N-acetyl-d-[\textsuperscript{2}H\textsubscript{7}]leucine was hydrolyzed with hydrochloric acid to give d-[\textsuperscript{2}H\textsubscript{7}]leucine. The optical purities of the d- and l-[\textsuperscript{2}H\textsubscript{7}]leucine were determined by GC-MS after methyl esterification of those with hydrochloric acid in methanol, and the subsequent N-acylation with (+)-MTPA-Cl. The respective (+)-MTPA-OMe derivatives of d- and l-[\textsuperscript{2}H\textsubscript{7}]leucine gave single peaks as shown in Fig. 2. The respective optical purities were found more than 99.8% e.e.

After an i.v. administration of a near equimolar mixture of d-[\textsuperscript{2}H\textsubscript{7}]leucine and d-leucine (1 mg/kg b.wt. each), the plasma concentrations of the enantiomers of the labeled and nonlabeled leucines were determined by GC-MS-SIM. Figure 3 shows the representative SIM profiles that were obtained from the plasma sample at 5 min after dosing. There was no interference from endogenous compounds in the vicinity of the peaks of analytes in the mass fragmentograms. Figure 4 shows the plasma concentration-time profiles of d-[\textsuperscript{2}H\textsubscript{7}]leucine, d-leucine, l-[\textsuperscript{2}H\textsubscript{7}]leucine, and l-leucine. Both administered compounds declined biexponentially and the plasma concentrations could be followed up to 6 h. No appreciable differences between the plasma concentrations of d-[\textsuperscript{2}H\textsubscript{7}]leucine and d-leucine were observed. Table 1 shows the pharmacokinetic parameters of individual d-leucine. No statistically significant differences (P > .05) were detected between the values of t\textsubscript{1/2}, MRT, AUC, Cl\textsubscript{ret}, and Vd\textsubscript{ss} of d-[\textsuperscript{2}H\textsubscript{7}]leucine and those of d-leucine. After administration of the d-enantiomer, l-[\textsuperscript{2}H\textsubscript{7}]leucine quickly appeared in the plasma, reached a maximum concentration (40.4 ± 14.0 ng/ml) at approximately 3 min, and then gradually decreased to be less than the limit of quantitation (0.02 μg/ml) at 120 min after dosing. Plasma concentration of endogenous l-leucine just before the administration of d-enantiomer was 15.73 ± 2.09 μg/ml. Plasma concentration of endogenous l-leucine tended to fall gradually, reaching a minimum value of 9.06 ± 1.06 μg/ml at 90 min after the administration. Thereafter, there was an upward trend in the plasma to reach the plasma concentration before dosing.

Figure 5 shows the plasma concentration-time profiles of d-[\textsuperscript{2}H\textsubscript{7}]leucine, l-[\textsuperscript{2}H\textsubscript{7}]leucine, and l-leucine after an i.v. administration of d-[\textsuperscript{2}H\textsubscript{7}]leucine (10 mg/kg b.wt.). Considerable amounts of

![Fig. 1. Resolution of dl-[\textsuperscript{2}H\textsubscript{7}]leucine.](image1)

![Fig. 2. SIM profiles of (+)-MTPA-OMe derivatives of d-[\textsuperscript{2}H\textsubscript{7}]leucine (A), dl-[\textsuperscript{2}H\textsubscript{7}]leucine (B), and l-[\textsuperscript{2}H\textsubscript{7}]leucine (C).](image2)

![Fig. 3. Representative SIM profiles of extracts from rat plasma after the i.v. administration of a near equimolar mixture of d-[\textsuperscript{2}H\textsubscript{7}]leucine and d-leucine (1 mg/kg b.wt. each).](image3)

![Fig. 4. Plasma concentration versus time profiles for d-leucine (■), d-[\textsuperscript{2}H\textsubscript{7}]leucine (○), l-[\textsuperscript{2}H\textsubscript{7}]leucine (□), and l-leucine (□) in rats after an i.v. administration of an equimolar mixture of d-leucine and d-[\textsuperscript{2}H\textsubscript{7}]leucine (1 mg/kg b.wt. each; mean ± S.D., n = 6).](image4)
l-[2H7]-leucine were detected in plasma. The concentration reached a maximum (613.4 ± 127.7 ng/ml) at 5 min after dosing and declined in a biexponential manner. Table 1 shows the pharmacokinetic parameters. The values of $t_{1/2}$, MRT, CL_\text{tot}, and Vd_\text{ss} of d-[2H7]-leucine were almost the same as those of d-[2H7]-leucine after an i.v. administration 1 mg/kg b.wt., suggesting linearity within 1 to 10 mg/kg b.wt. Plasma concentration of endogenous l-leucine just before the administration of d-[2H7]-leucine was 14.19 ± 1.43 μg/ml. Plasma concentration of endogenous l-leucine tended to fall gradually, reaching a minimum value of 8.53 ± 1.57 μg/ml at 90 min after d-[2H7]-leucine administration. Thereafter, there was an upward trend in the plasma to reach the plasma concentration before dosing.

Figure 6 shows the plasma concentration-time profiles of l-[2H7]-leucine and endogenous l-leucine in rats after an i.v. administration of d-[2H7]-leucine (10 mg/kg b.wt.; mean ± S.D., n = 6).

### Discussion

Stable isotopically labeled d-leucine can be used to investigate the inversion of d-leucine, differentiating endogenous l-leucine from the labeled l-leucine formed from labeled d-leucine administered exogenously. It is necessary for this study to use the compounds labeled at positions that are chemically and biologically inert. d-Leucine labeled with deuterium at the 4, 5, and 6 positions (d-[4,5,5,5,6,6,6-2H7]-leucine; d-[2H7]-leucine) was chosen for a tracer because the deuterium labels were placed at a sufficient distance from the amino group of leucine to avoid loss of the label under the transamination process.

In applying the stable isotope dilution analysis methodology for the pharmacokinetic study, it is essential to establish the bioequivalence of labeled and unlabeled forms of compounds. Bioequivalence of labeled and unlabeled forms of d-leucine was determined by dosing a near equimolar mixture of d-[2H7]-leucine and d-leucine. No appreciable differences between the plasma concentrations of d-[2H7]-leucine and d-leucine were observed (Fig. 4). The pharmacokinetic parameters of d-[2H7]-leucine were practically the same as those of the unlabeled compound (Table 1). These results indicated that pharmacokinetics of d-[2H7]-leucine could be considered to be equivalent to that of d-leucine. On the other hand, it is impossible to conduct such an experiment with l-[2H7]-leucine and l-leucine, because l-leucine is already present in the body fluids as a biological substance. Isotope effects of the natural substrates in the enzyme reactions are usually small because of higher commitments of substrate to the enzyme (Hermes et al., 1985; Furuta et al., 1996). Because the labels of...
D-[-2H\textsubscript{7}]leucine were placed at a sufficient distance from the metabolic site, it would not lead to erroneous conclusions in interpreting the pharmacokinetics of L-leucine.

The stereoselective pharmacokinetics of leucine were studied after bolus injection of stable isotopically labeled enantiomers in rats so that we could investigate the chiral inversion from D-leucine to L-leucine. The fractions of D-[-2H\textsubscript{7}]leucine that inverted to the L-enantiomer were 29.8 ± 15.3% (1 mg/kg b.wt.) and 35.5 ± 13.2% (10 mg/kg b.wt.), respectively (Table 1). Dose dependence within 1 to 10 mg/kg b.wt. was not observed in the chiral inversion. The values are comparable with those results observed previously for the inversion of D-phenylalanine to L-phenylalanine in humans (33%) (Lehmann et al., 1983) and the inversion of N\textsuperscript{2}-nitro-D-arginine to the L-isomer in rat (40%) (Wang et al., 1999).

D-Leucine is considered to be inverted to the L-enantiomer by two steps as shown in Fig. 7 (Ratner et al., 1940). First, it is oxidatively deaminated to \(\alpha\)-ketoisocaproate (KIC) by D-amino acid oxidase. Subsequently, the \(\alpha\)-keto acid is asymmetrically reaminated by transaminase to form the L-enantiomer. KIC may be decarboxylated by branched-chain \(\alpha\)-keto acid dehydrogenase, resulting in an irreversible loss of leucine. The incomplete inversion of D-[-2H\textsubscript{7}]leucine to L-[-2H\textsubscript{7}]leucine might depend on the irreversible decarboxylation of \([2H\textsubscript{7}]\)KIC formed from D-[-2H\textsubscript{7}]leucine (Cruz and Berg, 1970; Chawla et al., 1975; Nissen and Haymond, 1981; Imura et al., 1988). Urinary excretion of \([2H\textsubscript{7}]\)KIC and D-[-2H\textsubscript{7}]leucine might also have caused the incomplete inversion, although we did not measure those concentrations.

Inversion of D-leucine to L-leucine may also occur via racemases. These enzymes have not been endogenous produced in mammals but may be present in contaminating bacteria. Wolosker et al. (1999) have recently purified serine racemase from rat brain. The racemase is highly selective toward serine, failing to racemize other amino acids, e.g., alanine, threonine, and aspartate. Hamase et al. (1997) reported that D-leucine was present in the pineal gland and the hippocampus in rats, suggesting the biosynthesis of D-leucine in rats. Racemases catalyze interconversion between L- and D-enantiomer in both directions. Thus, the inversion of D-[-2H\textsubscript{7}]leucine to L-[-2H\textsubscript{7}]leucine in this study is unlikely to be attributable to the action of a racemase because no detectable amounts of D-[-2H\textsubscript{7}]leucine were found after administration of L-[-2H\textsubscript{7}]leucine.

The L-leucine levels just before an i.v. administration of D-[-2H\textsubscript{7}]leucine (10 mg/kg b.wt.) were 14.19 ± 1.43 \(\mu\)g/ml and fell gradually to 8.53 ± 1.57 \(\mu\)g/ml at 90 min after injection (Fig. 5). These findings seem to indicate that D-[-2H\textsubscript{7}]leucine suppressed the endogenous L-leucine levels. However, the decrease in plasma levels may be due to surgery or anesthesia because similar decreases in the plasma levels of the endogenous L-leucine occurred after an i.v. administration of saline (data not shown). The result is in agreement with that reported by Everson and Fritschel (1952), who found that the plasma levels of the individual essential amino acids were decreased below the preoperative plasma levels after surgery or ether anesthesia in men.

By using the technique described here, it should be possible to determine the degree that D-amino acid was inverted to the L-enantiomer. The methodology would be confirmed to be applicable for studying the stereoselective pharmacokinetics of chiral compounds as well as amino acids.

References


