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DETERMINATION OF LEUCINE FLUX IN VIVO BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY UTILIZING STABLE ISOTOPES FOR TRACE AND INTERNAL STANDARD

MOREY W. HAYMOND*, CAMPBELL P. HOWARD, JOHN M. MILES and JOHN E. GERICH

Endocrine Research Unit, Departments of Pediatric and Medicine, Mayo Medical School and Foundation, Rochester, MN 55901 (U.S.A.)

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SUMMARY

A simple and reliable method is described for the determination of leucine flux in vivo using two stable isotopes of leucine and gas chromatography—mass spectrometry (GC—MS). [6,6,6-$^2H_3$]Leucine is administered as a primed-dose constant infusion in vivo and DL-[$^2H_7$]-leucine is added to plasma as an internal standard. Plasma leucine concentration and moles per cent enrichment of [$^2H_3$]leucine can be determined simultaneously by GC—MS and selected ion monitoring. Leucine flux calculated from the [6,6,6-$^2H_3$]leucine data was nearly identical to that obtained with L-[U-^{14}C]leucine in dogs. This method is readily applicable to the study of leucine metabolism in humans of all ages and laboratory animals.

INTRODUCTION

The branched-chain amino acids (BCAA) leucine, isoleucine and valine, cannot be synthesized de novo in mammals. Therefore the only source of these essential amino acids for protein synthesis is dietary intake or proteolysis of endogenous protein. In addition to their essential role as components of protein, BCAA may play a direct role in the regulation of protein metabolism [1—3] and serve as a major source of nitrogen for non-essential amino acids [4—6]. Currently there is no simple and reliable method for the determination of BCAA flux, and little is known about the factors which regulate their metabolism in vivo. In the present report a simple and rapid method is described for the simultaneous quantitation of plasma leucine content and the moles per cent enrichment of stable isotope tracer, using gas chromatography—mass spectrometry (GC—MS) and simultaneous ion monitoring of the tracer (L-[$^2H_3$]leucine) and an internal standard (DL-[$^2H_7$]leucine).

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METHODS AND REAGENT PREPARATION

DL-[4,5,5,5,6,6,6-$^2H_7$]Leucine ([$^2H_7$]leucine) and L-[6,6,6-$^2H_3$]leucine ([$^2H_3$]leucine) were obtained from Merck, Sharp and Dohme Isotope Division (Point Claire/Duval, Quebec, Canada). The DL-[$^2H_7$]leucine and L-[$^2H_3$]leucine were 91 and 97 moles per cent enriched, respectively. The [$^2H_3$]- and [$^2H_7$]-leucine were determined to be >99% pure by amino acid analysis and gas chromatography. L-[U-^{14}C]Leucine (300 mCi/mmole) was obtained from New England Nuclear (Boston, MA, U.S.A.). Natural L-leucine was obtained from Sigma (St. Louis, MO, U.S.A.).

The [$^2H_3$]leucine and [U-^{14}C]leucine were dissolved in 0.9% sterile saline, passed through a 0.2-μm filter (Gelman Instrument Co., Ann Arbor, MI, U.S.A.) into sterile vials and autoclaved prior to use. The [$^2H_3$]leucine was determined to be pyrogen free by an FDA approved laboratory. An approximately 30 μM solution of [$^2H_7$]leucine was made by dissolving the stable isotope in deionized water and its content was determined using an amino acid analyzer (Beckman Model 119CL, Palo Alto, CA, U.S.A.).

Leucine concentrations in plasma were determined by ion-exchange chromatography (Beckman Model 119CL). For determination of [^{14}C]leucine radioactivity, plasma was processed in a manner identical to that for quantitation of the plasma leucine concentration, except that water was substituted for ninhydrin and the analyzer effluent was collected in 2.0-ml aliquots. These aliquots were quantitatively transferred to 20-ml scintillation vials (Curtin-Matheson Industries, Houston, TX, U.S.A.) and suspended in 15 ml of scintillation medium (Aquasol®, New England Nuclear) for counting in a liquid scintillation spectrometer (Searle Corporation, Des Plaines, IL, U.S.A.). Quench correction was accomplished by the use of a ^{133}Ba external standard. The dpm/ml plasma were divided by the concentration (μmoles/ml) to obtain [^{14}C]leucine specific activity.

AG 50W-X8 and AG 1-X8 ion-exchange resins (Bio-Rad Labs., Richmond, CA, U.S.A.) were rinsed ten times with an equal volume of deionized water and stored at 4°C as a 1:1 (v/v) suspension in deionized water. Separate disposable 1×8 cm plastic columns (Quik-Sep columns with plastic filter disc; Isolab, Akron, OH, U.S.A.) were filled with 2.0 ml of the respective resin suspensions and rinsed with 2—4 ml of deionized water immediately before use. Screw-cap vials (4 ml) were obtained from Kimble (Toledo, OH, U.S.A.) and their caps fitted with Teflon liners (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.). Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Regisil®) was obtained from Regis (Morton Grove, IL, U.S.A.) and acetonitrile (silylation grade) was obtained from Pierce (Rockford, IL, U.S.A.). Ammonium hydroxide was obtained from Mallinckrodt (Davis, KY, U.S.A.). OV-11 was obtained from Supelco (Bellefonte, PA, U.S.A.).

PROCEDURE

One to two milliliters of blood are collected in an iced tube containing sodium heparin (Kimble-Terumo, Elkton, MD, U.S.A.) during infusion of [$^2H_3$]-leucine (or [^{14}C]leucine). Following centrifugation at 4°C for 10 min, the

plasma is separated and stored at −80°C until the time of assay.

At the time of analysis, 200 μl are accurately transferred to a 10 mm × 75 mm test-tube and 40 μl of DL-[$^2H_7$]leucine are added making an approximately 6.0 μ*M* solution. The sample is deproteinized with 200 μl of 3 *M* perchloric acid; after mixing, the sample is centrifuged for 10 min, and 300 μl of supernatant are transferred to a second 10 mm × 75 mm test-tube. This latter solution is neutralized to a pH of 7 with 10% (w/v) potassium hydroxide solution, and centrifuged for 10 min. The resultant supernatant is applied to the AG1-X8 anion-exchange resin and the effluent is directly applied to the AG 50-X8 cation-exchange resin column to remove organic acids and to isolate plasma amino acids. The AG 50W-X8 column is rinsed with 4 ml of deionized water and the column effluent discarded. The amino acids are eluted from the AG 50W-X8 column with 2 ml of freshly prepared 4 *N* ammonium hydroxide solution. This latter eluate is collected in 4-ml reaction vials, frozen and taken to dryness by lyophilization. Trimethylsilyl derivatives are made by the addition of 50 μl each of BSTFA and acetonitrile; the reaction vial is tightly capped and allowed to stand at room temperature for 14—24 h.

One to two microliters of the derivatized sample are injected into a 6 m × 6 mm column packed with 3% OV-11 interfaced by a jet separator to a 5985B Hewlett-Packard gas chromatograph—mass spectrometer. The injection temperature is 250°C. Following 1 min at 90°C the column heat-bath temperature is increased at 4°C/min to 200°C. Utilizing positive electron-impact ionization (70 eV) and simultaneous ion monitoring the peak heights of 158, 161, and 165 *m/e* ions are determined using relative dwell times of 50, 250, and 100 msec, respectively.

The peak height ratios of (*m/e* 161/*m/e* 158) × 100 and (*m/e* 165/*m/e* 158) × 100 are calculated; the molar ratios are determined from the standard curve, and moles per cent enrichment calculated: moles per cent enrichment = 100/[1 + (1/molar ratio)]. Natural leucine concentration in plasma is calculated from the moles per cent enrichment of [$^2H_7$]leucine [MPE($^2H_7$)] by the formula

$$\mu\text{moles leucine/liter plasma} = \frac{6.0\ \mu\text{moles } [^2H_7]\text{leucine per liter plasma}}{[\text{MPE}(^2H_7)] \times \frac{1}{100}}$$

At steady-state, leucine flux was calculated by the formula [6]

$$R_a = R_d = \left(\frac{100}{\text{MPE}_e} - 1\right) r^s$$

where R_a and R_d are the rates of appearance and disappearance of leucine, respectively, MPE_e is the moles per cent enrichment of [$^2H_3$]leucine at steady-state and r^s is the rate of infusion of [$^2H_3$]leucine (moles per cent enrichment × μmoles/min). r^s is subtracted from the apparent flux since it is infused at 1—2% of the estimated endogenous production rate.

Leucine flux utilizing [^{14}C]leucine was calculated by the formula

$$R_a = R_d = \frac{r^*}{\text{SA}_e} - r^s$$

where r^* is the rate of [^{14}C]leucine infused (dpm/min) and SA_e is the specific activity of leucine at steady-state.

STANDARD CURVES

To determine the effects of [$^2H_7$]leucine on the $^2H_3:{}^2H_0$ leucine standard curve, two separate sets of standards were made, one with various molar ratios (0—0.06) of $^2H_3:{}^2H_0$ leucine, and a second set of standards identical to the first set except that [$^2H_7$]leucine was added to create varying $^2H_7:{}^2H_0$ leucine molar ratios. These $^2H_7:{}^2H_0$ leucine ratios (0.015—0.12) would accommodate the physiologic range of plasma leucine concentrations (for example, 400 to 50 μM, respectively [7]).

To maximize any artifacts introduced by ion fragments of the [$^2H_7$]leucine at *m/e* 158 and 161, the standard with the highest molar ratio of $^2H_7:{}^2H_0$ leucine contained no [$^2H_3$]leucine. The contribution of the [$^2H_7$]leucine to *m/e* fragments 158 and 161 was 0.6% and 0.2% of the base peak (*m/e* 165). The contribution of [$^2H_7$]leucine to the *m/e* 158 ion, assuming the molar ratio of $^2H_7:{}^2H_0$ leucine was within our standard curve, would be $<0.1\%$, below the level of sensitivity of the assay method. The two $^2H_0:{}^2H_3$ leucine standard curves derived from $^2H_0:{}^2H_3:{}^2H_7$ leucine and $^2H_0:{}^2H_3$ leucine mixtures were essentially identical (Fig. 1).

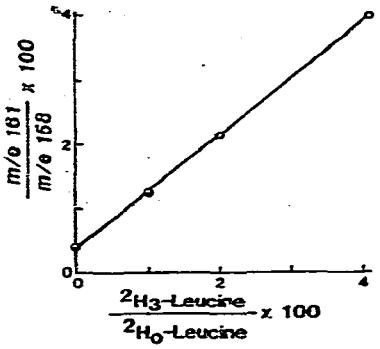
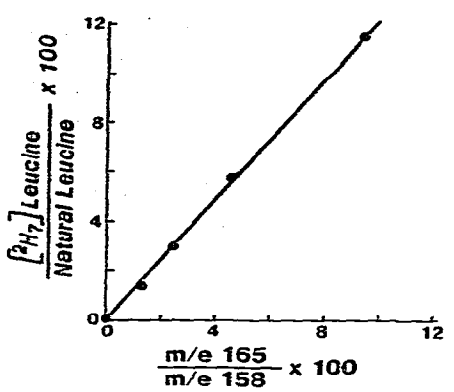


Fig. 1. The peak height ratio of *m/e* 161/*m/e* 158 and the molar ratio of L-[6,6,6-$^2H_3$]-leucine/natural leucine ($^2H_3:{}^2H_0$) in the presence (○) and absence (●) of DL-[4,5,5,5,6,6,6-$^2H_7$]leucine. Triplicate points are included within the area of each symbol.

The simultaneously derived $^2H_7:{}^2H_0$ leucine standard curve is depicted in Fig. 2. The linear relationship between peak height ratio and molar ratio suggests no significant interference from other potential contaminating ions at *m/e* 158 since the lowest enrichment of [$^2H_7$]leucine was in the standard with the highest enrichment of [$^2H_3$]leucine.

Fifteen plasma samples were analyzed for their leucine content both by amino acid analyzer and by GC—MS using [$^2H_7$]leucine as an internal standard (Fig. 3). The correlation coefficient between the two methods was $r = 0.99$. The coefficient of variation of ten replicate injections of the same sample was 0.95%. The coefficient of variation of leucine content determined on ten separate preparations of the same sample was 1.4%.



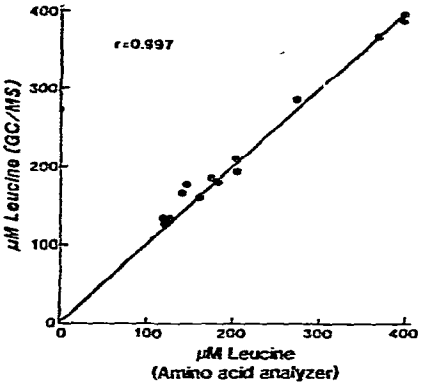


Fig. 2. The molar ratio of DL-[4,5,5,5,6,6,6-$^2H_7$]leucine to natural leucine and peak height ratio of *m/e* 165/*m/e* 158. Triplicate points are included within the area of each symbol.

Fig. 3. Plasma leucine content determined by an amino acid analyzer and by the addition of DL-[4,5,5,5,6,6,6-$^2H_7$]leucine to plasma as an internal standard (ca. 6 μmoles [$^2H_7$]leucine per l) and selected ion monitoring by GC—MS.

IN VIVO VALIDATION

Leucine production rates were estimated simultaneously with [^{14}C]leucine and [$^2H_3$]leucine in three dogs. The animals were fasted for 24 h and placed in a standing sling. Two plastic venous catheters were placed, one in a forelimb vein (isotope infusion) and a second in the saphenous vein (blood sampling). The animals were administered primed, continuous infusions of [^{14}C]leucine (0.225 μCi/kg, 0.015 μCi/kg per min for 4 h) and [$^2H_3$]leucine (60 μg/kg, 8 μg/kg per min for 4 h). Two hours were allowed for isotopic equilibrium and, subsequently, plasma samples were obtained at half-hourly intervals over the last 2 h of study for determination of plasma leucine content, moles per cent enrichment of [$^2H_3$]leucine and specific activity of [^{14}C]leucine.

Over the last 2 h of study, isotope and substrate equilibrium was observed. Leucine flux was essentially the same with both isotopes (4.00 ± 0.20 vs. 4.10 ± 0.27 μmoles/kg per min from [^{14}C]leucine and [$^2H_3$]leucine, respectively, Table I).

TABLE I

STEADY-STATE FLUX RATES OF LEUCINE SIMULTANEOUSLY DETERMINED BY [U-^{14}C]LEUCINE AND [$^2H_3$]LEUCINE

Dog	[$^2H_3$]Leucine (μmoles/kg per min)	[U-^{14}C]Leucine (μmoles/kg per min)
1	3.64	3.62
2	4.32	4.57
3	4.03	4.11
Mean ± S.E.	4.00 ± 0.20	4.10 ± 0.27

Following an overnight fast, five normal male volunteers received a primed-dose (ca. 60 μg/kg) continuous infusion (ca. 4 μg/kg per min) for 4 h to enrich the circulating leucine to 1—2 moles per cent. Following 2 h to allow for isotopic equilibration, venous blood was obtained at 30-min intervals over the last 2 h of study. The plasma leucine concentrations and moles per cent [$^2H_3$]-leucine enrichment reflect substrate and isotopic equilibration (Fig. 4). The leucine flux (production = utilization at steady-state) in these subjects was 2.2 ± 0.2 μmoles/kg per min.

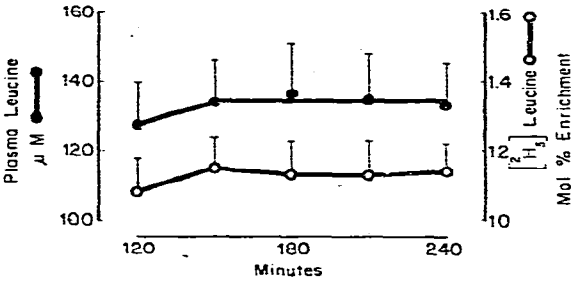


Fig. 4. Plasma leucine concentration and moles per cent enrichment of L-[6,6,6-$^2H_3$]leucine in five normal male volunteers during the last 2 h of a 4-h infusion of L-[6,6,6-$^2H_3$]leucine. Data are displayed as mean ± S.E.

DISCUSSION

Little information is currently available on the regulation of branched-chain amino acid production and utilization in vivo [8—11]. A previously described isotopic method for determination of leucine flux in vivo requires the use of [^{14}C]leucine and is tedious and time-consuming [9]. With the increased reliability and availability of GC—MS systems and the custom synthesis of stable isotopes, it is now possible to examine the metabolic regulation of a number of compounds in man with isotope dilution methodology without the use of ionizing radiation.

The method described herein utilizes two deuterium-labeled leucine molecules; one for in vivo infusion (L-[$^2H_3$]leucine) and one as an internal standard (DL-[$^2H_7$]leucine). Selection of the [6,6,6-$^2H_3$]leucine places the ^2H at a sufficient distance from the amino nitrogen group of leucine that transamination of the isotopically labeled leucine does not result in loss of enrichment. Subsequent metabolism of the leucine carbon chain by branched-chain α-keto acid dehydrogenase is irreversible [12], thus preventing re-entry of the deuterium label into the leucine pool. As a result, use of [$^2H_3$]leucine as tracer should accurately estimate the flux of the leucine carbon. This was confirmed in the present studies since simultaneous estimation of leucine flux with [$^2H_3$]- and [U-^{14}C]leucine gave identical flux rates in dogs. Moreover, the rates of leucine flux observed in normal adult humans utilizing [$^2H_3$]leucine in the present studies were not appreciably different from those found by Golden and Waterlow [9] in adults using [U-^{14}C]leucine as trace.

Stable isotopes have been widely used as internal standards for the quantitation of a number of compounds in plasma, including amino acids [13]. In the

present method, careful selection of the internal standard is required to avoid artifactual contamination of the fragments of interest from the unlabeled leucine (*m/e* 158) and [$^2H_3$]leucine (*m/e* 161). Therefore, the internal standard must be of sufficient purity and enrichment to permit accurate quantitation of the unlabeled leucine and to avoid interference with the determination of the mole per cent enrichment of the infused trace (L-[$^2H_3$]leucine, *m/e* 161; 1—1.5 moles per cent enrichment). The DL-[$^2H_7$]leucine used in the present method as the internal standard has less than 1% the base peak at mass fragments of *m/e* 158 and 163. These fragments could potentially interfere with the quantitation of leucine and the determination of the [$^2H_3$]leucine enrichment. Addition of [$^2H_7$]leucine to the plasma sample, creating a plasma [$^2H_7$]leucine concentration equal to the plasma concentration of unlabeled leucine, increased the molar ratio of [$^2H_3$]leucine to natural leucine by 0.4%. At the concentration (6 μM) selected in the described methods, no significant error was introduced (Fig. 1).

The assessment of substrate flux requires the determination of both substrate concentration and isotopic enrichment (moles per cent enrichment or specific activity). The method described above provides a rapid and accurate technique for the simultaneous quantitation and determination of the moles per cent enrichment of the infused trace in large numbers of samples. It eliminates time-consuming procedures involving ion-exchange resin chromatography for the determination of leucine specific activity and the exposure of subjects and laboratory personnel to radioactive compounds. Moreover, since it requires small volumes of blood, it can be readily used for the determination of leucine flux in children and laboratory animals where the volume of blood available for sampling may be severely limited.

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REFERENCES

1 R.M. Fulks, J.B. Li and A.L. Goldberg, J. Biol. Chem., 250 (1975) 290.
2 M.G. Buse and S.S. Reid, J. Clin. Invest., 56 (1975) 1250.
3 M.G. Buse and D.A. Weigand, Biochim. Biophys. Acta, 475 (1977) 81.
4 A.J. Garber, I.E. Karl and D.M. Kipnis, J. Biol. Chem., 251 (1976) 836.
5 R. Odessey, E.A. Khairallah and A.L. Goldberg, J. Biol. Chem., 249 (1974) 7623.
6 T.W. Chang and A.L. Goldberg, J. Biol. Chem., 253 (1978) 3677.
7 D.M. Bier, K.J. Arnold, W.R. Sherman, W.H. Holland, W.F. Holmes and D.M. Kipnis, Diabetes, 26 (1977) 1005.
8 S.A. Adibi, A.L. Drash and E.D. Livi, J. Lab. Clin. Med., 76 (1970) 722.
9 M.H.N. Golden and J.C. Waterlow, Clin. Sci. Mol. Med., 53 (1977) 277.
10 R. Sherwin, J. Rosenzweig, V. Soman, R. Handler and P. Felig, Diabetes, 25 (1976) 332.
11 R.S. Sherwin, J. Clin. Invest., 61 (1978) 1471.
12 C.R. Scriver and L.E. Rosenberg, Amino Acid Metabolism and Its Disorders, W.B. Saunders, Philadelphia, PA, 1973, p. 258.
13 E.E. Kingston and A.M. Duffield, Biomed. Mass Spectrom., 5 (1978) 621.