Measurement of Very Low Stable Isotope Enrichments by Gas Chromatography/Mass Spectrometry: Application to Measurement of Muscle Protein Synthesis

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Measurement of muscle protein synthesis using stable isotopically labeled tracers usually requires isotope ratio mass spectrometry (IRMS) because of the need to measure very low enrichments of stable isotopically labeled tracers (tracer to tracee ratio [TTR], 0.005% to 0.10%). This approach is laborious, requiring purification of the metabolite of interest and combustion to a gas for IRMS analysis, and is best suited for use with ^{13}C tracers. We have developed an approach whereby low enrichments can be conveniently measured by a conventional gas chromatography/mass spectrometry (GC/MS) instrument. The approach includes three critical elements: (1) use of a highly substituted tracer containing three or more labeled atoms, to measure enrichment above a very low natural abundance of highly substituted isotopomers; (2) use of a highly substituted natural abundance isotopomer as a base ion for comparison rather than the most abundant m + 0 isotopomer, to reduce the dynamic range of the isotopomer ratio measurement; and (3) a sensitive mass spectrometric analysis that measures the natural abundance of the isotopomer used as a tracer with a high signal to noise ratio (>100:1). This approach was used to measure the rate of synthesis of muscle protein following a primed continuous infusion of L-[$^{13}C_6$]-phenylalanine (PHE) in eight fasted dogs and L-[$^2H_3$]-leucine in five fasted human subjects. Values for [$^{13}C_6$]-PHE enrichment by GC/MS rates were virtually identical to those obtained by a conventional approach using high-performance liquid chromatography (HPLC) to isolate PHE, combustion to CO_2, and measurement of $^{13}CO_2$ enrichment by IRMS (IRMS enrichment = 0.9988 × GC/MS enrichment, R^2 = .891), resulting in identical values for muscle fractional synthesis rates ([FSRs] mean ± SEM: 2.7 ± 0.2 and 2.5 ± 0.2%/d for GC/MS and IRMS, respectively). Human muscle synthesis rates measured by GC/MS analysis of [$^2H_3$]-leucine enrichment (1.90 ± 0.17%/d) were similar to published values based on IRMS analysis using a 1-^{13}C-leucine tracer. We conclude that compared with the IRMS approach, the GC/MS approach offers faster throughput, has a lower sample requirement, and is suitable for a wider variety of tracers such as ^2H. The principles outlined here should be applicable to the measurement of low enrichments by GC/MS in a wide variety of stable isotope tracer applications.

STABLE ISOTOPICALLY LABELED tracers have been used to evaluate diverse areas of protein, lipid, and carbohydrate metabolic kinetics in vivo. Two types of instruments are conventionally available to measure isotopic enrichment for such studies,[1] the gas chromatograph/mass spectrometer (GC/MS) and the isotope ratio mass spectrometer (IRMS). The GC/MS is suited to a wide variety of analyses through a choice of derivatization reactions, GC columns, and MS ionization modes. Furthermore, this instrument requires little sample for analysis (≤1 nmol). Reliable isotopic enrichment measurements by GC/MS are typically limited to tracer to tracee ratios (TTRs) greater than 0.5%, since the limited accuracy and precision of isotope ratio measurements by GC/MS requires a significant enrichment above the natural abundance of stable isotopes. In contrast, the IRMS is specifically designed to measure small TTRs (0.001% to 0.1%) accurately and precisely, but can only analyze purified gases (eg, CO_2 or H_2) and requires greater sample size (>10 nmol).

The IRMS in conjunction with ^{13}C-labeled amino acids is traditionally used to measure muscle protein synthesis rates because of the very low enrichments (TTR < 0.10%) customarily encountered in muscle protein following a constant infusion of a stable isotopically labeled amino acid. We have used ring-$^{13}C_6$-phenylalanine (PHE) to measure muscle protein synthesis,[2-4] because the large number of carbon atoms that are isotopically labeled in this tracer (six of nine) generate a higher TTR following combustion to $^{13}CO_2$ than if a singly substituted tracer such as 1-^{13}C-leucine (one of six carbon atoms) was used. However, the approach with $^{13}C_6$-PHE is slow and laborious, as it requires isolation of the amino acid from a protein hydrolysate by high-performance liquid chromatography (HPLC) and combustion to CO_2 for subsequent IRMS analysis. Furthermore, this approach is limited by a slow HPLC throughput and requires both HPLC and IRMS instrumentation. Recently, an IRMS connected to a GC through an on-line combustion interface[5] has been used to measure ^{13}C enrichment of muscle protein hydrolysate amino acids. However, the derivatization of amino acids for gas chromatography dilutes the ^{13}C label such that it is necessary to use a higher tracer infusion rate for a longer period to achieve measurable enrichments.

Measurement of muscle protein synthetic rates would be greatly simplified if very low enrichments could be measured by a GC/MS, thereby eliminating the need to combust samples for IRMS analysis. In the present report, we detail a new procedure whereby very low enrichments (tracer to tracee ratio [TTR], 0.005% to 0.10%) of labeled amino acids incorporated into muscle protein can be accurately determined by a GC/MS. The approach is based on three critical elements: use of a highly substituted isotopomer as a tracer (containing three or more isotopically labeled atoms); use of an isotopomer with a very low natural abundance as a base for comparison rather than the

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m + 0 isotopomer, to avoid the large dynamic range associated with measuring an m + 0 isotopomer; and a mass spectrometric detection system that achieves a signal to noise ratio greater than 100:1 for the natural abundance isotopomer used as a tracer. These principles were used to measure muscle protein synthesis using either $^{13}C_6$-PHE or $^{2}H_3$-leucine (LEU) tracers.

SUBJECTS AND METHODS

Preparation of Isotope Enrichment Calibration Standards

Stock solutions (nominally 20 mmol/L) of the unlabeled tracees PHE or LEU (Sigma Chemical, St Louis, MO) and the isotopically labeled tracers L-ring-$^{13}C_6$-PHE (99% ^{13}C) or L-methyl-$^{2}H_3$-LEU (98% ^{2}H) (Cambridge Isotope Laboratories, Woburn, MA) were prepared to known weight percent compositions and mixed gravimetrically to obtain final solutions with TTRs of 0% to 0.10%.

Biological Tracer Studies

Two study protocols were performed. In one study, eight dogs were anesthetized and administered a primed (4.0 μmol/kg) continuous intravenous infusion (0.10 μmol/kg · min) of ring-$^{13}C_6$-PHE using a calibrated syringe pump. Hindquarter muscle biopsies were obtained from each leg before and after 5 hours of tracer infusion. Samples were immediately frozen in liquid N_2 and stored at −70°C for subsequent processing. The protocol was approved by the Animal Research Committee of the University of Texas Medical Branch. In the second study, five adult male human subjects were placed on a liquid formula diet (1.5 g/kg/d protein and 36 kcal/kg/d; Ensure, Ross Laboratories, Columbus, OH) for 3 days. On the fourth day, the liquid formula was divided into equal aliquots administered at 30-minute intervals starting 2 hours before and continuing throughout the isotope infusion study. Intravenous catheters were placed in an antecubital vein, and a primed (6 μmol/kg) continuous infusion (0.10 μmol/kg · min) of $^{2}H_3$-LEU was infused for 8 hours. Muscle biopsies were obtained from the quadriceps femoris muscle using a 4-mm Bergström biopsy needle[6] with local anesthesia under sterile conditions at 5 and 8 hours of isotope infusion. One subject was studied a second time during the postabsorptive state (overnight fast and fasted throughout tracer study). All subjects provided informed consent before participating in this study, which was approved by the Human Studies and General Clinical Research Center Scientific Review committees of Washington University School of Medicine.

Muscle Sample Processing

Muscle biopsy samples were thawed, weighed (typically 20 to 50 mg wet weight), and extracted three times with 5% perchloric acid by grinding with a Teflon pestle in a microfuge tube. Intracellular free amino acids from the pooled supernatant were recovered by cation-exchange chromatography. Intracellular free PHE was purified by HPLC and converted to *N*-acetyl-*n*-propyl esters, and intracellular PHE enrichment was measured by chemical ionization GC/MS on a Hewlett Packard (Palo Alto, CA) 5985 system[1,2,4]; enrichment of intracellular free LEU was measured by electron-impact ionization (EI) GC/MS on a Hewlett Packard 5971A mass selective detector using a *tert*-butyldimethylsilyl derivative.[7] Different instruments and derivatives were used for intracellular PHE and LEU based on routine procedures at the two facilities where these studies were conducted.

The muscle protein pellet was washed three additional times with 2% perchloric acid, followed by two ethanol washes and one ether wash. All grinding and washing procedures were performed on ice. The protein was then hydrolyzed in 6N constant boiling HCl at 110°C for a minimum of 24 hours. Following hydrolysis, the amino acids were divided such that an aliquot corresponding to approximately 10 mg starting wet weight tissue was analyzed by a conventional approach using HPLC/combustion/IRMS,[2,4] and an aliquot corresponding to 4 mg wet tissue weight was analyzed by the experimental GC/MS procedure. For the latter, a cation-exchange column (Dowex AG 50W-X8; Bio-Rad Laboratories, Richmond, CA) was used to recover and purify the amino acids before GC/MS analysis. Isotopic dilution standards (~100 nmol) were dried in separate tubes.

GC/MS Analysis

N-heptafluorobutyryl-*n*-propyl ester (HFBPr) derivatives of samples and isotopic enrichment standards of known TTR were prepared by first heating the samples at 110°C for 1 hour with 0.5 mL 3.5N HBr in propanol (Alltech Associates, Deerfield, IL) in a screw-capped tube, drying the sample under N_2, and then heating at 60°C for 20 minutes with 0.1 mL heptafluorobutyric anhydride (Sigma Chemical). The samples were briefly dried under N_2 (evaporated just to dryness, since the samples are volatile; only a few minutes are required) and dissolved in 0.1 to 0.5 mL ethyl acetate.

One-microliter aliquots were analyzed by GC/MS using one of two techniques. $^{13}C_6$-PHE was determined on either an MD800 (Fisons Instruments, Beverly, MA) or a VG 12-250 (VG Biotech, Altrincham, UK) GC/MS system using a 15-m × 0.32-mm DB5-MS capillary column (GC conditions: initial temperature, 80°C [held for 1.2 minutes]; rapid ramp [50°C/min] to 140°C; slow ramp [5°C/min] to 175°C; and a final rapid ramp [60°C/min] to 290°C [held for 3 minutes]). Mass spectrometry was performed using 70-eV EI with selected ion monitoring of *m/z* 95 and 97 (m + 4 and m + 6 isotopomer ions, respectively, of the *m/z* 91 ion from the phenyl-CH_2-side chain of PHE; Fig 1). $^{2}H_3$-LEU was determined on a model 5988 GC/MS (Hewlett Packard) using a 30-m × 0.32-mm DB-17 column (GC conditions: initial temperature, 70°C [held for 1 minute]; rapid ramp [70°C/min] to 115°C; slow ramp [3°C/min] to 125°C; and a final rapid ramp [70°C/min] to 260°C [held for 2.5 minutes]). Mass spectrometry was performed using methane-negative chemical ionization (NCI) with selected ion monitoring of *m/z* 352 and 351 (m + 3 and m + 2 isotopomer ions, respectively, of the *m/z* 349 ion corresponding to loss of HF from the molecular ion). Both procedures used approximately 1 nmol target amino acid injected in splitless mode; an aliquot of protein hydrolysate equivalent to 4 mg wet weight starting tissue produced similar GC/MS peak areas for PHE and LEU compared with the 1-nmol standards. The TTR of samples was determined by regression analysis of the instrument measurement isotopomer ratio against standards of known TTR. This standard-curve approach is necessary because TTRs cannot be directly derived from the measured ion abundance ratios unless the m + 0 ion abundance is measured.[1] Furthermore, the standard-curve approach corrects for numerous sources of error in the analysis, including biases in the mass spectrometric ratio measurement and purity of the tracer.

IRMS Analysis

PHE from additional aliquots of dog muscle protein hydrolysates (corresponding to ~10 mg initial wet weight tissue) was purified by HPLC in a carbon-free buffer system and combusted in sealed tubes to produce CO_2.[1] Aliquots (~40 μg) of isotopic dilution standards were also combusted for IRMS analysis. Isotopic enrichment of $^{13}CO_2$ was measured by IRMS on a dual-inlet SIRA-12 system (VG Biotech).

Calculation of Muscle Protein Fractional Synthetic Rate

Muscle protein fractional synthetic rate (FSR) was determined by dividing the rate of increase in muscle protein enrichment (percent per hour) by the intracellular free amino acid enrichment (percent).[1]

RESULTS

The EI spectrum of HFBPr derivatives of PHE and ring-$^{13}C_6$-PHE is shown in Fig 1. The intact molecular ion (*m/z* 403) does

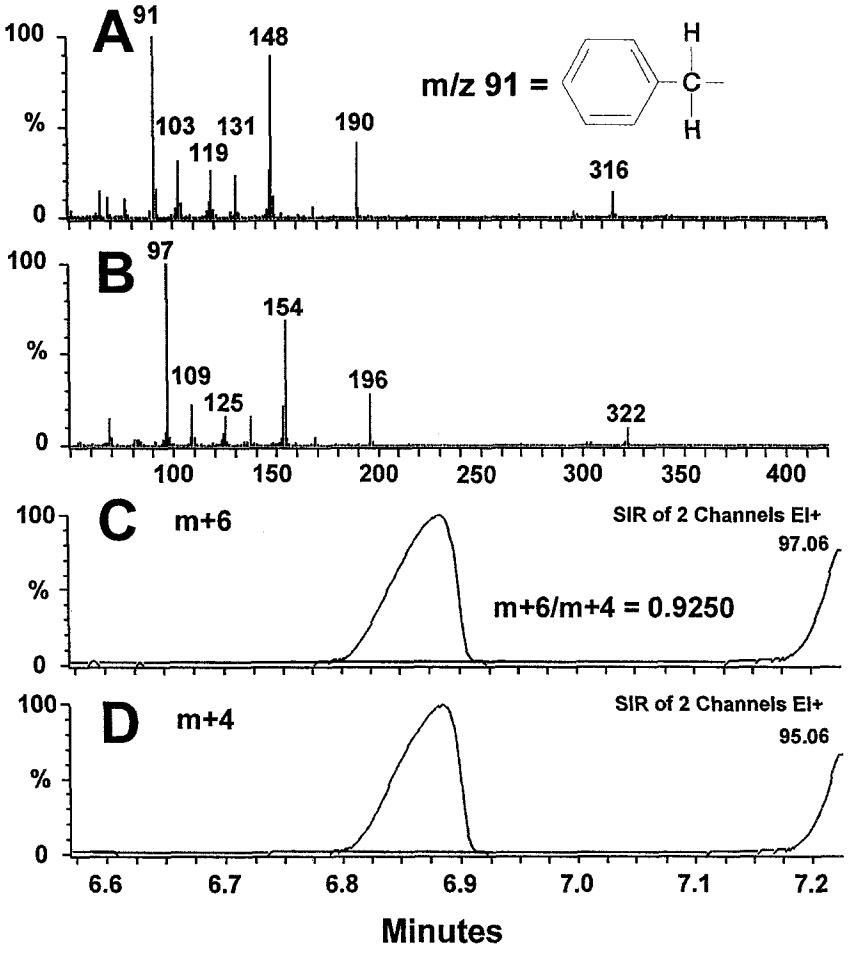

Fig 1. Mass spectrum and chromatogram for PHE. EI spectrum (*m/z* 50 to 420) of HFBPr derivative of PHE: (A) natural abundance PHE; (B) $^{13}C_6$-PHE. (C and D) Selected ion monitoring of PHE from muscle protein hydrolysate; the m + 6 and m + 4 isotopomer ions (*m/z* 97 and 95, respectively) are shown. The ratio of integrated isotopomer peak areas is compared with ratios measured on standards of known isotopic enrichment to determine the true TTR, which is approximately 0.02% in this sample.

not appear in the 70-eV EI spectrum. The most abundant ion in the spectrum, which corresponds to the phenyl-CH_2-side chain (*m/z* 91), was used to monitor ring-$^{13}C_6$-PHE enrichment. Selected ion monitoring of the m + 4 and m + 6 isotopomers (*m/z* 95 and 97) of PHE for a muscle protein sample is also shown in Fig 1. Both ions are well resolved from other chromatographic peaks (the peak beginning to elute at the end of the display range was identified as HFBPr-glutamate). Although the PHE peak shows evidence of column overloading, the tracer ion exactly coelutes with the tracee ion and the m + 6/m + 4 ratio was virtually unaffected by the amount of sample injected (0.4 to 2 nmol). The signal to noise (peak to peak) ratio of the PHE m + 6 ion (*m/z* 97) was estimated to be greater than 200:1 for 1 nmol of natural abundance PHE analyzed on the Fisons MD800 instrument with EI.

The methane NCI spectrum of HFBPr derivatives of LEU and $^2H_3$-LEU is shown in Fig 2. The ion corresponding to loss of HF from the molecular ion (*m/z* 349) was the most abundant fragment in the spectrum and was used to measure muscle LEU enrichment. A chromatogram of the m + 2 and m + 3 ions of LEU is also illustrated in Fig 2 for a typical muscle protein sample. Isoleucine was well resolved from the desired LEU peak. The signal to noise ratio of the LEU m + 3 ion (*m/z* 352) was estimated to be 100:1 for 1 nmol of natural abundance LEU analyzed on the Hewlett Packard 5988 instrument with methane NCI.

Accuracy of the PHE isotope enrichment standards used in GC/MS was validated by measuring ^{13}C enrichment by IRMS. The regression line of measured enrichment versus expected enrichment had an intercept not significantly different from zero and a slope of 0.989 (results not shown). Isotope enrichment standard curves for GC/MS measurements are shown in Figs 3 and 4. The measured m + 6/m + 4 isotope ratio for $^{13}C_6$-PHE was linear between TTRs of 0% to 0.10% with excellent precision of triplicates (Fig 3A). Enrichment of 0.001% above natural abundance was detectable (Fig 3B). Essentially identical standard curves were obtained on both the Fisons MD 800 and VG 12-250 instruments. The m + 6/m + 4 ratio was independent of the amount of PHE analyzed over the range of 0.1 to 2 nmol both at natural abundance and at 0.1% enrichment for both GC/MS instruments (results not shown). The measured m + 3/m + 2 ratio for $^2H_3$-LEU enrichment standards was linear between TTRs of 0% to 0.07% with excellent precision of triplicates (Fig 4). It was necessary to adjust the concentration of all standards and biological samples to produce approximately equivalent peak areas for the m + 2 peak (within a factor of ~2) because the measured m + 3/m + 2 ratio for $^2H_3$-LEU was slightly dependent on the amount of sample injected.

A comparison of $^{13}C_6$-PHE enrichments obtained by GC/MS with values obtained by IRMS in dog skeletal muscle protein after 5 hours of $^{13}C_6$-PHE infusion is shown in Fig 5. There was excellent agreement between the two methods, with a slope of

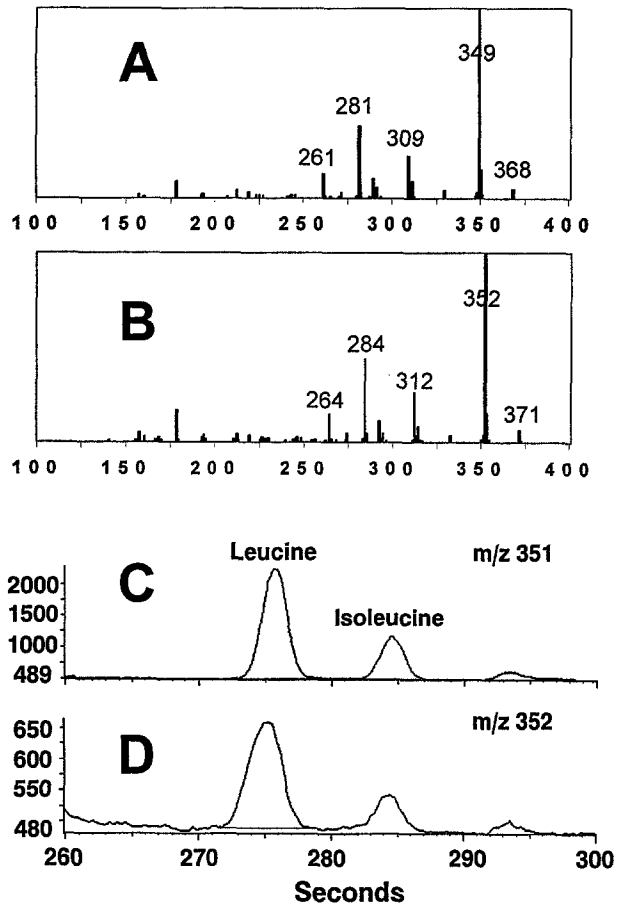

Fig 2. Mass spectrum and chromatogram for LEU. Methane-negative chemical ionization spectrum (*m/z* 100 to 400) of HFBPr derivative of LEU: (A) natural abundance LEU; (B) $^2H_3$-LEU. (C and D) Selected ion monitoring of LEU from muscle protein hydrolysate; the m + 2 and m + 3 isotopomer ions (*m/z* 351 and 352, respectively) are shown.

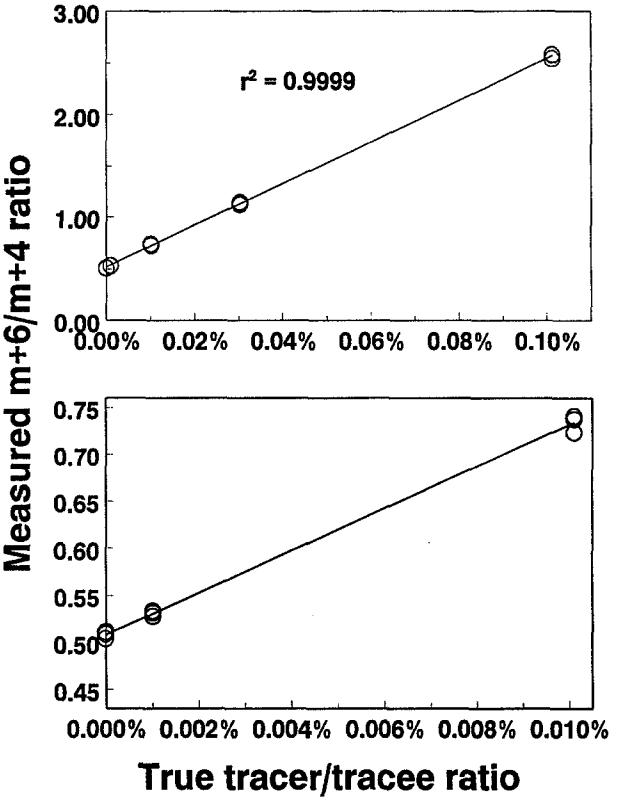

Fig 3. Isotopic enrichment standard curve for $^{13}C_6$-PHE. (A) The m + 6/m + 4 isotope ratio was measured in triplicate on standards ranging from natural abundance to a TTR of 0.10%. Regression line: m + 6/m + 4 ratio = (20.26 ± 0.0923) × TTR% + (0.5164 ± 0.0044). (B) Magnification of scale from 0% to 0.01% $^{13}C_6$-PHE.

0.9988 and an intercept not significantly different from zero. The FSR was virtually identical for muscle protein enrichments measured by GC/MS and IRMS (mean ± SEM: 2.7 ± 0.2 and 2.5 ± 0.2 %/d for GC/MS and IRMS, respectively). Human muscle protein FSR measured with $^2H_3$-LEU was 1.90 ± 0.17 %/d for five subjects in the fed state. (It was not feasible to directly validate $^2H_3$-LEU enrichment measurement by IRMS as it was for ^{13}C-labeled PHE.) One subject was studied in both the fed and postabsorptive condition: muscle FSR decreased from 1.93 %/d during the fed state to 1.45 %/d in the postabsorptive state.

During the course of these studies, we examined several variables that impact the processing of muscle samples. For example, we observed that muscle protein hydrolysates produced with ACS grade 6N HCl, in contrast to the traditional use of the more expensive constant boiling HCl,[2,4] did not introduce chromatographic impurities in the GC/MS analysis (Fig 2). We did not find it necessary to perform chloroform/ether lipid extractions from muscle homogenates. However, when acid-washed Celite (Johns-Manville Products, Manville, NJ) columns were used to remove particulates from the acid hydrolysates,[2,4] we observed that samples frequently exhibited anomalous chromatographic behavior resulting in dramatic drift in peak retention times and reduced yields. We therefore recommend the use of a cation-exchange column to effect both removal of particulates and purification of amino acids from protein hydrolysates.

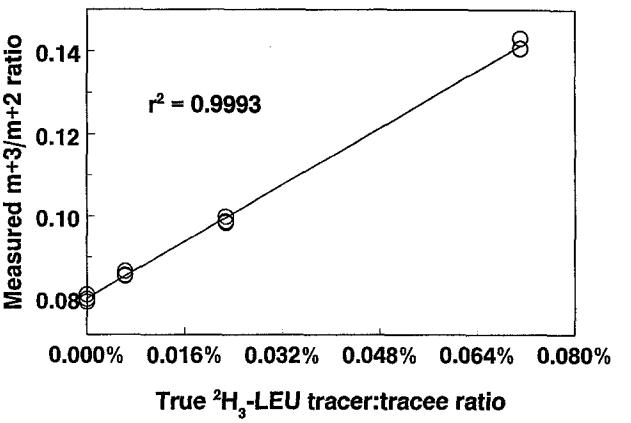

Fig 4. Isotopic enrichment standard curve for $^2H_3$-LEU. The m + 3/m + 2 ratio was measured in triplicate on standards ranging from natural abundance to a TTR of 0.07%. Regression line: m + 6/m + 4 ratio = (0.8660 ± 0.0101) × TTR% + (0.0799 ± 0.0004).

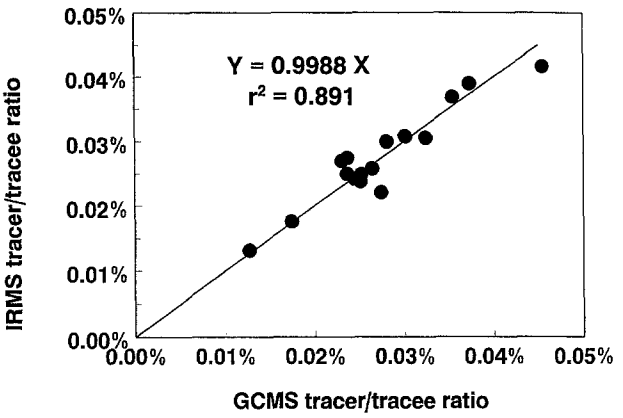

Fig 5. Correlation between measurements of $^{13}C_6$-PHE enrichment by IRMS and GC/MS. Aliquots of hydrolysates from 16 separate muscle samples were divided between the GC/MS method and conventional analysis involving HPLC, combustion, and IRMS. GC/MS values reflect the mean of triplicate analyses; IRMS analyses are based on a single analysis per sample.

DISCUSSION

The results of the present study demonstrate that very low stable isotope enrichments such as those encountered in studies of muscle protein synthesis can be reliably measured by GC/MS with the proper choice of isotopically labeled tracers and instrument operation conditions. Identical enrichments were measured by GC/MS and IRMS in dog muscle samples labeled with $^{13}C_6$-PHE. Furthermore, the values for muscle protein FSR in fasted human subjects using $^2H_3$-LEU were in good agreement with those summarized by Smith and Rennie[8] based on a primed constant infusion of 1-^{13}C-LEU and analysis by IRMS.

Our method to measure very low enrichments by GC/MS offers several advantages over traditional methods of analysis requiring IRMS. Dozens of samples can be processed per day by GC/MS, compared with a throughput of about four per day by the HPLC/combustion/IRMS approach, because the GC/MS method eliminates the slow and laborious task of purification of desired components by HPLC. Furthermore, the GC/MS method requires much less sample than the IRMS method: a 4-mg sample of muscle tissue provides material for approximately 100 replicate analyses by GC/MS, compared with a single IRMS analysis for a 10-mg muscle sample. In addition, the GC/MS approach is suitable for a larger variety of tracers, including ^{2}H as demonstrated by measuring protein synthesis with $^2H_3$-LEU, whereas the IRMS approach is most directly applicable to ^{13}C tracers.

The ability to measure very small enrichments by GC/MS is contingent on three critical elements. First, it is necessary to use a highly substituted tracer such as $^{13}C_6$-PHE (m + 6) or $^2H_3$-LEU (m + 3), because it is not feasible to measure such small enrichments above natural abundance using a singly (m + 1) or doubly (m + 2) substituted tracer. Second, it is necessary to use some other ion that is much less abundant than the m + 0 ion as a basis for comparison to reduce the dynamic range of the measurement (such as the m + 4 ion for $^{13}C_6$-PHE or the m + 2 ion for $^2H_3$-LEU). The third critical element concerns the sensitivity of the GC/MS analysis. The mass spectrometric analysis chosen must measure the natural abundance of the isotopomer to be used as a tracer with a sufficiently high signal to noise ratio to provide a high precision in isotopomer ratio measurements (we prefer to operate with a signal to noise ratio ≥ 100:1). The Fisons MD 800 instrument we used for measurement of $^{13}C_6$-PHE enrichment by EI had exceptional sensitivity and measured the m + 6 ion of PHE (1 nmol injected) at natural abundance with a signal to noise ratio of approximately 200:1. In contrast, our Hewlett Packard 5988 instrument had considerably lower sensitivity; the m + 6 ion of PHE at natural abundance was virtually undetectable in either EI or NCI operation (1 nmol injected). However, the m + 3 ion of LEU (considerably more abundant than the m + 6 ion at natural abundance) was detectable at natural abundance with a signal to noise ratio of 100:1 by NCI, thereby necessitating our choice of $^2H_3$-LEU for studies using this instrument. Many elements critical for GC/MS sensitivity are features of instrument design beyond the control of the investigator (eg, design of the ion source, type of detector, and quality of vacuum). However, the operator may achieve higher signal to noise ratios for a given instrument by increasing the amount of material injected (we typically apply 1 nmol splitless) and selecting a more sensitive mass spectrometric analysis (eg, choice of derivatization and ionization method, such as using highly fluorinated derivatives with negative chemical ionization).

The method reported here is similar to that of Calder et al,[9] who measured very low enrichments (0.002% to 0.09%) of $^2H_5$-PHE by GC/MS. However, we were able to validate the accuracy of our method by measuring the enrichment of our $^{13}C_6$-PHE standards by IRMS and by direct comparison of muscle protein enrichment measured by GC/MS and IRMS (Fig 5). Our approach is readily adapted to the use of $^2H_5$-PHE, as we have observed excellent chromatographic separation for biological samples (similar to Fig 2) and isotopic calibrations (similar to Fig 3) with $^2H_5$-PHE by measuring the m + 5/m + 4 ratio rather than the m + 6/m + 4 ratio (A.A. Ferrando and B.W. Patterson, unpublished observation). A practical consideration in using $^2H_5$-PHE rather than $^{13}C_6$-PHE is a 75% reduction in tracer cost.

There are a few notable differences between our method and that reported by Calder et al.[9] First, their method was specific for PHE, as it used an enzymatic decarboxylation of PHE to form phenylethylamine to eliminate chromatographic interference[9]; introduction of this enzymatic process increases the possibility of contamination of the tracer-labeled biological samples with unlabeled amino acid from extraneous sources. In contrast, our method uses the propyl ester of the carboxyl group, which is appropriate for virtually all amino acids (as we demonstrated by measuring muscle protein synthesis with $^2H_3$-LEU). Second, in the EI fragmentation of phenylethylamine previously reported, the ion chosen for measurement of m + 5 enrichment (*m/z* 104) was present only at a reduced electron energy (22 eV), thereby requiring a nonconventional tuning condition for the mass spectrometer, whereas our analysis of PHE used a conventional 70-eV EI. Finally, Calder et al[9] reported that both the slope and intercept of their standard curve for $^2H_5$-PHE were dependent on the amount of sample injected into the analyzer. In contrast, we have observed virtually no effect of concentration on our calibration curve for

$^{13}C_6$-PHE with either of two instruments examined, although we have observed concentration effects with other applications.[10] This difference is not due to instrumentation, as we have used a VG 12-250 instrument identical to that in the previous report.[9] However, the difference may be explained by the dynamic range of isotope ratios monitored by the two methods. Although both methods measure isotopic enrichment over the same range of PHE enrichment, we chose the m + 4 ion as a base for isotope ratio determinations rather than the m + 2 ion used in the previous report.[9] The m + 5/m + 2 ratio reported by Calder et al[9] varied from 0.015 to 0.15 for TTRs between 0% and 0.1% (ie, numerator and denominator peak intensities differed by 67- and 6.7-fold at the low and high end of the calibration curve, respectively). In contrast, the numerator and denominator peak intensities differ no more than 2.5-fold over the entire range of our m + 6/m + 4 calibration curve (Fig 3). Furthermore, we observed a concentration dependency for the m + 3/m + 2 ratio for the $^2H_3$-LEU standard curve, which has approximately a 10-fold difference in the minor and major peak areas (Fig 4). The narrower dynamic range of our $^{13}C_6$-PHE m + 6/m + 4 ratio reduces sources of nonlinear instrumental bias inherent to wider dynamic ranges.[11,12] A particular advantage of our method is that at TTRs typical of muscle protein PHE (~0.03%; Fig 5), the measured instrument response is approximately 1:1 (Fig 3); concentration dependencies in isotope ratios caused by nonlinearities should be minimized when the numerator and denominator signals are present at the same intensity, because a nonlinearity bias would operate identically on both signals.

In summary, very low isotopic enrichments (TTR, 0.005% to 0.10%) can be reliably measured by GC/MS with the proper choice of tracers and instrument operation conditions. Compared with a conventional approach using IRMS with ^{13}C-labeled tracers, the GC/MS approach offers faster throughput, has a lower sample requirement, and is suitable for a wider variety of tracers such as ^2H. The principles outlined here can facilitate the use of GC/MS for measuring enrichment in a variety of applications other than muscle protein synthesis when it is necessary to measure very low enrichments in studies using stable isotopically labeled tracers.

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