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Determination of Low Isotopic Enrichment of L-[1-^{13}C]Valine by Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry: a Robust Method for Measuring Protein Fractional Synthetic Rates *In Vivo*

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A method was developed for measuring protein fractional synthetic rates using the *N*-methoxycarbonylmethyl ester (MCM) derivative of L-[1-^{13}C]valine and on-line gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). The derivatization procedure can be performed rapidly and GC separation of valine from the other branched-chain amino acids, leucine and isoleucine, is easily obtained. A good lineair relationship was observed between the increment of the $^{13}C/^{12}C$ isotope ratio in CO_2 gas derived from the combustion of derivatized valine and the tracer mole ratio of L-[1-^{13}C]valine to unlabelled valine. The limit of quantitation was at an L-[1-^{13}C]valine tracer mole ratio of 0.0002. The method was used to measure the isotopic enrichment of L-[1-^{13}C]valine in standard mixtures and in skeletal muscle of six growing piglets infused with L-[1-^{13}C]valine (2 mg kg^{-1} h^{-1} for 6 h). After infusion of L-[1-^{13}C]valine the mean tracer mole ratio in plasma of L-[1-^{13}C]valine at the isotopic steady state was 0.0740 ± 0.0056 (GC/MS, mean ± SEM) and the mean tracer mole ratio of valine in muscle protein fraction at 6 h was 0.000 236 ± 0.000 038 (GC/C/IRMS). The resulting mean protein fractional synthetic rate in piglet skeletal muscle was 0.052 ± 0.007% h^{-1}, which is in good agreement with literature data obtained with alternative, more elaborate techniques. By this method protein fractional synthetic rates can be measured at low isotopic enrichment levels using L-[1-^{13}C]valine, the MCM derivative and on-line GC/C/IRMS.

KEYWORDS: stable isotopes; gas chromatography/combustion/isotope ratio mass spectrometry; amino acids; valine; protein fractional synthetic rate

INTRODUCTION

For ethical reasons, radioactive isotopes have been replaced by stable isotopes. Therefore, the use of stable isotopes in human *in vivo* studies has increased dramatically. This is also true in the area of protein synthesis and breakdown. Recently this has been extensively discussed by various authors for the field of protein metabolism in health and disease.[1] Fractional synthetic rates of proteins are now commonly determined by measuring the rate of incorporation of a stable isotope-labelled amino acid.[1] In general, the degree of labelling was found to be low.[1] Several mass spectrometric approaches have been developed to deal with the problem of analytical sensitivity.[2–5]

Using a gas chromatographic/mass spectrometric (GC/MS) approach, highly substituted tracer amino acids containing three or more labelled atoms have been used.[2,3] In these methods the authors showed that the natural abundance of the isotopomer of interest in the relevant fragment is very low. In the GC/MS measurement of isotopic enrichment, the isotopomer chosen for comparison is the natural abundance isotopomer with one mass unit less than the highly substituted isotopomer. Using such methods, the authors were able to measure small isotopic enrichments of tracer amino acid, highly substituted with stable isotopes of C or H, in protein hydrolysates.

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Another approach is to measure the enrichment of a ^{13}C-labelled amino acid in the protein hydrolysate with the aid of isotope ratio mass spectrometry (IRMS).[4,5] Until recently this approach was cumbersome and time consuming. A large sample size was necessary together with purification of the labelled amino acid from a protein hydrolysate by GC[4] or high performance liquid chromatography[5] and subsequent conversion of the labelled carbon atoms of the amino acid to CO_2, either by combustion[5] or oxidative decarboxylation of the amino acid with ninhydrin.[4]

An alternative to this method is the on-line combination of GC and an IRMS, interfaced by means of a combustion furnace (GC/C/IRMS).[6] In this method, the labelled derivatized amino acid is separated from other amino acids by GC, eliminating the need for a preparative step to isolate the amino acid of interest. The GC effluent is directed into the furnace, where organic matter is combusted to CO_2, the ^{13}C enrichment of which is measured by IRMS. In this method, the derivative should be fully combustible and should allow baseline separation of the amino acid of interest from the other amino acids in the hydrolysate. Such a GC/C/IRMS method has been described recently.[6] In this method, the enrichment of L-[1-^{13}C]leucine in muscle protein was measured using its *N*-acetyl-*n*-propyl (NAP) derivative. However, this method has several drawbacks. The derivatization procedure is a two-step method and therefore time consuming. Furthermore, the derivatization reagent adds five extra carbon atoms to the six carbon atoms of leucine. Upon combustion of the leucine derivative, the ^{13}C carbon atom of the L-[1-^{13}C]leucine tracer is diluted by 10 carbon atoms at natural abundance. Finally, baseline separation of the NAP derivative of leucine from isoleucine may be difficult to achieve.

Hušek[7] described a rapid derivatization procedure and a GC method for the determination of amino acids. Amino acids are converted into their corresponding *N*(*O*,*S*)-alkoxycarbonylalkyl esters. Complete separation of protein-derived amino acids by GC was obtained on a capillary OV-1701-type column for both the *N*(*O*,*S*)-methoxycarbonylmethyl ester (MCM) and the *N*(*O*,*S*)-ethoxycarbonylethyl ester derivatives. In this method, amino acids are derivatized in a single step. Possible problems in the separation of leucine from isoleucine can be avoided by replacement of L-[1-^{13}C]leucine with L-[1-^{13}C]valine. Furthermore, the resulting dilution of the ^{13}C by additional carbon atoms at natural abundance during combustion of the derivatized amino acid will be less for the MCM derivative of valine than for the NAP derivative of leucine (eight instead of 11 C atoms). Replacement of leucine with valine was validated and discussed by Rennie and co-workers.[8,9] They have shown that the values for muscle protein turnover as measured with L-[1-^{13}C]leucine or L-[1-^{13}C]valine were identical.

In this paper, a rapid method is presented for the determination of protein fractional synthetic rates by means of the incorporation of L-[1-^{13}C]valine and by using an MCM derivative. The method was applied to the determination of the fractional synthetic rate of muscle protein from skeletal muscle in growing piglets.

EXPERIMENTAL

Animal studies

Animal studies were approved by the Animal Ethics Committee of the University of Utrecht. Female crossbred (Landrace × Yorkshire) piglets, with an average mass of 13.9 kg (11.2–16.4 kg), were used. The piglets were fitted with arterial and venous catheters and prepared for the experiments as described previously.[10] L-[1-^{13}C]Valine (>99% MPE, MassTrace, Woburn, MA, USA) was dissolved in sterile 0.9% saline solution and the solution was sterilized by filtration through a 0.22 μm filter (Millipore, Eschborn, Germany). The tracer was administered as a primed (2 mg kg^{-1}), constant infusion (2 mg kg^{-1} h^{1} for 6 h). Heparinized blood samples for valine isotopic enrichment determinations in plasma were drawn immediately before and hourly after the start of the priming dose. Blood samples were immediately centrifuged and plasma was stored at −20 °C. At the end of the experiment the piglets were killed by injection of 1 g of sodium pentobarbital and immediately thereafter small tissue samples (10–30 mg) were removed from skeletal muscle (hind leg), frozen in liquid nitrogen and stored at −20 °C.

Isolation of amino acids

The muscle protein fraction was isolated as follows: 2 ml of 0.2 M perchloric acid (PCA) at 4 °C were added to tissue samples immediately after the samples had been taken out of the freezer and the suspensions were homogenized. Particulate material, containing denatured proteins, was precipitated by centrifugation at 1800 *g* for 20 min. Pellets were washed three times with 3 ml of 0.2 M PCA to remove remaining free amino acids. The plasma mixed protein fraction was isolated after adding 2 ml of 0.2 M PCA to an aliquot of plasma (100 μl) by centrifuging the resulting suspension at 1800 *g* for 20 min. The pellets were washed three times with 3 ml of 0.2 M PCA to remove remaining free amino acids.

Pellets containing the protein fractions were hydrolysed by adding 2 ml of 6 M HCl and incubated at 110 °C overnight in sealed tubes. An aliquot (0.5 ml) was dried under a stream of N_2 at 50 °C and the residue was resuspended in 1 ml of 0.2 M PCA.

Prior to isolation of the plasma free amino acids, plasma was deproteinized (1 ml of plasma + 2 ml of 0.2 M PCA) and the denatured protein was precipitated by centrifugation (1800 *g* for 20 min).

Amino acids were isolated from protein hydrolysates or deproteinized plasma by passing the amino acid-containing solutions through a cation-exchange column (Maxi-Clean IC-H, 0.5 ml bed volume; Alltech, Deerfield, IL, USA). These columns were conditioned prior to use by washing them twice with 3 ml of 2 M HCl. The solutions containing the isolated amino acids (500 μl) were applied to the columns. Columns were washed with 5 ml of aqua dest and the amino acids were eluted with 0.5 ml of 25% ammonia solution. The eluate was

dried under a stream of N_2 at 60 °C. The dried sample was kept at room temperature until derivatization.

Derivatization of amino acids

The MCM derivatives of amino acids were prepared according to Hušek.[7] Dried samples containing the isolated amino acids were dissolved in 100 μl of a mixture of water, methanol, acetonitrile and pyridine (60:16:16:8, v/v). Then 5 μl of methyl chloroformate were added with vigorous mixing on a Vortex mixer. Subsequently, 100 μl of chloroform containing 1% (v/v) methyl chloroformate was added and mixed vigorously on a Vortex mixer. The water and organic phases were separated by centrifugation, after which the organic phase was transferred into a GC injection vial. Within 1 week after preparation of the derivative the samples were analysed. MCM derivatives of the isotopically enriched valine standards were prepared simultaneously.

Gas chromatography/mass spectrometry

L-[1-^{13}C]valine isotopic enrichment in the free plasma amino acids was measured by capillary GC/MS. The GC/MS instrument consisted of a Hewlett-Packard 5890A Series II capillary gas chromatograph, coupled to a JEOL JMS-AX505W double-focusing mass spectrometer of normal geometry, at a resolution of 1000, using NH_3 positive-ion chemical ionization. The gas chromatograph was fitted with a capillary column (CP Sil 19CB, 25 m × 0.25 mm i.d., 0.2 μm film thickness; Chrompack, Middelburg, The Netherlands). The column was directly inserted into the ion source of the mass spectrometer. An aliquot (2 μl) of the chloroform layer containing the derivatized amino acids was injected using the split mode (1:20). The carrier gas was He (99.996%), maintained at a column head pressure of 19 psi. The injector temperature was 280 °C and the oven temperature was programmed starting at 140 °C for 2 min, then increased from 140 to 280 °C at 25 °C min^{-1} and held at 280 °C for 2 min. Valine eluted at ~4 min. The mass spectrometer was operated in the selected ion monitoring mode. The MS conditions were as follows: interface temperature, 280 °C; ion source temperature, 220 °C; ionization energy, 70 eV. Selected ion monitoring was carried out at m/z 190 and 191 ($[M + H]^+$ and $[M + H + 1]^+$ ion, respectively, with $M = 189$ for the MCM derivative of unlabelled valine, $C_8H_{15}NO_4$) with a dwell time of 100 ms for each of the selected m/z values.

On-line gas chromatography/combustion/isotope ratio mass spectrometry

L-[1-^{13}C]Valine isotopic enrichments in mixed protein hydrolysates were measured using a Finnigan MAT Delta S IRMS interfaced to a Varian 3400 gas chromatograph via a ceramic combustion furnace[11] (Finnigan MAT, Bremen, Germany). The furnace, encased in a concentric metal tube, consists of a 0.5 mm i.d. ceramic tube containing an oxidized Cu wire and Pt as a catalyst. The furnace is maintained at 800 °C, at which temperature CuO exists in equilibrium with metallic Cu and O_2, and compounds eluting into the furnace are combusted, catalysed by Pt at this temperature. The combustion gases containing CO_2 and H_2O are directed into a Nafion (DuPont) tube where H_2O but not CO_2 diffuses through the Nafion membrane. The stream of dried CO_2 in He carrier gas passes an open split from which the mass spectrometer samples continuously (split ratio 1:3).

For the measurement of the isotopic enrichment of protein-derived valine, an aliquot (2 μl) of the chloroform layer containing the derivatized amino acids was injected into the gas chromatograph fitted with a capillary column (CP Sil 19CB, 25 m × 0.32 mm i.d., 0.2 μm film thickness), using the splitless mode. The carrier gas was helium (99.996%) maintained at a column head pressure of 19 psi. The injector temperature was 275 °C and the oven temperature was programmed from 80 °C (held for 1 min) to 100 °C at 5 °C min^{-1}, then to 275 °C at 30 °C min^{-1} and held at 275 °C for 2 min. Valine eluted at ~5.7 min.

Standard mixtures

Weighed amounts of L-[1-^{13}C]valine were mixed with weighed amounts of commercially available unlabelled valine (Sigma Chemical, St Louis, MO, USA) to make serial standard dilutions with a tracer mole ratio for L-[1-^{13}C]valine ranging from 0 to 0.25 for the GC/MS analysis of plasma free valine and from 0 to 0.004 for the GC/C/IRMS analysis of protein-derived valine.

Calibration curves

For the GC/MS measurement of the isotopic enrichment of L-[1-^{13}C]valine in plasma, a calibration curve was used to convert the GC/MS peak area ratio of m/z 191 to 190 into the tracer mole ratio of L-[1-^{13}C]valine to unlabelled valine.

For the GC/C/IRMS measurement the approach was as follows. First, prior to the elution of the valine derivative from the GC column, two pulses of a CO_2 reference gas of known isotopic enrichment were introduced into the mass spectrometer. The isotopic enrichment of the different lots of the laboratory CO_2 reference gas were calibrated against a certified standard (AGA, Amsterdam, The Netherlands). Second, measured signal ratios of m/z 45 to 44 were corrected for the ^{17}O natural abundance, based on the signal at m/z 46.[12] The corrected signal ratios of derivatized valine were converted into isotope ratios of $^{13}C/^{12}C$ in CO_2 (R_{CO_2}) using a conversion factor derived from the measurement of the CO_2 reference gas. Third, calibration curves were constructed. Since the method is highly sensitive and accurate, the R_{CO_2} after combustion of derivatized unlabelled valine used for the calibration curve generally differs from the R_{CO_2} after combustion of derivatized valine in protein hydrolysates prior to L-[1-^{13}C]valine infusion. To allow the use of a calibration curve, the increment in R_{CO_2} over natural abundance (ΔR_{CO_2}) was related to the

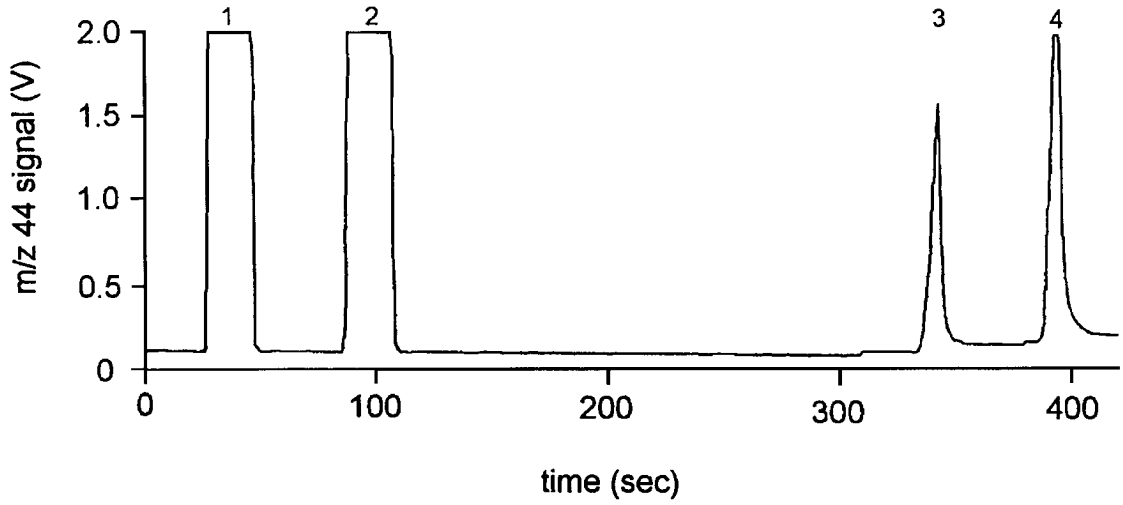

Figure 1. Typical GC/C/IRMS trace of the *m/z* 44 signal of CO_2 gas derived from the on-line combustion of the *N*-methoxycarbonylmethyl ester of valine (3) at 341 s (5.68 min), leucine + isoleucine (4) at 393 s (6.55 min) together with two CO_2 reference gas pulses (1) and (2). The CO_2 reference gas pulses are off-scale, because the scale has been adjusted to show the peaks of the amino acids more clearly.

tracer mole ratio of L-[1-^{13}C]valine to unlabelled valine. For the calibration curve, the sample used for the measurement of natural abundance was unlabelled valine. In the animal experiments, the sample used for natural abundance measurements was valine present in the hydrolysate of the plasma mixed protein fraction obtained prior to L-[1-^{13}C]valine infusion. The use of plasma mixed protein fraction for the determination of natural abundance has been validated by Nakshabendi *et al.*[13]

Calculations

In order to calculate the fractional synthetic rate of proteins, the isotopic enrichments of L-[1-^{13}C]valine in plasma and protein hydrolysates were expressed as tracer mole ratios (Z) as discussed by Cobelli *et al.*[14] The use of tracer mole ratio instead of tracer mole fraction makes it possible to link stable isotope measurements to the kinetic formalism developed for radioactive tracers. The fractional synthetic rate (*FSR*, % h^{-1}) of muscle protein in each animal was calculated according to[15]

$$FSR = \frac{Z_{\text{Val:Pr}}}{\overline{Z_{\text{Val:Pl}}}\Delta t} \times 100 \qquad (1)$$

where $\overline{Z_{\text{val:Pl}}}$ denotes the mean tracer mole ratio of L-[1-^{13}C]valine to unlabelled valine in plasma, $Z_{\text{val: Pr}}$ is the tracer mole ratio of L-[1-^{13}C]valine to unlabelled valine in muscle protein at $t = 6$ h and Δt is the duration of the experiment (6 h). $\overline{Z_{\text{val:Pl}}}$ was obtained by calculating the mean of $Z_{\text{val: Pl}}$ at the individual time points at the isotopic steady state. $Z_{\text{val: Pl}}$ at each time point was obtained by conversion of the ratio of the peak areas at *m/z* 190 and 191 to Z_{val} using the GC/MS calibration curve. $Z_{\text{val: Pr}}$ was obtained from the GC/C/IRMS calibration curve using the following equation to calculate ΔR_{CO_2}:

$$\Delta R_{CO_2} = [R_{CO_2}(t = 6)] - [R_{CO_2}(t = 0)] \qquad (2)$$

where $R_{CO_2}(t = 6)$ is the R_{CO_2} after combustion of derivatized valine in the hydrolysate of muscle mixed protein fraction at $t = 6$ h and $R_{CO_2}(t = 0)$ is the R_{CO_2} after combustion of derivatized valine in the hydrolysate of the plasma mixed protein fraction at $t = 0$ prior to infusion of L-[1-^{13}C]valine.

Statistical analysis

Data are expressed as mean, standard error of the mean (SEM) and coefficient of variation (CV). Lineair regression analysis was applied to the calibration curves.

RESULTS

In the calculation of the *FSR* by Eqn (1), measurement of the isotopic enrichment of L-[1-^{13}C]valine is necessary in both the plasma free amino acid fraction by GC/MS and the mixed protein fractions by GC/C/IRMS. Since MCM derivatives of amino acids have not been used before in isotope dilution GC/MS, the variation introduced by the instrument and the analytical day-to-day variation were estimated for the GC/MS determination of the isotopic enrichment of L-[1-^{13}C]valine as an MCM derivative. The CV of the instrumental variation was 0.47% measured by repeated analysis of a sample of the MCM derivative of unlabelled valine. The overall CV of the analytical day-to-day variation was 3.9 $\pm$ 0.3% for the GC/MS determination of the peak area ratio of *m/z* 191 to 190 over the range of tracer mole ratios of L-[1-^{13}C]valine from 0 to 0.25 over a period of several months. The CV decreased with increasing GC/MS peak area ratio of *m/z* 191 to 190 from 4.2% at $Z_{\text{val}} = 0$ to 3.5% at $Z_{\text{val}} = 0.25$. The GC/MS peak area ratio was linearly related to Z_{val} with a slope of 0.93 $\pm$ 0.02 and a y-intercept of 0.100 $\pm$ 0.003 ($r^2 = 0.9995$). The observed y-intercept was not significantly different from the theoretical value (0.0951), calculated from the natural abundance of C, H, N and O in the MCM derivative of unlabelled valine.

In Fig. 1, a typical GC/C/IRMS trace is shown of the MCM derivatives of the branched-chain amino acids of valine, leucine and isoleucine. The MCM derivative of

Table 1. Calculation of fractional synthetic rate (FSR) of leg muscle protein in growing piglets

Piglet No.	$\overline{Z_{val:Pl}}$ [a] ($n = 6$) ($\times 10^{-2}$)	$Z_{val:Pr}$ [b] ($\times 10^{-2}$)	CV (%)	Muscle protein FSR (% h^{-1})
1	7.70	0.0333	13.1	0.072
2	8.25	0.0319	4.1	0.064
3	7.74	0.0280	7.7	0.060
4	5.73	0.0107	27.1	0.031
5	5.82	0.0140	20.2	0.040
6	9.16	0.0234	2.5	0.043
Mean	7.40	0.0236		0.052
SEM	0.56	0.0038		0.007

[a] GC/MS measurement. CV = 4.9 ± 0.8%.
[b] GC/C/IRMS measurement.

valine is baseline separated from the MCM derivatives of leucine and isoleucine, which co-elute.

The variation introduced by the analytical instrument was evaluated for the values of R_{CO_2} after repeated analyses from the same vial of the MCM derivative of unlabelled valine. The value for the R_{CO_2} of the particular batch of unlabelled valine was $(1.081\,97 \pm 0.000\,16) \times 10^{-2}$, resulting in a CV of the instrument of 0.015%.

For the evaluation of the analytical day-to-day variation of the measurement of R_{CO_2} and ΔR_{CO_2}, serial standard mixtures of L-[1-^{13}C]valine with unlabelled valine at different values of Z_{val} ranging from 0 to 0.4×10^{-2} were measured over a period of several months. Prior to each measurement aliquots of the standard mixtures were derivatized. The mean CV was 0.09 ± 0.01%. The CV was independent of the R_{CO_2} in the range $1.082\,03 \times 10^{-2}$–$1.130\,22 \times 10^{-2}$. As a consequence, the day-to-day variation of the determination of ΔR_{CO_2} decreased from 25% at $\Delta R_{CO_2} = 0.001 \times 10^{-2}$ to 5% or less at $\Delta R_{CO_2} \geq 0.004 \times 10^{-2}$. Furthermore, ΔR_{CO_2} was linearly related to Z_{val} with $Z_{val} = (0.001 \pm 0.003) + (8.299 \pm 0.029)\Delta R_{CO_2}$ ($r^2 = 0.9999$).

The reproducibility of the measurement of R_{CO_2} and ΔR_{CO_2} was estimated for *in vivo* samples. Six growing piglets were infused with L-[1-^{13}C]valine. Prior to infusion, two blood samples were drawn for preparation of plasma mixed protein fraction and after 6 h of infusion two muscle biopsies were taken from each animal. Samples from each animal were processed separately in the same series. The differences in R_{CO_2} between the two plasma mixed protein samples, expressed as a percentage of the mean, varied between 0.01 and 0.08% (0.05 ± 0.01%). Similar calculations for the differences in R_{CO_2} of derivatized valine between the two muscle protein samples showed a variation between 0.02 and 0.14% (0.06 ± 0.02%). From ΔR_{CO_2}, calculated according to Eqn (2), $Z_{val:Pr}$ values were calculated for each animal, using the GC/C/IRMS calibration curve. In Table 1 the values of $Z_{val:Pr}$ and the CV are given for each animal, together with $\overline{Z_{val:Pl}}$ and the muscle protein FSR, calculated according to Eqn (1). It is clear from this table that at values of $Z_{val:Pr}$ of $\sim 0.01 \times 10^{-2}$ the CV is high ($\sim$25%) and decreases with increasing values of $Z_{val:Pr}$. The skeletal muscle fractional protein synthesis rate had a mean value of 0.052 ± 0.007% h^{-1}.

DISCUSSION

The results show that the rapid MCM derivatization method of Hušek[7] for amino acids could be applied successfully for the quantification of the tracer mole ratio of L-[1-^{13}C]valine in protein hydrolysates by on-line GC/C/IRMS at low enrichments. Furthermore, for the range of tracer mole ratios tested, the MCM derivative can be used for the determination of the isotopic enrichment of L-[1-^{13}C]valine by GC/MS. Valine was chosen as a tracer amino acid. Baseline separation of the MCM derivative of leucine from that of isoleucine could not be achieved owing to the hardware requirements for the GC/C/IRMS measurements.

From the presented data, a limit of quantification (LOQ) of Z_{val} can be estimated for GC/C/IRMS, by calculating the minimum difference of R_{CO_2} over natural abundance which can be determined with 95% confidence. According to Whitby *et al.*,[16] this minimum difference equals 2.8SD, where SD is the standard deviation of the analytical day-to-day variation. At natural abundance with $R_{CO_2} = 1.082\,03 \times 10^{-2}$ and CV = 0.09%, this results in a minimum value for $\Delta R_{CO_2} = 0.002\,73 \times 10^{-2}$. Using the GC/C/IRMS calibration curve this results in $Z_{val} = 0.02 \times 10^{-2}$. From the *in vivo* data presented in Table 1, it is clear that, above the LOQ, Z_{val} can be measured with an acceptable CV < 15%. Below the LOQ the CV increases considerably and as a consequence samples have to be analysed repeatedly to obtain a reasonable estimation of Z_{val} at these very low isotopic enrichments of L-[1-^{13}C]valine.

The LOQ of the tracer mole ratio of L-[1-^{13}C]valine by GC/C/IRMS is well below the LOQ of the tracer mole ratio of L-[1-^{13}C]valine by capillary GC/MS. In our hands, for capillary GC/MS the LOQ of the MCM derivative of L-[1-^{13}C]valine is at $Z_{val} = 0.8 \times 10^{-2}$. The application of GC/C/IRMS leads to an improvement in the LOQ by a factor of 40. When highly substituted tracer amino acids are being used, LOQs were observed with GC/MS[2,3] similar to those obtained in this study with singly substituted ^{13}C-labelled amino acids and GC/C/IRMS.

From the data presented, it is clear that the LOQ of the tracer mole ratio of L-[1-^{13}C]valine is determined by the analytical day-to-day variation of the measurement of R_{CO_2}. The instrumental error explains only a minor part of the observed limit. The mean analytical day-to-day variation of 0.09% is almost one order of magnitude larger than the instrumental error of $\sim$0.01%.

Yarasheski *et al.*[6] suggested that dilution of the ^{13}C by carbon atoms at natural abundance of the derivatized amino acid is a major determinant of the observed LOQ. The use of a smaller precursor amino acid and a derivative adding less carbon atoms was thought to be preferable. A comparison of the present data with those published by Yarasheski *et al.*[6] shows, however, that the observed reproducibilities were not different when using the MCM derivative of valine (eight C atoms) instead of the NAP derivative of leucine (11 C atoms). They observed an analytical day-to-day variation above 15%

at L-[1-^{13}C]leucine enrichments $\leqslant$0.02 atom% excess (L-[1-^{13}C]leucine tracer mole ratio = 0.019×10^{-2}) and $\leqslant$3% at or above values of 0.05 atom% excess (L[1-^{13}C]leucine tracer mole ratio = 0.048×10^{-2}). This indicates that dilution of the ^{13}C atom by carbon atoms at natural abundance of the amino acid derivative might not be a major reason. Also, the constant absolute error irrespective of the tracer mole ratio of L-[1-^{13}C]valine to unlabelled valine contradicts this. The large difference between the instrumental variation and the analytical variation indicates that sample preparation is a major determinant. This most likely explains the large difference in precision that Yarasheski *et al.*[6] observed between the on-line GC/C/IRMS method and an off-line method combining preparative GC, ninhydrin-induced decarboxylation and dual-inlet IRMS. The off-line method involves a major clean-up of the sample with isolation of the leucine from the protein hydrolysate and the production of CO_2 gas by the ninhydrin-induced decarboxylation. Furthermore, in this procedure the use of the dual-inlet IRMS adds to a smaller CV in comparison with the on-line method in which continuous-flow IRMS is used.

The calculated value *FSR* for leg muscle protein synthesis in growing piglets compared well with those published previously.[17,19] Because of the low level of isotopic enrichment of L-[1-^{13}C]valine observed in muscle protein, comparison of the GC/MS and GC/C/IRMS methods on the same samples was not possible. For an estimate of the accuracy of the method for the calculation of *FSR* of muscle protein, the data of Watt *et al.*[18] can be used. They performed similar experiments on growing piglets using a primed continuous infusion protocol with L-[1-^{13}C]leucine. They measured the isotopic enrichments of L-[1-^{13}C]leucine in the plasma free amino acid fraction by GC/MS and in leg muscle protein by dual-inlet IRMS after isolation of leucine from the protein hydrolysate and decarboxylation with ninhydrin. The calculated value for *FSR* of leg muscle protein was $0.041 \pm 0.004\%\ h^{-1}$, which is in good agreement with the value reported in this study, $0.052 \pm 0.007\%\ h^{-1}$.

In conclusion, capillary GC/C/IRMS analysis of the valine-MCM derivative in protein hydrolysates offers a rapid method for measuring the tracer mole ratio of L-[1-^{13}C]valine to unlabelled valine in proteins after a primed continuous infusion of L-[1-^{13}C]valine. This method permits the calculation of the fractional synthetic rate of muscle protein isolated from small ($\sim$20 mg) tissue samples, compatible with a needle biopsy.

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