ELSEVIER

Journal of Chromatography B, 710 (1998) 37–47

JOURNAL OF CHROMATOGRAPHY B

Determination of $\delta^{13}C$ values of valine in protein hydrolysate by gas chromatography–combustion isotope ratio mass spectrometry

W. Kulik*, J.A.N. Meesterburrie, C. Jakobs, K. de Meer

University Hospital Vrije Universiteit, Department of Clinical Chemistry, Metabolic Laboratory, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

Received 18 November 1997; received in revised form 12 February 1998; accepted 13 February 1998

Abstract

A gas chromatographic–combustion isotope ratio mass spectrometric (GC–C-IRMS) method for the determination of [1-^{13}C]valine enrichments in protein hydrolysates is described. Using a quick derivatization method, $\delta^{13}C$ values of the N-methoxycarbonyl methyl ester of valine can be determined from baseline separated GC peaks. Evaluation studies with respect to precision, accuracy, linearity, reduction capacity of the CuO combustion furnace and isotope dilution as a result of derivatization, showed that our GC–C-IRMS system allows robust measurement of enrichments of [1-^{13}C]valine in the range 0 to 1.5 MPE (S.D.±0.01 MPE, $n=3$). Therefore this method is suited to determine fractional synthetic rates (FSRs) of proteins as low as one-tenth of the FSR of human albumin, in studies using a primed, continuous (6 h) infusion with [1-^{13}C]valine plasma enrichments of approximately 15 MPE and an hourly sampling schedule.

Keywords: $\delta^{13}C$ values; Valine

1. Introduction

For an estimation of the rate of protein synthesis in vivo, on basis of infusions of stable isotope labelled amino acids, generally two different approaches are in use. One method calculates rates of whole body protein synthesis by determining the difference between the flux of an individual free amino acid and oxidation of this amino acid e.g. [1–6]. This method requires assumptions with respect to the relation between tracer amino acid and amino acid composition of body proteins and results in different flux rates for different amino acids. The other method determines the rate of incorporation of a labelled amino acid (usually [1-^{13}C]leucine) into a specific protein with known amino acid sequence, resulting in an estimation of the fractional synthetic rate (FSR) of this protein, e.g. [7–15]. In the latter case enrichment of the amino acid tracer used as precursor is usually determined in derivatized plasma samples with gas chromatography–mass spectrometry (GC–MS). The incorporation of the precursor into protein is obtained by measuring the enrichment of the labelled amino acid in hydrolysate of the isolated and purified protein. For proteins with a slow production half-life generally low enrichments are found (0.001–0.05 atom percentage excess) in the hydrolysate. This requires the use of isotope ratio mass spectrometry (IRMS) [7]. By

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00090-5

coupling a gas chromatograph directly to the IRMS via an on-line combustion furnace it is possible to measure low enrichments with a high precision using small sample size [16,17]. This allows the measurement of FSR values of protein with a low production half-life (e.g. human albumin: FSR=0.25%/h [18,19], using acceptable precursor enrichments at relatively low tracer costs. In this paper we have focused on the description and validation of a GC–C-IRMS method for the determination of [1-^{13}C]valine enrichments in human albumin hydrolysate. We discuss precision, accuracy, linearity, interferences of nitrogen oxides, the effect of isotope dilution as a result of derivatization and a working method.

Many derivatization methods of amino acids have been described for GC. We use the method of Hušek [20] because it is a quick derivatization method; it results in a gas chromatogram in which valine is well separated from the other amino acids; and only three carbon atoms are added by derivatization by which the dilution of the $^{13}CO_2$ after combustion will be minimal. We demonstrate that for human albumin low ^{13}C-enrichments of valine can still be measured after a brief primed, continuous infusion period, within acceptable limits of expansion of the plasma valine pool by the tracer.

2. Experimental

2.1. Instrumentation

A schematic of the GC–C-IRMS system (VG Optima Isochrom, Micromass, Manchester, UK) employed for the determination of δ^{13}C values of CO_2 is shown in Fig. 1. Individual compounds were separated chromatographically and by closing the heart split (HS) valve, only selected parts of the effluent were introduced via a heated interface into the combustion furnace. By opening the HS valve, the other part of the chromatographic effluent was diverted to the flame ionization detector (FID). From the effluent stream of combustion gases H_2O was removed by a cryogenic trap. A portion of the remaining effluent stream (mainly He, CO_2 and possibly N_2) was introduced into the ion source through a capillary. Reference CO_2 gas (of known isotopic composition) was delivered from a reference gas injection system and was measured before and after the introduction of the combustion gases.

2.2. Gas chromatography

The instrument was equipped with a HP 5890A gas chromatograph (series II, Hewlett-Packard, Palo

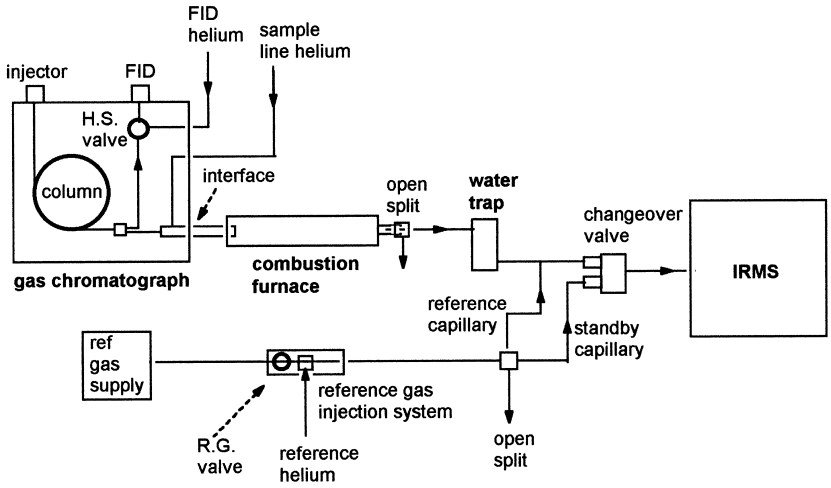

Fig. 1. Schematic presentation of the GC–C-IRMS system.

Alto, CA, USA). Samples were injected using a heated split/splitless injector (T=290°C). A CP-SIL-19CB, 25 m×0.25 mm (I.D.), 0.20 μm (film thickness) column (Chrompack International, Middelburg, Netherlands) was used for analyses of valine in protein hydrolysate and standards with helium as a carrier gas (100 kPa). Typically, 1 μl of sample was injected with a splitflow of 15 ml/min. The GC oven was programmed to increase from 80°C (1 min) to 100°C at a rate of 5°C/min and to increase from 100°C to 290°C at a rate of 30°C/min where its temperature was kept constant for 5 min. The FID was at a constant temperature of 340°C. For the combined analyses of valine and its dipeptide a CP SIL-8CB/MS, 30 m×0.25 mm (I.D.), 0.25 μm (film thickness) column (Chrompack International) was used. For these analyses the GC oven was programmed from 70°C (1 min) to 300°C at a rate of 25°C/min. The column was kept at 300°C for 10 min. Other conditions were similar.

The GC column was connected, via a splitter union, to the combustion furnace via a heated interface at 350°C.

2.3. Combustion

The GC effluent entered the combustion furnace which consisted of a quartz tube (600 mm×0.7 mm I.D.×6 mm O.D.) partly filled with copper granulate oxidized to cupric oxide (CuO). At the end of the quartz tube over a length of 1 cm it was filled with silver wool. The combustion oven was kept at 850°C. Water was removed from the combustion gases by passing the gas stream through a cryogenic trap which was kept at a temperature of −100°C.

2.4. Mass spectrometer

CO_2 entered the ion source of the mass spectrometer through a capillary. Ions were generated by electron impact ionization with an electron energy of 100 eV. The mass spectrometer was operated with an accelerating voltage of 3.5 kV and a magnetic current of approximately 3.3 A. Ion currents were measured continuously for m/z 44, 45 and 46 using a set of three Faraday cups. The (analogue) ion currents were integrated and digitized at intervals of 100 ms. The resulting (chromatographic) peak areas were integrated and background corrected. Using Craig corrections [21], ^{13}C content was calculated and, using the standard convention, expressed as $\delta^{13}C$ values (‰) relative to Peedee Belemnite (PDB). The sample CO_2 was measured against a laboratory CO_2 working standard calibrated against NBS 19 reference material.

2.5. Chemicals

Methylchloroformate (MCF) was obtained from Merck-Schuchhardt (Hohenbrunn, Germany), pyridine and methanol from Merck (Darmstadt, Germany) and chloroform from J.T. Baker (Deventer, Holland). L-Valine was purchased from Fluka (Buchs, Switzerland) and L-[1-^{13}C]valine (98.9 APE) from Cambridge Isotope Laboratories (Andover, USA).

2.6. Protein hydrolysate

Plasma albumin samples were obtained from a healthy volunteer, using a primed, continuous infusion of [1-^{13}C]valine (98.9 APE); enrichment in plasma at plateau approximately 12 APE. The infusion protocol, sampling procedure, isolation and hydrolysis of the human albumin samples have been described elsewhere [22]. The dried samples of protein hydrolysate used in the present study, originated from 400 μg protein isolated from 0.5 ml of serum.

2.7. Derivatization

Valine was converted to its N-methoxycarbonyl methyl ester (Fig. 2) according to the procedure of Hušek [20]. Dried protein hydrolysate and valine standards were dissolved in 100 μl of a mixture of water–methanol–acetonitrile–pyridine (60:16:16:8, v/v). A 5 μl volume of MCF was added and mixed by briefly shaking the reaction vial. The resulting derivatized compounds were extracted with 100 μl chloroform containing 1% MCF. The derivatization and extraction process can be performed within a minute per sample, allowing GC–C-IRMS processing on the same day.

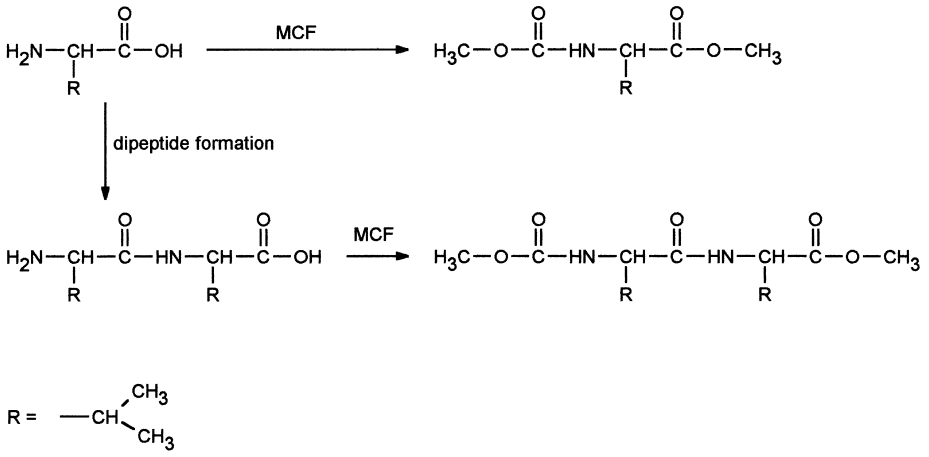

Fig. 2. Derivatization of valine (and its dipeptide) to the N-methoxy methyl ester.

The extent of dipeptide formation depended on the total amount of amino acids in the reaction mixture. In the preparation of valine standards the formation of Val–Val was enhanced by exceeding the limit of 200 μg per 100 μl of reaction mixture.

2.8. Standards

Standards were prepared by weighing an amount of L-[1-^{13}C]valine followed by addition of a weighed amount of L-valine to obtain the required enrichment. The combined mixture was dissolved completely in (heated) water. Thereafter the mixture was dried, the resulting powder was stored at room temperature. This procedure was repeated to obtain independently standards with different [1-^{13}C]valine enrichments in the range between 0 and 2 mol percent excess (MPE). Small portions of these standard mixtures were derivatized and stored in chloroform at 4°C under which conditions the samples proved to be stable. Standard solutions of approximately 10 nmol/μl were used.

2.9. Calculations and presentation of results

The δ per mil value (δ^{13}C ‰) is related to the ^{13}C/^{12}C ratio of the samples and reference by the formula

$$\delta^{13}C_{sample} = (^{13}C/^{12}C\text{sample} - {}^{13}C/^{12}C\text{standard})/(^{13}C/^{12}C\text{standard}) \times 1000 \quad (1)$$

where the standard is referenced to the international PDB standard; its CO_2 obtained from the carbonate shell of a cretaceous mollusc, *Belmnitella americana*, from the Pee Dee formation in South Carolina. The δ^{13}C value can be transformed to ^{13}C atom percentage (AP) with the formula

$$AP = 100 \cdot R[(\delta^{13}C_{sample}/1000) + 1]/1 + R[(\delta^{13}C_{sample}/1000 + 1] \quad (2)$$

where R is the ^{13}C/^{12}C ratio of the international PDB reference ($R = 0.0112372$)

Values are expressed as mean±standard deviation (S.D.).

3. Results and discussion

3.1. Procedure

Fig. 3 shows a representative GC–C-IRMS chromatogram from a derivatized albumin hydrolysate with the collector signal of m/z 44. After identification of the peak corresponding to valine, valves were programmed in such a way that only the GC effluent with well separated valine entered the combustion furnace. Oven and helium flow restrictions were adjusted to obtain a maximum yield of CO_2 in the mass spectrometer. Before and after the 'valine peak' pulses of CO_2 reference gas were measured. The intensity of the signal resulting from the reference

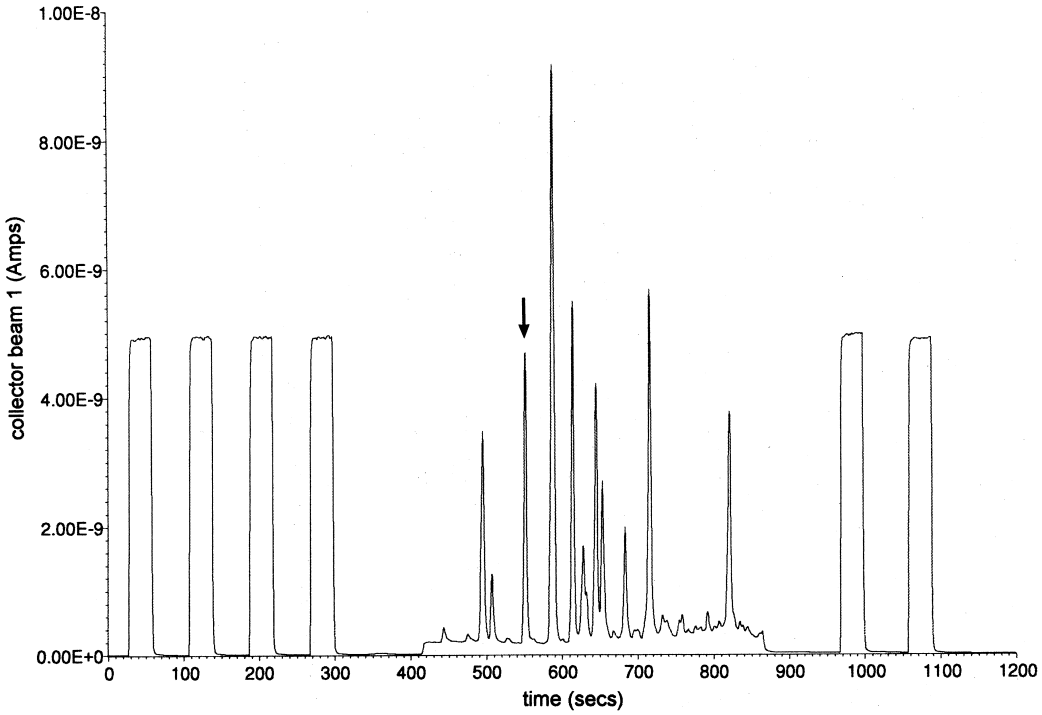

Fig. 3. Typical GC–C-IRMS chromatogram from a derivatized human albumin hydrolysate with the collector signal for m/z 44.

pulses was adjusted at similar ion beam intensities to the ion beam intensity of valine. The optimized GC–C-IRMS chromatogram, depicted in Fig. 4 shows a well separated symmetrical valine peak.

3.2. Linearity

In order to establish which range of valine concentrations were allowed for GC–C-IRMS analyses, we evaluated measured $\delta^{13}C$ values as a function of the collector signal of beam 1 for m/z 44 (see Fig. 5). Two standards ($\delta^{13}C = -36.1$‰ and $\delta^{13}C = +182.0$‰) were injected repeatedly. By variation of the split ratio, the collector signal of beam 1 (m/z 44) was varied between $0.5 \cdot 10^{-9}$ and $10 \cdot 10^{-9}$ A. The light valine standard started to show stable $\delta^{13}C$ values with a collector beam 1 signal $> 0.5 \cdot 10^{-9}$ A ($\delta^{13}C = -36.1 \pm 0.1$‰, $n = 10$). The heavy valine standard showed constant $\delta^{13}C$ values between $1.6 \cdot 10^{-9}$ and $7.4 \cdot 10^{-9}$ A ($\delta^{13}C = 182.0 \pm 0.2$‰, $n = 5$). Therefore $\delta^{13}C$ values of samples were only accepted if the collector beam signal was within the range $2 \cdot 10^{-9}$ to $7 \cdot 10^{-9}$ A.

3.3. Precision

The standard deviations, characteristic of the distribution of $\delta^{13}C$ values observed after n repeated injections, of both standards and hydrolysates, are summarized in Table 1 (hydrolysates correspond to samples taken during plateau enrichment in plasma at $t = 1$ h, $t = 5.5$ and $t = 7.5$ h). From these data it follows that the $^{13}CO_2/^{12}CO_2$ ratio, or atom percentage (AP) of the N-methoxycarbonylmethyl ester can be determined with a coefficient of variation (C.V.) $< 0.030\%$; as a result the mol percentage (MP) of [1-^{13}C]valine can be determined with a C.V. $< 0.045\%$. This means that with respect to the within-day precision [1-^{13}C]valine enrichment can be determined ± 0.003 MPE.

Single point measurements of both hydrolysates and standards, repeated on 3 different days, showed that in the range of measured values of $\delta^{13}C =$

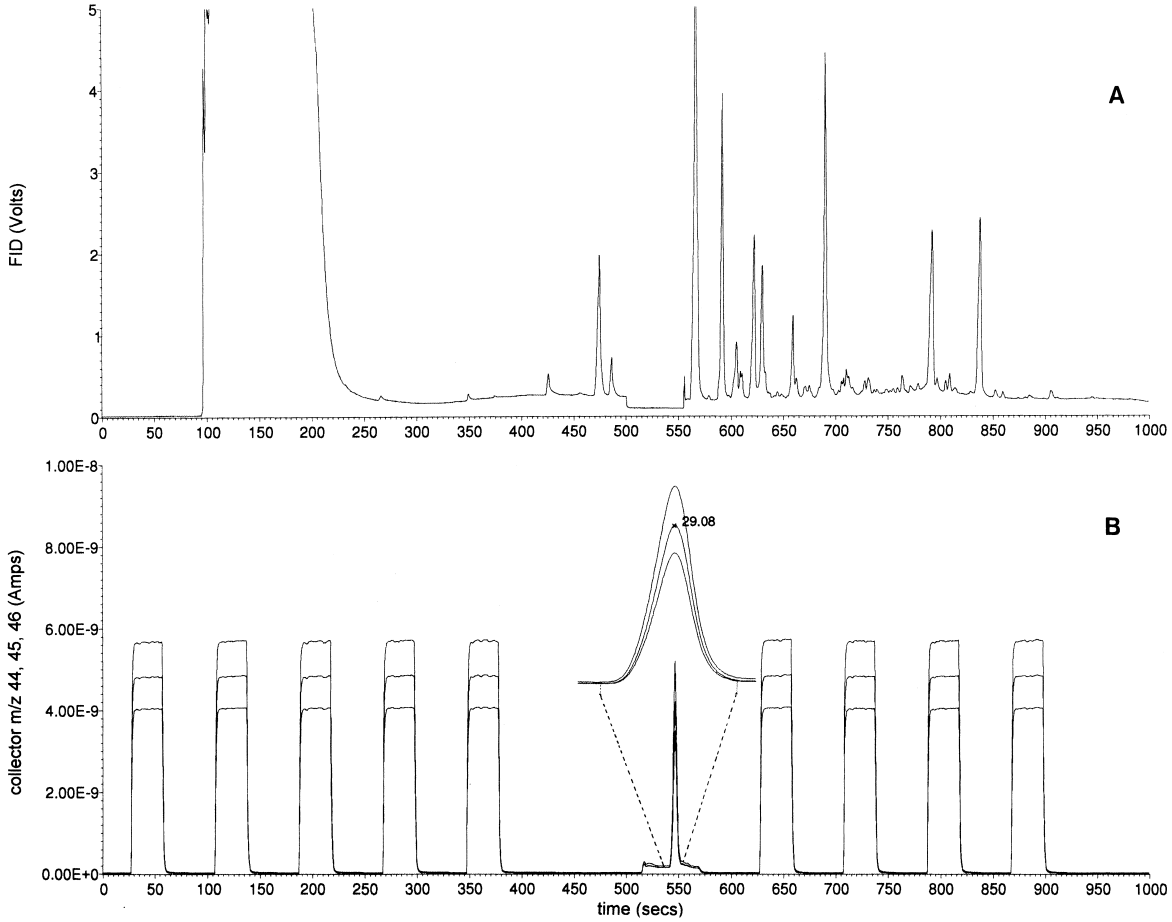

Fig. 4. Optimized GC–C-IRMS chromatogram of albumin hydrolysate for the determination of $\delta^{13}C$ values of valine with signals for the FID (panel A) and collector signals for m/z 44, m/z 45 and m/z 46 (panel B).

−32‰ to +147‰, AP of the N-methoxycarbonylmethyl ester can be determined with a C.V.< 0.052%; as a result the MP of [1-^{13}C] valine can be determined with a C.V.<0.078%. This means that with respect to the between-day precision [1-^{13}C]valine enrichment can be determined ±0.005 MPE.

3.4. Accuracy

As a measure of the accuracy of the GC–C-IRMS system we determined the difference between the MPE value of valine obtained from the GC–C-IRMS measurements and the MPE values of valine calculated from the weighed amounts of valine at natural abundance and [1-^{13}C]-enriched valine (98.9 APE).

In Table 2 the mean results (±S.D.) of five series of separate, single point, measurements are given, using calibration based upon L-valine of known natural abundance. The weighing procedure accounts for standards with a weighing error ±0.005 MPE and differences between calculated and measured values for independently prepared standards are close to zero and within analytical error boundaries (95% confidence interval). Therefore, it seems probable that the observed differences originate mainly from limitations in the preparation of derivatized stan-

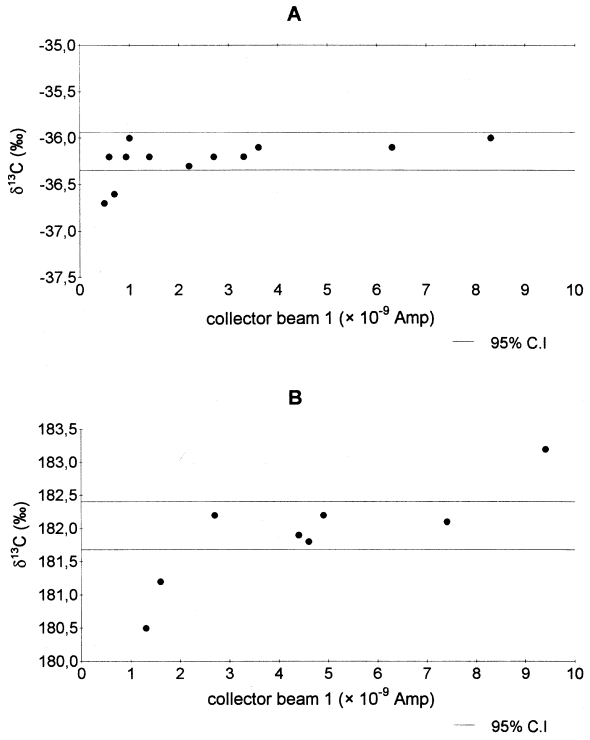

Fig. 5. δ^{13}C values as a function of the collector signal for m/z 44 of two valine standards (Panel A: −36.1‰; Panel B: 182.0‰).

Table 1
Precision of δ^{13}C values (‰) at different levels of enrichment (n repetitive injections)

δ^{13}C±S.D.	n	Sample
−32.57±0.03	2	Hydrolysate
−11.64±0.14	3	Hydrolysate
−1.28±0.14	4	Hydrolysate
−34.33±0.15	4	Standard
−1.30±0.22	4	Standard
23.31±0.25	4	Standard
81.86±0.33	4	Standard
147.52±0.30	4	Standard

Table 2
Differences between calculated and measured enrichments of [1-C^{13}]valine standards (injections: $n=5$)

Weighted[a] MPE±S.D.	Measured MPE±S.D.	Difference (mean)
0.515	0.5078±0.0034	0.0060
1.016	1.0184±0.0031	−0.0028
1.586	1.5912±0.0035	−0.0050

[a] Estimated error due to microbalance±0.005 MPE.

dards. Even when the highest difference is used, accuracy is at least ±0.006 MPE.

3.5. Memory effect

For samples within the acceptable collector beam 1 signal range no memory effect was observed. If consecutive samples with different δ^{13}C values <150‰ were measured alternately the mean δ^{13}C values did not deviate from mean δ^{13}C values after repeated measurement of the same sample.

3.6. Nitrogen oxide gases

Depending on combustion oven conditions, it was noted that during the combustion process nitrogen containing compounds, like valine, are converted to CO_2, H_2O and/or nitrogen oxide gases (N_xO_y) [23–25]. H_2O is removed from the combustion gases by the water trap (−100°C). However, nitrogen oxide gases cannot be trapped separately and have to be removed by reduction to N_2. Removal of N_xO_y is necessary because N_2O produces ions at m/z 44 and m/z 45 which interfere with the measurement of the $^{12}CO_2$ and $^{13}CO_2$ ions. Since the instrument is not equipped with an on-line reduction furnace it is crucial that the conditions of the combustion furnace are optimized for both oxidation and reduction, because otherwise too much NO_2 will be formed resulting in either too low δ^{13}C values in case of valine with natural nitrogen or too high δ^{13}C values in case of ^{15}N-labelled (tracer) valine. Thus, measurements were optimized (for O_2-pressure, temperature, limits of sample size) to gain sufficient reducing power of the combustion furnace, using traces of elemental copper and silver wool at the end of the reactor. It was demonstrated that δ^{13}C values for CO_2 of valine are free from N_xO_y interference under optimized conditions (see Appendix A). These findings are in agreement with other reports. Beylot et al. [24] stated that the GC–C-IRMS analyses of the dimethylaminomethylene derivative of urea (containing four nitrogen atoms per seven carbon atoms) can be performed with a similar CuO combustion furnace without interferences of nitrogen oxides. Merritt et al. [25] concluded also that a normal CuO combustion furnace (without silver wool) has reducing power: 2 nmol of derivatized (^{15}N-labelled) alanine

and glycine (N-acetyl, *n*-propyl) was found to produce enough N_2 to obtain reliable $\delta^{15}N$ values.

3.7. $\delta^{13}C$ values of the constituent C atoms in derivatized valine

The mean $\delta^{13}C$ value of the C atoms in underivatized valine can be calculated on basis of the stoichiometric mass balance [26]:

$$\delta^{13}C_{measured} = (5/8 \cdot \delta^{13}C_{valine}) + (3/8 \cdot \delta^{13}C_{derivatization}) \quad (3)$$

where $\delta^{13}C_{measured}$ is the measured $\delta^{13}C$ value of the eight C atoms in derivatized valine, $\delta^{13}C_{valine}$ is the mean $\delta^{13}C$ value of the five C atoms of underivatized valine and $\delta^{13}C_{derivatization}$ is the mean $\delta^{13}C$ value of the three C atoms introduced by derivatization. The literature concerning $\delta^{13}C$ values of derivatized and underivatized amino acids, suggests that $\delta^{13}C_{derivatization}$ values have to be regarded as a $\delta^{13}C$ value of a putative effective stable carbon isotope composition: in the $\delta^{13}C_{derivatization}$ value an assumed isotopic fractionation factor has been incorporated which is not related to the actual carbon isotope composition of the reagent [26]. The constancy of the derivatization fractionation however, allows that the original amino acid stable carbon isotope composition can be derived from the measured $\delta^{13}C$ values of the derivatized amino acid. Thus $\delta^{13}C_{derivatization}$ can be determined empirically. This $\delta^{13}C$ value can be determined in two ways: (i) from the on-line GC–C-IRMS measurement of derivatized valine, if the $\delta^{13}C$ value of underivatized valine is known; and (ii) from GC–C-IRMS measurements of derivatized valine, and its derivatized dipeptide from the same valine source solving two mass balance equations with two unknowns for $\delta^{13}C_{valine}$ and $\delta^{13}C_{derivatization}$ (see Appendix B). The results of these two approaches are summarized in Table 3. Although the results of both empirical methods show close agreement, precision is better for method (i). If facilities for off line oxidation–reduction are not available, method (ii) can be used to establish $\delta^{13}C$ values of the constituting C atoms of derivatized valine.

Table 3
$\delta^{13}C$ values of the constituent C atoms in derivatized valine[a]

Method	$\delta^{13}C_{valine} \pm$S.D. (‰)	$\delta^{13}C_{derivatization} \pm$S.D. (‰)
(i)	-27.9 ± 0.2	-45.7 ± 0.2
(ii)	-28.0 ± 1.3	-45.3 ± 0.6

[a] Determined by two methods (i) off-line preparation of valine (ii) amino acid vs. dipeptide. See Appendix B for full description of procedures.

3.8. Standard curves for practical use

For applications, i.e. when determination of ^{13}C-enrichment above baseline (e.g natural abundance) is the point of interest, $\delta^{13}C_{derivatization}$ is not required. Since the latter value is assumed to be a constant value (within a series of measurements) it cancels out from equations for the measurement of differences [27]. For the same reason the $\delta^{13}C$ value for valine at natural abundance ($\delta^{13}C_{valine,b}$) is not a requirement.

In this case, a standard curve of calculated [1-^{13}C]valine enrichments (MPE) vs. measured $\delta^{13}C$ values can be used. A representative standard curve is depicted in Fig. 6.

Given the strong linear relationship between theoretical and measured enrichments (for the example in Fig. 6: $n=6$; $r^2=0.999$) we use as a working method only four standards with known enrichments. From the measured $\delta^{13}C$ values we calculate a mean 'effective' $\delta^{13}C_{derivatization}$. Based upon this value,

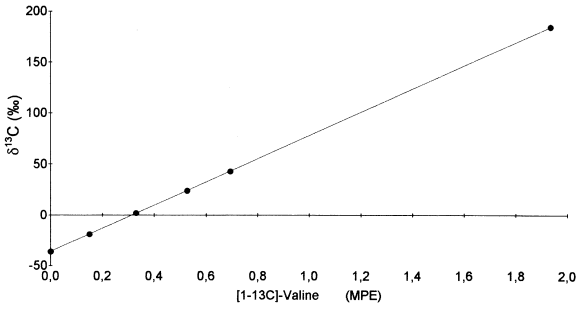

Fig. 6. Representative standard curve of calculated [1-^{13}C]valine enrichments (MPE) vs. measured $\delta^{13}C$ values of single point analyses (regression: $y=114.07x-36.12$‰; $r^2=0.999$).

$\delta^{13}C$ values of samples are converted to atom percentages and MPE values for [1-^{13}C]valine.

3.9. Application

Based upon the reported precision and accuracy, we are confident that the measured enrichment values for [1-^{13}C]valine can be determined within ± 0.01 MPE. This is generally regarded to be within limits of biological fluctuation.

Therefore the method described is suited to determine FSR values of proteins which are 1/10 of the normal FSR of albumin in healthy human volunteers (0.25%/h, [18,19]) using an hourly sampling scheme for 6 h at precursor enrichment plateau of approximately 15 MPE.

4. Conclusion

We conclude that enrichment of [1-^{13}C]valine in albumin hydrolysate can be determined reliably by determination of $\delta^{13}C$ values of CO_2 generated from its N-methoxycarbonyl methyl ester using a GC–C-IRMS system. The Hušek derivatization procedure is quick, allowing sample derivatization and processing with GC–C-IRMS on the same day. After optimization of conditions of the gas chromatography and the CuO combustion furnace, we found that enrichments in the range of 0 to 1.5 MPE of [1-^{13}C]valine can be determined with an accuracy and precision better than 0.01 MPE. For practical purposes, calibration curves using four standards can be used without loss of robustness of the method.

Acknowledgements

We are grateful to Mrs. M. de Sain-van der Velden for providing the samples of hydrolysed human albumin. The authors are grateful to Dr. D.-J. Reijngoud and Dr. F. Stellaard for proposing the use of valine and the derivatization protocol of Hušek for the measurement of protein turnover.

Appendix A

Reduction capacity of CuO combustion furnace

The cupric oxide (CuO) furnace of our GC–C-IRMS system derives it reducing power from silver wool at the end of the reactor and traces of elemental copper in the oven. In order to be sure that conditions were optimal for accurate CO_2 measurements, we carried out the following experiments.

Without precautions against formation of N_xO_y, valine was oxidized in an off-line CuO combustion furnace under nonoptimized conditions ($T=900$°C, without silver wool, high O_2 pressure). The combustion gases were collected. A sample of these gases was measured by IRMS in the dual inlet mode under conditions for a normal CO_2 measurement. The same sample of these gases was reduced with silver wool ($T=450$°C, 0.5 h) and thereafter its $\delta^{13}C$ value was also measured with IRMS.

Repeated measurements showed a small but significant change from $\delta^{13}C = -28.22 \pm 0.02$‰ to $\delta^{13}C = -27.93 \pm 0.04$‰ without and with silver wool respectively ($P=0.0004$; $n=3$). Since the ratio for m/z 45: m/z 44 for N_20 (0.0078) is smaller than the m/z 45: m/z 44 ratio for CO_2 (0.012), any presence of N_2O will decrease the $\delta^{13}C$ value. Optimal removal of N_2O by reduction increases the $\delta^{13}C$ value measured; scanning the mass range m/z 25–m/z 35 showed also a decrease of the intensity of m/z 30 (NO^+ as fragment of $N_2O^{+\cdot}$) after reduction; the origin of the interference was confirmed by repeating the experiment using ^{15}N-valine, in which case $\delta^{13}C$ values are decreased after reduction [$\delta^{13}C = -13.63$ (before reduction); $\delta^{13}C = -21.10$‰ (after reduction)].

Hence, with precautions against formation of N_xO_y, oven parameters were adjusted for on-line measuring conditions and no shift in the measured $\delta^{13}C$ values was observed resulting from the sequential reduction process. Scanning of the mass range m/z 25–m/z 35 did not show increased intensities for fragments of nitrogen oxide.

On-line measurement of derivatized valine over the acceptable concentration range for collector beam 1 showed no significant change in the $\delta^{13}C$ value.

These results indicate, under these conditions, the oxidation–reduction capacity of the oven was not exceeded.

Appendix B

Experiments for the determination of $\delta^{13}C$ values of the constituent C atoms

Method (i)

The off-line oxidation (and reduction) of underivatized valine, followed by IRMS measurement in the dual inlet mode gives the value of $\delta^{13}C_{valine}$. The on-line GC–C-IRMS measurement of derivatized valine gives the value of $\delta^{13}C_{measured}$. From Eq. (1) for the mass balance, the value for $\delta^{13}C_{derivatization}$ is derived.

From two repeated series of experiments with valine at natural abundance we found $\delta^{13}C_{valine} = -27.9 \pm 0.2$‰ and $\delta^{13}C_{derivatization} = -45.7 \pm 0.2$‰.

The same experiment was performed with tracee valine enriched with [1-^{13}C]valine. In this case, we measured after off-line oxidation and reduction $\delta^{13}C_{valine} = 55.5 \pm 0.3$‰, from the measured value for the derivatized valine we calculated $\delta^{13}C_{derivatization} = -45.6 \pm 0.2$‰. Both values of $\delta^{13}C_{derivatization}$ calculated from method (i) experiments are in close agreement.

Method (ii)

On-line measurements of derivatized valine and the derivatized dipeptide of valine (Val–Val) from the same source were conducted, as these should give the same results as those obtained with method (i). The incorporation of C atoms resulting from derivatization is assumed to be the same for valine and its dipeptide (we observed no fractionation in the $\delta^{13}C$ values of valine as a result of dipeptide formation). Dipeptide formation from [1-^{13}C]valine enriched valine will result in a dipeptide with the same mean $\delta^{13}C$ value for its C atoms as the original valine sample. This results in two mass balance equations with two unknowns, which can simply be solved for $\delta^{13}C_{valine}$ and $\delta^{13}C_{derivatization}$:

$$\delta^{13}C_{valine} = 13/5 \cdot \delta_2 - 8/5 \cdot \delta_1 \quad (4)$$

$$\delta^{13}C_{derivatization} = 16/3 \cdot \delta_1 - 13/3 \cdot \delta_2 \quad (5)$$

in which δ_1 and δ_2 stand for the measured $\delta^{13}C$ value of the derivatized valine and its dimer respectively. These equations can simply be generalized for other amino acids.

We performed experiments with four standard solutions with different [1-^{13}C]valine enrichments (range 0 to 1.6 MPE). Each sample contained both derivatized valine and its dipeptide. From the four sets of δ_1, δ_2 values we calculated from Eqs. (4) and (5): $\delta^{13}C_{derivatization} = -45.3 \pm 0.6$‰. For $\delta^{13}C_{valine}$ we calculated values which corresponded with the expected ^{13}C-enrichments of the standards. Since the [1-^{13}C]valine enrichment of these standards was known, the $\delta^{13}C$- value of unenriched valine (i.e. valine at natural abundance) could be calculated at $\delta^{13}C_{valine} = -28.0 \pm 1.3$‰.

It should be noted that the $\delta^{13}C_{derivatization}$ can vary with different batches of derivatization chemicals.

References

[1] G.N. Thompson, P.J. Pacy, H. Merritt, G.C. Ford, M.A. Read, K.N. Cheng, D. Halliday, Am. J. Physiol. 256 (1989) E631–E639.
[2] D.E. Matthews, K.J. Motil, D.K. Rohrbaugh, J.F. Burke, V.R. Young, D.M. Bier, Am. J. Physiol. 238 (1980) E473–E479.
[3] K.J. Storch, D.A. Wagner, J.F. Burke, V.R. Young, Am. J. Physiol. 258 (1990) E790–E798.
[4] S.C. Denne, E.M. Rossie, S.C. Kalhan, Pediatr. Res. 30 (1991) 23–27.
[5] S.E. Daley, A.D. Pearson, A.W. Craft, J. Kernahan, R.A. Wyllie, L. Price, C. Brock, C. Hetherington, D. Halliday, K. Bartlett, Arch. Dis. Child. 75 (1996) 273–281.
[6] M. Krempf, R.A. Hoerr, L. Marks, V.R. Young, Metabolism 39 (1990) 560–562.
[7] P. Balagopal, G.C. Ford, D.B. Ebenstein, D.A. Nadeau, K.S. Nair, Anal. Biochem. 239 (1996) 77–85.
[8] K.S. Nair, D. Halliday, R.C. Griggs, Am. J. Physiol. 254(2) (1988) 17.
[9] A. Egerland, J.P. Reynier, O. Ballevre, J. Dicostanzo, C. Obled, M. Arnal, Clin. Chim. Acta 252 (1996) 51–60.
[10] K.E. Yarasheski, K. Smith, M.J. Rennie, D.M. Bier, Biol. Mass Spectrom. 21 (1992) 486–490.

[11] K.N. Cheng, P.J. Pacy, C. Hicks, G.C. Ford, H. Merritt, D. Halliday, Proc. Nutr. Soc. 47 (1988) 54A.
[12] E.J. Barrett, J.H. Reukin, L.H. Young, B.L. Zaret, R. Jacob, R.A. Gelfand, J. Clin. Invest. 80 (1987) 1–6.
[13] P.J. Pacy, K.N. Cheng, G.N. Thompson, D. Halliday, Ann. Nutr. Metab. 33 (1989) 65–78.
[14] W.H. Hartl, H. Demmelmair, K.W. Jauch, H.L. Schmidt, B. Koletzko, F.W. Schildberg, Am. J. Physiol. Endocrinol. Metab. 35 (1997) E796–E802.
[15] B.W. Patterson, X.J. Zhang, Y.P. Chen, S. Klein, R.R. Wolfe, Met. Clin. Exp. 46 (1997) 943–948.
[16] D.E. Matthews, J.M. Hayes, Anal. Chem. 50 (1978) 1465–1473.
[17] A. Barrie, W.A. Coward, Biomed. Mass Spectrom. 12 (1985) 535–541.
[18] K. Smith, S. Downie, J.M. Barua, P.W. Watt, C.M. Scrimgeour, M.J. Rennie, Am. J. Physiol. 266 (1994) E640–644.
[19] O.S. Olufemi, P. Humes, P.G. Whittaker, M.A. Read, T. Lind, D. Halliday, Eur. J. Clin. Nutr. 44 (1990) 352–361.
[20] P. Hušek, J. Chromatogr. 552 (1991) 289–299.
[21] H. Craig, Geochim. Cosmochim. Acta 12 (1957) 133–149.
[22] M.G.M. de Sain-van der Velden, G.A. Kaysen, K. de Meer, F. Stellaard, H.A.M. Voorbij, H.A. Koomans, D.J. Reijngoud, T.J. Rabelink, Kidney International 53(1) (1998) 181–188.
[23] W.W. Wong, D.L. Hachey, S. Zhang, L.L. Clarke, Rapid Commun. Mass Spectrom. 9 (1995) 1007–1011.
[24] M. Beylot, Y. Khalfallah, S. Normand, V. Large, H. Brunengraber, Biol. Mass Spectrom. 23(8) (1994) 510–513.
[25] D.A. Merritt, J.M. Hayes, J. Assoc. Soc. Mass Spectrom. 5 (1994) 387–397.
[26] J.A. Silver, M.H. Engel, S.A. Macko, E.J. Jumeau, Anal. Chem. 63 (1991) 370–374.
[27] Y. Khalfallah, S. Normand, S. Tissot, C. Pachiaudi, M. Beylot, J.P. Riou, Biol. Mass Spectrom. 22 (1993) 707–711.