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Determination of extracellular and intracellular enrichments of [1-^{13}C]-α-ketoisovalerate using enzymatically converted [1-^{13}C]-valine standardization curves and gas chromatography–mass spectrometry

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Abstract

We report a validated method for the determination of extra- and intracellular [1-^{13}C]-α-ketoisovalerate ([1-^{13}C]-KIV) enrichments by gas chromatography–mass spectrometry. Standardization curves were prepared by enzymatic oxidation of [1-^{13}C]-valine enriched standards of known composition. Slopes of [1-^{13}C]-valine standardization curves (mean±SD: 0.99±0.02, n=5) and [1-^{13}C]-KIV standardization curves (mean±SD: 0.98±0.01, n=7) were not significantly different. The method was applied for the determination of [1-^{13}C]-KIV enrichments in plasma and tissues during [1-^{13}C]-valine infusion in a piglet. [1-^{13}C]-KIV enrichment could be determined±0.1 MPE (C.V. 1%), and extracellular [1-^{13}C]-KIV enrichment was a reliable estimate of intracellular (skeletal muscle, bone growth plate) [1-^{13}C]-KIV enrichment. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: α-Ketoisovalerate; Valine

1. Introduction

In humans and in experimental animals protein metabolism on the whole body level has been studied with stable isotope labelled amino acids using blood samples; more recently production of hepatic-derived proteins has been measured. In order to determine fractional synthetic rates of specific plasma proteins, with stable isotope infusion methods, it is important to measure enrichments of labelled intracellular precursors accurately [e.g. 1–8]. The keto-acid of valine, α-ketoisovalerate (KIV), is formed exclusively from transamination of intracellular valine, and enrichment of [1-^{13}C]-KIV in the accessible (plasma) pool is assumed to reflect [1-^{13}C]-valine enrichments in tissues.

The use of [1-^{13}C]-valine as a tracer for the measurement of the production of specific proteins has been shown to be reliable [9] and several applications have been published [e.g. 5,7]. KIV can be analysed by different established methods using mass spectrometry [e.g. 10–13]. For accurate measurement of [1-^{13}C]-KIV enrichments it is necessary

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to use a reliable [1-^{13}C]-KIV calibration curve. The preparation of accurate [1-^{13}C]-KIV standards depends on the availability of (labelled) compounds with exactly known chemical purity, but these are not available from commercial or non-profit sources. Therefore, a simple and cost-effective way to prepare independent standards is by enzymatic conversion of chemically pure (and more stable) [1-^{13}C]-valine and unlabelled valine, which are readily available from commercial sources.

This paper describes the method of reliable determination of [1-^{13}C]-KIV enrichments from converted [1-^{13}C]-valine standards, and the applicability of the method in a [1-^{13}C]-valine tracer infusion in a biological model.

2. Experimental

2.1. Animal experiments

Piglets (female crossbred Landrace×Yorkshire; 10 kg average weight) were studied in the fasting ($t=-90$ to $t=240$ min) and the fed state ($t=240$–480 min) with a primed (21 μmol kg^{-1}) continuous intravenous infusion of [1-^{13}C]-valine (21 μmol kg^{-1} h^{-1}). Blood samples were taken at regular intervals from an arterial catheter. Isotopic enrichment in plasma of KIV was used as an indicator for intracellular precursor enrichment during protein synthesis. Based on pilot experiments, in the fed state the rate of infusion was adjusted to flatten out the effect of increased exogenous/endogenous tracee amino acid. Piglets were terminated at the end of the experiments by injection of 1 g of Pentothal. Using this piglet model, we showed that the plasma precursor enrichment was constant over the time period between $t=150$ min until the termination at $t=480$ min. Details of the model and study results are described elsewhere [8]. For the present study, plasma samples were obtained before administration of the isotopes ($t=0$) and just before termination ($t=480$ min) in one piglet. Immediately thereafter (<20 min after death), tissues (hindleg skeletal muscle, liver, humerus growth plate) were sampled, frozen with liquid nitrogen and stored at −80°C until analysis.

2.2. Instrumentation

Samples were analysed by gas chromatography–mass spectrometry (GC–MS) using a HP Engine (Hewlett-Packard, Palo Alto, CA, USA) which consisted of a 5890-series II gas chromatograph (GC) and a 5989B mass spectrometer (MS). The heated split/splitless injector (280°C) was connected via a precolumn of non-polar fused-silica, 1 m×0.20 mm (I.D.) (Supelco, Bellefonte, PA, USA) with a CP Sil 19 CB, 25m×0.25 mm (I.D.)×0.20 μm (film thickness) GC-column (Chrompack International, Middelburg, The Netherlands). Helium was used as carrier gas with a constant column pressure of 0.7 bar. Typically 1 μl of sample was injected with the GC programmed to increase from 80°C (1 min) to 180°C with a rate of 15°C min^{-1}, to 240°C with a rate of 8°C min^{-1} and with 30°C min^{-1} to 300°C where the temperature was kept constant for 3 min.

The MS was operated with an interface temperature of 290°C and a source temperature of 300°C for KIV analyses and 200°C for valine analyses using chemical ionization (CI) conditions with NH_3 as reaction gas. The KIV derivatives were monitored at m/z 303.19 and m/z 304.19; the valine derivatives were monitored at m/z 190.11 and m/z 191.11 using dwell times of 70 ms.

2.3. Chemicals

L-Valine was purchased from Fluka (Buchs, Switzerland), L-[1-^{13}C]-valine (98.9 APE) from Cambridge Isotope Laboratories (Andover, USA); L-amino acid oxidase and *o*-phenylene diamine (OPDA) from Sigma (Zwijndrecht, The Netherlands); *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) from Pierce (Rockford, IL, USA).

Methyl chloroformate (MCF) was obtained from Merck–Schuchhardt (Hohenbrunn, Germany); pyridine, hexane, methanol, ethanol, anhydrous sodium sulphate, tris(hydroxymethyl) aminomethane from Merck (Darmstadt, Germany); chloroform and hydrochloric acid (36–38%) from J.T. Baker B.V. (Deventer, The Netherlands); ethyl acetate from Vel N.V. (Leuven, Belgium). All chemicals were of analytical grade.

The anion-exchange resin AG 1-X8 (100–200 mesh, chloride form) was purchased from BioRad Laboratories (Richmond, CA, USA).

2.4. Derivatization

Valine was analysed as its *N*-methoxycarbonyl methyl ester according to the procedure of Hušek [14]. Valine standards and biological residues were dissolved in 200 μl of a mixture of water–methanol–acetonitrile–pyridine (60–16–16–8, v/v). 10 μl MCF was added and mixed by briefly shaking the reaction vial. The resulting derivatized compounds were extracted with 200 μl chloroform containing 1% of MCF.

KIV was analysed as the *O*-*t*-butyldimethylsilyl-quinoxalinol derivative based on the procedure of Rocchiccioli et al. [10]. Plasma samples of 250 μl were deproteinized with 2 ml ethanol. After separation by centrifugation the ethanol layer was evaporated to dryness (50°C, N_2). Tissue samples (0.2 g) were pulverized, homogenized and deproteinized [15,16] with ethanol (4 ml). After 10 min the mixture was centrifuged and the ethanolic layer was evaporated to dryness (N_2, 50°C).

The biological residues were dissolved in 1 ml of a freshly prepared *o*-phenylene diamine (OPDA) solution (1% in 2 *M* hydrochloric acid) and heated for 1 h. at 90°C. The solution was extracted with 4 ml ethyl acetate and dried with anhydrous sodium sulphate. After separation by centrifugation, the dried solution was evaporated to dryness (40°C, N_2). The residue was derivatized with 40 μl of a 3:1 mixture of MTBSTFA–pyridine (15 min, RT). The resulting mixture was diluted with 100 μl hexane.

2.5. Preparation of standards

Independent valine standards were prepared by repeatedly weighing an amount of L-[1-^{13}C]-valine followed by addition of a weighed amount of unlabelled L-valine to obtain the required enrichments (expressed in mole percent excess (MPE): 0%, 2%, 4%, 6%, 9%, 13%, 16%). To obtain a homogeneous mixture it was dissolved in (heated) water. Thereafter the mixture was dried and the resulting white powder was stored at room temperature.

[1-^{13}C]-KIV enriched standards were prepared by enzymatic conversion of the previously prepared [1-^{13}C]-valine enriched standards. A sample of each valine standard was dissolved in 500 μl Tris buffer (=tris(hydroxymethyl) aminomethane buffer; 1 *M*, pH=7.7) and 50 μl L-amino acid oxidase solution (6 mg ml^{-1} Tris buffer). The mixtures were incubated for 1.5 h at 37°C. The resulting KIV standards were converted to the quinoxalinol derivative in a similar way as described for the biological samples (0.5 ml of OPDA, 2% in 4 *M* hydrochloric acid was added to the incubated mixture). Thereafter standards were stored at 4°C. Shortly before GC–MS analyses quinoxalinol standards were converted to the TBDMS derivatives. For GC–MS analyses the concentrations of the analyte in standard solutions were levelled with sample concentrations.

3. Results and discussion

3.1. Linearity

Typical calibration curves of ^{13}C-enriched KIV and valine expressed in MPE(%) are shown in Fig. 1; for the working range of 0–16 MPE good linear relationships were obtained ($r=0.9995\pm0.0003$; $n=7$ and $r=0.9994\pm0.0003$; $n=5$ respectively).

Measured on separate days over a period of half a year, the slopes of the linear regression lines of KIV, enzymatically prepared from the valine standards, and valine were not significantly different ($n=5$; 0.98 ± 0.01 and 0.99 ± 0.02 respectively; paired *t*-test; NS).

3.2. Precision and accuracy

Standard deviations, characteristic for the distribution of enrichment values after *n* repeated injections, of standards, plasma and piglet tissue (skeletal muscle, humerus growth plate) at different levels of enrichment are summarized in Table 1.

With respect to within-day precision, ratios of areas under the curve in the ion-chromatograms were determined with a C.V. $<0.25\%$ ($n=3$). As a result,

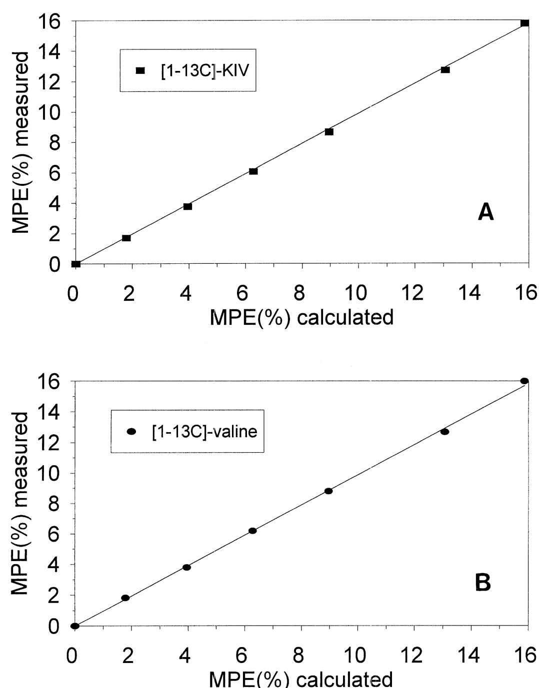

Fig. 1. Representative calibration curves for [1-^{13}C]-KIV (A) and [1-^{13}C]-valine (B), calculated vs measured values in MPE (%).

[1-^{13}C]-KIV enrichment could be determined with a within-day precision<0.1 MPE.

Measurement of standards in the working range of 0–16 MPE and biological samples on different days, over a period of six months, showed between-day precision for [1-^{13}C]-KIV enrichment equal to or less than ±0.16 MPE (n=7) for standards and plasma samples; equal to or less than ±0.18 MPE (n=3) for tissue samples.

As a measure of accuracy for the measurement of [1-^{13}C]-KIV enrichment we determined the difference between MPE of [1-^{13}C]-KIV and MPE of [1-^{13}C]-valine established for corresponding standards (Table 2). Differences between corresponding [1-^{13}C]-KIV and [1-^{13}C]-valine enrichments were <0.1 MPE and not significant (paired t-test, NS).

Table 1
Within-day precision for [1-^{13}C]-KIV[a] enrichments at different levels of enrichment (n repetitive injections) in analytical standards and biological materials

MPE (%)±SD	n	Sample
0.00±0.07	4	Standard
3.86±0.06	3	Standard
6.00±0.07	3	Standard
12.28±0.08	3	Standard
0.00±0.06	4	Piglet plasma (t=0)
9.54±0.09	3	Piglet plasma (t=480 min)
9.52±0.11	3	Piglet skeletal muscle

[a] [1-^{13}C]-KIV enrichments in piglet plasma and skeletal muscle were obtained before (t=0) and after (t=480 min) intravenous administration of [1-^{13}C]-valine during 8 h.

3.3. Stability

The O-t-butyldimethylsilyl-quinoxalinol derivative of KIV in standards and plasma, stored under nitrogen at room temperature, showed a half-life time ranging between 1 and 4 months. The quinoxalinol derivative of KIV and the N-methoxycarbonyl methyl ester of valine did not show any decay over a period of 1 year.

3.4. Degree of conversion

The degree of enzymatic conversion of valine standards, for the conditions described, was determined. Several converted standards were analysed for remaining valine relative to valine present in corresponding non-converted valine standards. These experiments showed a mean degree of conversion of 83%±4%, n=3.

In principle, for the preparation of [1-^{13}C]-KIV enriched standards, the relative yield was not very important; the ratio between [1-^{13}C]-KIV and KIV remained the same since labelled and unlabelled compounds were mixed before the enzymatic conversion.

Table 2
Differences between measured enrichments of the corresponding [1-^{13}C]-KIV and [1-^{13}C]-valine standards (separate analyses: n=5)

[1-^{13}C]-KIV MPE(%)±SD	[1-^{13}C]-Val MPE(%)±SD	Mean difference MPE(%)±SE
1.76±0.11	1.85±0.12	−0.09±0.07
3.81±0.14	3.90±0.11	−0.09±0.08
8.80±0.11	8.86±0.13	−0.06±0.08
12.80±0.16	12.73±0.09	0.07±0.08
16.01±0.10	16.10±0.08	−0.10±0.06

Table 3
[1-^{13}C]-KIV and [1-^{13}C]-valine enrichments in plasma and tissues during in vivo [1-^{13}C]-valine infusion in piglet

	Plasma	Skeletal muscle	Bone growth plate	Liver
KIV (MPE)	9.54±0.11	9.57±0.18	9.53±0.17	N.D.
Valine (MPE)	[a]N.D.	8.35±0.14	8.95±0.14	9.75±0.15

[a] Results are means±SD (n=3 separate analyses); N.D.=not determined.

3.5. Biological measurements

Measurements of [1-^{13}C]-KIV enrichments during the in vivo [1-^{13}C]-valine infusion are summarized in Table 3. It showed that [1-^{13}C]-KIV plasma enrichment was in close agreement with [1-^{13}C]-KIV tissue enrichment. Liver [1-^{13}C]-valine enrichment was also in agreement with plasma [1-^{13}C]-KIV enrichment, but muscle and bone samples showed 6–13% lower [1-^{13}C]-valine enrichments. These results support the assumption that [1-^{13}C]-KIV plasma enrichment is in isotopic equilibrium with the intracellular compartments. Valine enrichments show variations between the tissues and this cannot be attributed to analytical error. Biological explanations (e.g. post mortem proteolysis) may be put forward for the lower [1-^{13}C]-valine enrichments in the muscle and bone samples.

In the method used by Schadewaldt et al. [17], L-amino acid dehydrogenase was used to analyse serum branched chain amino acids as the corresponding 2-oxo acids, suggesting that values for ^{13}C-enrichments were not affected by enzymatic conversion. This hypothesis was supported by our findings that corresponding [1-^{13}C]-KIV and [1-^{13}C]-valine enrichments in the standards were not significantly different. The valid preparation of independent [1-^{13}C]-valine enriched standards, based on weighed amounts of labelled/unlabelled valine [9], allowed the use of the enzymatically prepared

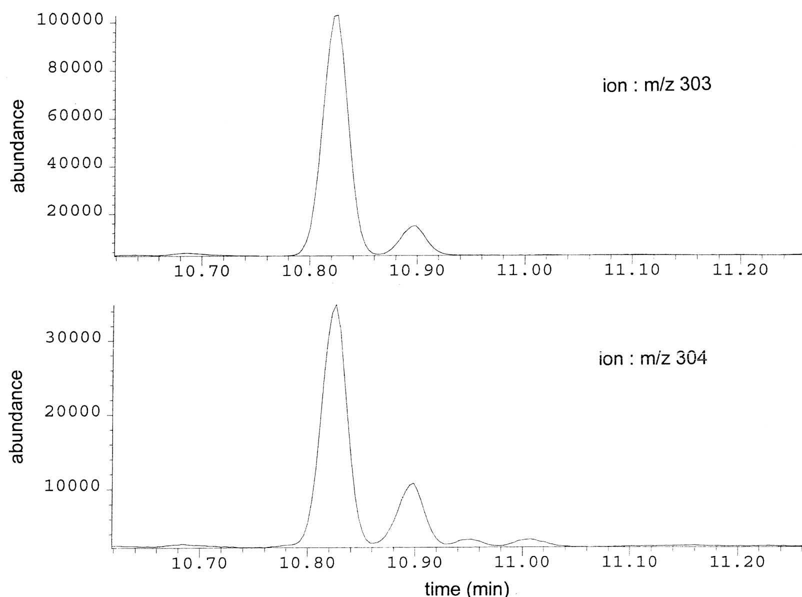

Fig. 2. Typical ion-chromatogram (m/z 303; m/z 304) for the O-t-butyldimethylsilyl-quinoxalinol derivative of KIV in skeletal muscle of a piglet after 8 h of intravenous [1-^{13}C]-valine infusion.

[1-^{13}C]-KIV calibration curves in the present study. This method is rapid and inexpensive.

For handling convenience, we used plasma samples of 250 μl. The signal to noise ratio of these samples analysed ($S/N>1000$ for the m/z 304 signal at natural abundance, end volume sample 140 μl) allows miniaturization of the method. Tissue sample sizes of 0.2 g resulted in lower but sufficient signal to noise ratios ($S/N>50$ for the m/z 304 signal at plateau enrichment, end volume 140 μl) under identical instrumental conditions (see Fig. 2).

4. Conclusion

The ^{13}C-enrichments in standards did not significantly change by enzymatic conversion of [1-^{13}C]-valine into [1-^{13}C]-KIV. Thus, the possibility of a measurable isotope effect from L-amino acid oxidase was excluded over the enrichment range (0–16 MPE) reported here. As a result, reliable [1-^{13}C]-valine standards can be used for the preparation of a [1-^{13}C]-KIV calibration curve for accurate determination of [1-^{13}C]-KIV enrichments in biological fluids (SD<0.1 MPE).

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