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Determination of Low ^{13}C-Glutamine Enrichments Using Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry

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Glutamine is an essential fuel for tissues with high rates of cell replication, such as enterocytes and lymphocytes. Infusion of ^{13}C-labeled glutamine tracers allows for measurement of the rates of production, utilization and oxidation of glutamine's carbon skeleton *in vivo*. The use of this tracer, however, has been limited by its high cost and/or the difficulty in measuring low enrichments in biological fluids using conventional gas chromatography–mass spectrometry (GC/MS) techniques. We have developed a method using gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) that allows for the determination of low ^{13}C enrichments (down to 0.06 mol.% excess) with a precision of 2% or better, and a within-day and between-day variability better than 5%, in plasma free glutamine. The method was applied to measuring the incorporation of ^{13}C in plasma glutamine over the course of infusion of ^{13}C-labeled acetate in a human subject. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Glutamine comprises more than 60% of the free amino acid pool in skeletal muscle. Maintenance of intracellular glutamine concentration plays an instrumental role in protein homeostasis, and glutamine becomes essential under conditions of critical illness.[1] In addition, glutamine plays a prominent role in energy metabolism, since (a) glutamine is the main fuel selected by cells with high rates of replication, such as lymphocytes[2] and enterocytes,[3] (b) glutamine is a major source of carbon for gluconeogenesis[4] and (c) glutamine may affect lipolysis.[5,6]

Infusion of ^{13}C-labeled glutamine tracers allows for measurement of the rates of production, utilization and oxidation of glutamine's carbon skeleton.[7,8] The use of this tracer, however, has been hampered by its high cost and/or the difficulty in measuring low (<0.5 mol.% excess) enrichments in biological fluids using conventional gas chromatography–mass spectrometry (GC–MS) techniques. Because it combines ease of separation of specific substrates in a complex matrix, a characteristic of GC–MS, with the ability to determine low ^{13}C enrichments in pure CO_2, a characteristic of isotope ratio mass spectrometry (IRMS), gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) seems, in theory, ideally suited to determine low ^{13}C enrichments in plasma amino acids. On the other hand, because most derivatization procedures add carbon atoms to the molecule, the analysis of an amino acid as CO_2 obtained from its combustion results in a severalfold dilution of the labeled carbon. For instance, in the case of a singly labeled glutamine tracer the label is diluted by a total of 12 carbons (four from other carbon atoms in the glutamine molecule plus eight from derivatization to NAP-glutamate). This could result in a significant loss in the ability of GC–C–IRMS to measure low ^{13}C enrichments in derivatized glutamine. Despite this carbon dilution, GC–C–IRMS has been shown to allow for detection of low levels of enrichments in other substrates such as glucose[9] or leucine.[10,11] Yet to our knowledge the use of GC–C–IRMS had not been tested in the case of glutamine.

The method described in this paper uses GC–C–IRMS to measure low ^{13}C-glutamine enrichments in human or animal plasma.

EXPERIMENTAL

Analytical methods

Five hundred microliters of plasma were deproteinized with 300 μl of ice-cold 10% (w/v) sulfosalicyclic acid

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and centrifuged at 3000 rev min^{-1} for 20 min at 4 °C. Glutamine was then separated from plasma glutamate by anion exchange chromatography as previously described.[12] Briefly, the protein-free supernatant was neutralized with 120 μl of 1 M KOH, diluted with 3 ml of pH 9 buffer (Prolabo) and applied to disposable plastic columns containing 2 ml of a 50/50 slurry of an anion exchange resin (AG1X8, 200–400 mesh, formate form, Biorad) in water. The eluate was collected and the column was further washed with 4 ml of pH 9 buffer. The collected glutamine fraction was then acidified to pH < 3 with 700 μl of 1 M HCl and applied to disposable plastic columns containing 1 ml of a 50/50 slurry of a cation exchange resin (AG50X8, 100–200 mesh, hydrogen form, Biorad) in water. Columns were rinsed with 10 ml of distilled water, and glutamine was then eluted with 2 ml of 3 M NH_4OH.

The glutamine-containing eluate was subsequently dried under nitrogen and derivatized to its *N*-acetyl, *n*-propyl (NAP)-ester derivative with slight modifications of the previously described procedure.[12,13] Each dried fraction was first spiked with 600 μl of a 1/5 (v/v) mixture of acetylchloride with ice-cold propanol and incubated at 110 °C for 30 min. Each sample was then cooled, evaporated under a stream of nitrogen, spiked with 400 μl of a 5/2/1 (v/v/v) mixture of acetone/triethylamine/acetic anhydride and incubated for 5 min at 70 °C. The derivatized glutamine was then dried under nitrogen and dissolved in 300 μl of ethylacetate until analysis of GC–C–IRMS. The choice of the NAP derivatization procedure was dictated by (a) the need for a very stable derivative that can be stored for weeks and (b) the fear that other convenient derivatives (such as fluoride-containing derivatives[11]) might shorten the lifespan of the combustion furnace in our specific system.

One microliter of the NAP-glutamine sample was injected into a gas chromatograph (5890 series II®, Hewlett–Packard) equipped with an Ultra-1® (25 m × 0.32 mm i.d., 0.17 μm film thickness, Hewlett–Packard) capillary column in the splitless mode. The temperature was set at 80 °C for 1 min, then ramped at 10 °C min^{-1} up to 200 °C and finally at 30 °C min^{-1} up to 250 °C. The eluted peak was transferred on-line to a 940 °C copper oxide combustion oven and the resulting CO_2 subsequently transferred to an on-line magnetic sector isotope ratio mass spectrometer (Delta-S®, Finnigan-Mat, Bremen, Germany) after water had been removed by passing through a Nafion membrane. The GC eluant was diverted to the IRMS approximately 200 s after the injection. The $^{13}CO_2/^{12}CO_2$ isotope ratio was determined from monitoring of the ions at *m/z* 44, 45 and 46.

A standard curve was prepared from mixtures of natural L-glutamine with L-[1-^{13}C]glutamine (99% ^{13}C, Cambridge Isotope Laboratories, Andover, MA, USA) at known enrichments between 0 and 0.25 mol.% excess (MPE).

Calculations

The isotopic abundance in ^{13}C is given by the system expressed as δ‰ versus the international standard (Pee Dee Belemnite, V-PDB) as follows:

$$\delta^{13}C‰ = 1000 \times (R_j - R_{PDB})/R_{PDB}$$

where R_j and R_{PDB} are the $^{13}C/^{12}C$ isotope ratios in sample j and the V-PDB international standard (0.011 237 2) respectively. Thus

$$R_j = R_{PDB}[1 + (0.001 \times \delta^{13}C‰)]$$

The observed ^{13}C fractional abundance (F_j, at.%) was calculated as $F_j = 100 \times R_j/(1 + R_j)$. Thus the observed ^{13}C enrichment in sample j (E_j, at.% excess) was $E_j = F_j - F_0$, where F_0 is the observed fractional abundance of ^{13}C in the GC–C–IRMS analysis of natural glutamine. The relationship between expected plasma ^{13}C-glutamine enrichment (mol.% excess) and observed E_j in the standards was described by a straight line ($y = kx + y_0$; see Fig. 2). Plasma ^{13}C-glutamine enrichment at time t (E_tGLN, mol.% excess) was therefore calculated as $E_t\text{GLN} = (E_t CO_2 - E_0CO_2)/k$, where $E_t CO_2$ and $E_0 CO_2$ are the enrichments observed in the GC–C–IRMS analysis of plasma glutamine at time t and time zero respectively and k is the slope of the standard curve.

In vivo experiment

After obtaining (a) approval from the Ethical Committee at the University of Nantes Medical Center and (b) informed consent from the subject, a 165 min, unprimed, continuous infusion of [1-^{13}C]acetate (99% ^{13}C, Isotec, Plaisir, France; 30 μmol kg^{-1} h^{-1}) was administered to a single human volunteer in the post-absorptive state through a short catheter placed in a superficial vein of the forearm. A second catheter was inserted in a superficial vein of the contralateral hand. The hand was warmed to about 50 °C with a heating pad to obtain arterialized venous blood at times 120, 135, 150 and 165 min. Blood samples were processed for measurement of ^{13}C-acetate enrichment by GC–MS as previously described[14] and of ^{13}C-glutamine enrichment by GC–C–IRMS as described above.

RESULTS

As shown in Fig. 1, the CO_2 peak resulting from the combustion of either natural glutamine (top) or plasma glutamine (bottom) was free of any interfering peak under the conditions used in the GC–C–IRMS system. Peaks of adequate amplitude could be obtained using injection volumes ranging from 0.2 to 1 nmol of glutamine.

Figure 2 displays a typical standard curve in which the measured enrichment in CO_2 resulting from the combustion of glutamine is plotted against the expected ^{13}C-glutamine enrichment in the standard solution (both expressed in mol.% excess). Enrichments as low as 0.02 mol.% excess could be detected and the calibration curve was linear over the range of enrichments tested. Obviously, the slope was much lower than unity, since singly labeled glutamine was analyzed after combustion of the entire molecule to CO_2 in the GC–C–IRMS

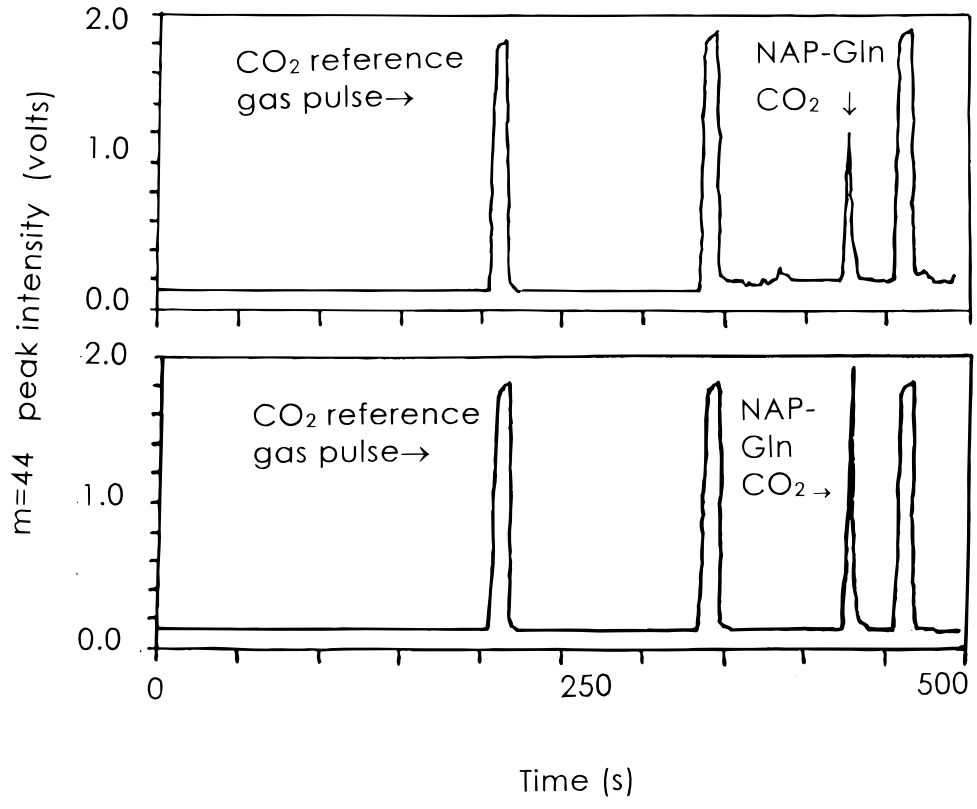

Figure 1. Typical mass spectrometer tracing for CO_2 gas (mass 44) obtained from combustion of NAP-glutamine isolated from an aqueous standard solution (bottom) and a sample of human plasma (top), as analysed by gas chromatography–combustion–isotope ratio mass spectrometry.

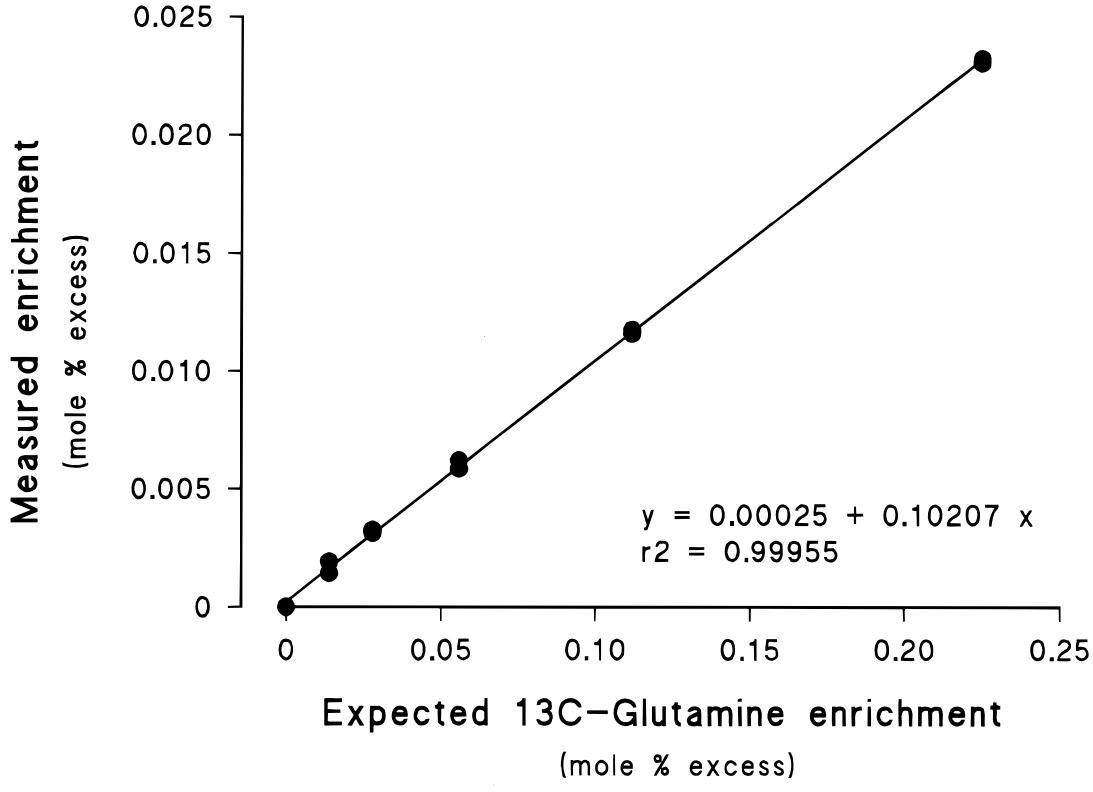

Figure 2. Standard curve of [^{13}C]glutamine enrichment measured by GC–C–IRMS: relationship between observed ^{13}C enrichment in CO_2 peak produced by analysis of glutamine and expected ^{13}C-glutamine enrichment (both expressed in mol% excess). Each standard was injected in triplicate and individual measurements are plotted.

Table 1. Run-to-run variability of GC–C–IRMS ^{13}C-glutamine assay[a]

Plasma #	Measured enrichment (mol% excess)	Mean	SD	CV (%)
Plasma #1				
a	0.2736			
b	0.2759			
c	0.2767			
d	0.2725	0.2747	0.0020	0.7
Plasma #2				
a	0.2880			
b	0.2892			
c	0.2871			
d	0.2867	0.2877	0.0011	0.4
Plasma #3				
a	0.2582			
b	0.2573			
c	0.2474			
d	0.2562	0.2548	0.0050	2.0
Plasma #4				
a	0.2624			
b	0.2727			
c	0.2717			
d	0.2690	0.2689	0.0046	1.7

[a] Each of four vials (numbered #1 through #4; prepared from four different human plasma samples) was injected four times (runs a, b, c and d) into the GC–C–IRMS system.

system. Differences in the ^{13}C content of the derivatization reagents and/or isotopic fractionation during the derivatization may account for the fact that the slope is about 1/10, in spite of a 1/13 'dilution' of the labeled carbon with the NAP-glutamate molecule.

Plasma samples spiked with known amounts of ^{13}C-glutamine were injected in quadruplicate to assess the run-to-run precision of the assay. As shown in Table 1, the run-to-run coefficient of variation (CV = 100 × SD/mean) for replicate injection of a given derivatized sample was less than 2%. Replicate aliquots of the same plasma prepared in parallel were used to determine the variability in ^{13}C-glutamine assay associated with the whole procedure, including extraction, derivatization, gas chromatographic separation and combustion: the coefficient of variation ranged between 2% and 5% (Table 2). The inter-assay coefficient of variation was assessed with plasma samples measured on separate days after storage as NAP derivatives at −20 °C for several weeks: day-to-day variability was below 5% (Table 3).

Table 2. Overall within-day reproducibility of GC–C–IRMS assay[a]

Plasma #	Measured enrichment (mol% excess	Mean	SD	CV (%)
Plasma #1				
a	0.06591			
b	0.06497			
c	0.06207			
d	0.06892	0.06547	0.00283	4.3
Plasma #2				
a	0.09814			
b	0.09633			
c	0.09360	0.09602	0.00228	2.4
Plasma #3				
a	0.13446			
b	0.14163			
c	0.13588	0.13730	0.00376	2.7
Plasma #4				
a	0.21755			
b	0.20619			
c	0.20899			
d	0.21534	0.21202	0.00532	2.5
Plasma #5				
a	0.26382			
b	0.26836			
c	0.28087	0.27102	0.00883	3.3

[a] Three or four aliquots (termed a, b, c and d) of five plasma samples (numbered #1 through #5) were extracted, derivatized and analysed in parallel.

Figure 3 shows the time course of plasma ^{13}C-glutamine enrichment in a human volunteer who received unprimed intravenous infusion of [1-^{13}C] acetate. Even though glutamine enrichment remained about 20 times lower than plasma ^{13}C-acetate enrichment (data not shown), the rise in plasma ^{13}C-glutamine was easily quantitated using the GC–C–IRMS method described above.

DISCUSSION

To our knowledge the present paper is the first to demonstrate that, despite the dilution of label intrinsic to the derivatization and combustion procedures intrinsic to the method, GC–C–IRMS can be used for the measurement of low ^{13}C enrichments in plasma glutamine. The method can detect enrichments that are

Table 3. Inter-assay variability of GC–C–IRMS assay[a]

Plasma	Day 1	Day 2	Difference	Relative day-to-day variation (%)
#1	0.2747 ± 0.0020	0.2877 ± 0.0011	0.0130	4.5
#2	0.2557 ± 0.0027	0.2688 ± 0.0047	0.0131	4.9
#3	0.2176 ± 0.0085	0.2160 ± 0.0065	0.0016	0.8

[a] Four aliquots (termed a, b, c and d) of three plasma samples (numbered #1 through #3) were extracted, derivatized and analysed on two occasions (day 1 and day 2), several weeks apart. Values (expressed in mol% excess) are means ± SD of four injections of same sample into the GC–C–IRMS.

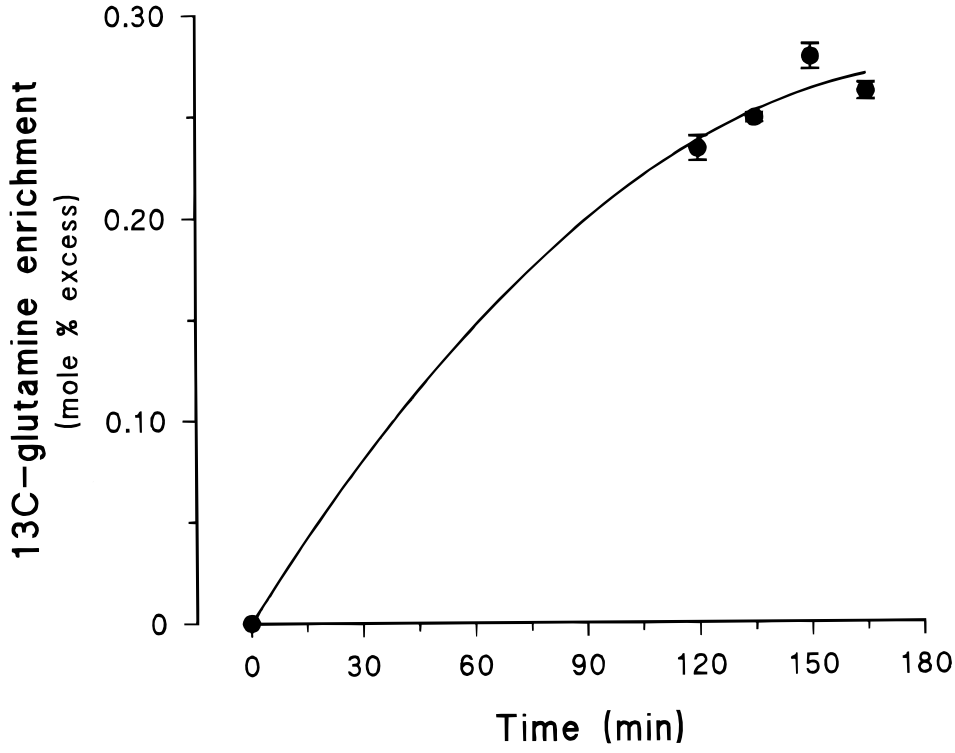

Figure 3. Time course of plasma [^{13}C]glutamine enrichment during an unprimed, 165 min infusion of [1-^{13}C]acetate (30 μmol kg^{-1} h^{-1}) in a single human volunteer in post-absorptive state. Values are means ± SD; error bars that are not apparent are within symbols.

10–20 times lower than those routinely measured using conventional GC–MS.[13] The inter-assay reproducibility (< 5%) is of the same order of magnitude as the approximately 2%–3% obtained in earlier reports on the GC–C–IRMS analysis of leucine[10,11] using either NAP[10] or heptafluorobutyryl methyl[11] derivatization.

The use of the described technique should facilitate investigation of whole-body glutamine kinetics. Indeed, assessment of glutamine appearance rate into plasma (typically about 350 μmol kg^{-1} h^{-1} in a 70 kg adult human[7]) routinely requires the infusion of about 540 mg of L-[1-^{13}C]glutamine for a 5 h, unprimed, continuous infusion designed to reach a steady state isotopic enrichment of about 3 mol% excess in plasma glutamine, which is easily measurable by GC–MS; at $800 per gram of tracer, the cost of each infusion therefore exceeds $400. By cutting the cost of tracer by a factor of 10 or 20, the use of GC–C–IRMS should result in significant savings compared with previous GC–MS techniques.

In the present paper the method was tested *in vivo* by monitoring the rise in plasma ^{13}C-glutamine enrichment during an unprimed infusion of ^{13}C-acetate in a single human volunteer. As a matter of fact, as acetate readily reaches the tricarboxylic acid (TCA) cycle through acetyl-coA, its carbon can appear in α-ketoglutarate; the latter can in turn be converted to glutamate and subsequently to glutamine through the combined action of glutamate dehydrogenase and glutamine synthetase. Even though the relatively low ^{13}C-glutamine enrichments expected would have been difficult to document using conventional GC–MS techniques, we indeed observed a significant incorporation of label in plasma glutamine during the infusion of labelled acetate using the current GC–C–IRMS assay (Fig. 3). Furthermore, the described method might be applied to the determination of the low ^{13}C enrichments in plasma free glutamine obtained *in vivo* upon infusion of other ^{13}C-labelled precursors (e.g. other amino acids or glucose) and thus facilitate the determination of glutamine *de novo* synthesis. Similarly, the method might be useful to follow incorporation of ^{13}C into specific proteins upon infusion of ^{13}C-labelled glutamine.

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