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Use of L-[^{15}N] glutamic acid and homoglutathione to determine both glutathione synthesis and concentration by gas chromatography-mass spectrometry (GCMS)

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A method for simultaneous measurement of both glutathione enrichment and concentration in a biological sample using gas chromatography mass spectrometry is described. The method is based on the preparation of *N*,*S*-ethoxycarbonylmethyl ester derivatives of glutathione, and the use of homoglutathione (glutamyl–cysteinyl–alanine) as an internal standard. A procedure for determination of glutamate concentration and enrichment is also reported. Both methods have within-day and day-to-day inter-assay coefficients of variation less than 5%, and recoveries of known added amounts of glutathione and glutamate are close to 100%. Taken together, these methods allowed determination of glutathione concentration and fractional synthesis rate in red blood cells using L-[^{15}N] glutamic acid infusion. This approach was applied *in vivo* to investigate the effects of a 72 h fast, compared with a control overnight fast, on erythrocyte glutathione in a single dog. The 72 h fast was associated with a 39% decline in erythrocyte glutathione level, (2.9 ± 0.4 versus 4.7 ± 0.5 mmol l^{-1}, fasting versus control) with no change in glutathione fractional synthesis (67.4 versus 71.3%d^{-1}, fasting versus control). Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: stable isotopes; glutathione; glutamate; erythrocyte; dog

INTRODUCTION

The tripeptide glutathione (γ-glutamyl–cysteinyl–glycine, GSH) is present at millimolar concentrations in the intracellular milieu in most tissues, and plays a prominent role in the defense against oxidative stress, as well as in the detoxification of many xenobiotics.[1] It arises from *de novo* synthesis from its constituent amino acids, glutamate, cysteine, and glycine, and has a very high turnover rate in most tissues.

GSH depletion has been described in various disease states,[2–4] and could arise from either a decreased rate of synthesis, increased rate of utilization, or a combination of both. To delineate the mechanisms responsible for the alterations of GSH concentrations, determination of its rate of synthesis is of physiological interest, and several groups have established methods to investigate the kinetics of GSH synthesis *in vivo* using stable isotopes over the course of intravenous infusions of stable isotope-labeled glycine,[4] cysteine,[5,6] or glutamate.[7]

Jahoor *et al*.[8] were first to use preparative high-performance liquid chromatography (HPLC) to separate blood GSH, then followed by hydrolysis of the tripeptide, to determine isotopic enrichments in GSH-bound glycine or glutamate by gas chromatography–mass spectrometry (GCMS). This approach was successful in determining the synthesis rate of GSH over the course of labeled glycine or glutamate infusion, and applied to the investigation of important aspects of the regulation of GSH homeostasis.[8,9] The method is, however, somewhat tedious as it involves the use of two separate analytical techniques, i.e. HPLC followed by GCMS. In addition, when labeled glutamate is used as a tracer to quantify GSH synthesis, accurate determination of the isotope enrichment in free glutamate is required to assess the isotope enrichment in the precursor pool used for GSH synthesis. As biological samples contain both glutamine and glutamate, an accurate determination of labeled glutamate enrichment is often hampered by the spontaneous hydrolysis

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of glutamine to glutamate *in vitro* during sample storage and processing. Although the method described by Reeds *et al*.[9] was aimed at minimizing the degradation of glutamine by using a ''soft'' derivatization procedure to reduce the ''contamination'' of glutamate by degraded glutamine, the assay did not provide a correcting mechanism in the case when glutamine degradation was not negligible.

More recently, Capitan *et al*.[5] used the direct derivatization of GSH extracted from blood and tissue to its ethoxylcarboxymethyl ester to measure the incorporation of labeled cysteine into blood and tissue GSH with a simple GCMS assay. The method, however, did not allow for a simultaneous determination of the stable isotope enrichment and concentration of blood GSH in the same sample.

The current report describes an improved approach, in as much as: (a) the GCMS assay makes use of an internal standard to allow for the simultaneous determination of the stable isotope enrichment and concentration of blood GSH; (b) the preparation of labeled GSH *in vitro* allows for the use of a standard curve in the determination of labeled GSH enrichments; and (c) the method can be used to measure accurately the ''true'' enrichment in the precursor free glutamate pool despite the potential ''contamination'' of glutamate by glutamine hydrolysis *in vitro* when L-[^{15}N] glutamic acid is used as a tracer to assess *in vivo* GSH kinetics.

EXPERIMENTAL

Reagents

Homoglutathione (hGSH) was obtained from BACHEM Biochimie (Voisins le Bretonneux, France). Reduced glutathione (GSH), ethyl chloroformate, homoglutamate (hglu), sulfosalicylic acid (SSA) and dithiothreitol (DTT) were obtained from Sigma (Sigma–Aldrich, Steinheim, Germany). L-[^{15}N] glutamic acid (^{15}N 95–99%) and L-[U-^{13}C] glutamine (^{13}C 99%) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). AG50 cation-resin was obtained from Aldrich (Sigma–Aldrich). Heptafluorobutyric anhydride (HFBA) was obtained from Fluka (Sigma–Aldrich).

GSH analysis

Preparation of red blood cells

Venous blood was collected in 5 ml (ethylenediaminetetraacetic acid) (EDTA) tubes, immediately put on ice, and centrifuged at 5000*g* for 10 min to separate the red cell pellet from plasma. After centrifugation of whole blood, a fine indelible ink line was drawn on the tube wall to mark the plasma level. Plasma was then removed, and replaced with cold distilled water drop by drop up to the mark, in order to achieve hemolysis of the red blood cells. After this step, samples could be stored at −80 °C for several weeks, until analysis. For analysis, 1.5 ml of hemolyzed sample was transferred into a 5 ml glass tube containing an 850 µl aliquot of 0.2 mol l^{-1} phosphate buffer with 1.5 µmol of hGSH as internal standard and 40 µmol of DTT; the pH was adjusted to 8.5 with 0.8 mol l^{-1} NaOH, and the mixture was left 15 min at room temperature. Samples were then deproteinized by adding 375 µl of 50% (w/v) SSA and 375 µl of 0.2 mol l^{-1} phosphate buffer. After centrifugation (3000 *g*, 15 min, 10 °C) the supernatant was removed and transferred into a 3 ml glass tube, and adjusted to pH 7.5 with 0.8 mol l^{-1} NaOH before derivatization.

Preparation of standard solutions

For GSH concentration determination, the tripeptide hGSH (glutamyl–cysteinyl–alanine)—an analogue of GSH that is exclusively synthesized in plants, and is not found in native human blood—was chosen as an internal standard because of its structure closely resembling that of GSH. To prepare a standard curve for the assay of GSH concentrations, graded amounts of natural GSH were added to 1 ml of phosphate buffer containing 1 µmol of hGSH and 80 µmol of DTT, to obtain GSH/hGSH molar ratios ranging between zero and two.

For GSH enrichment determination, a ^{15}N-labeled GSH was produced *in vitro* in the laboratory, by incubating 5 ml of hemolyzed human red blood cells, for 2 h at 37 °C with 300 µmol L-[^{15}N] glutamic acid, 200 µmol L-cysteine, 200 µmol L-glycine, 200 µmol ATP, 200 µmol D-glucose, 1.5 mmol Tris–HCl, 300 µmol magnesium chloride, in 10 ml distilled water at pH 7.5.[10] After incubation, protein was precipitated by adding 1.5 ml of 50% (w/v) SSA. The mixture was then centrifuged for 15 min at 3000 *g* and at 10 °C, the supernatant removed and filtered, and its pH adjusted up to 7.5 with 10 mol l^{-1} NaOH before the solution could be stored at −80 °C. The concentration of this final solution was assayed before each use by adding hGSH as internal standard as described above. Its GSH concentration was 623 ± 25 µmol l^{-1}, and, assuming a slope of unity for the determination of the 364/363 ion ratio on GCMS, the ^{15}N-GSH/natural GSH mole ratio was 0.365 ± 0.007.

GSH derivatization

The derivatization procedure was adapted from Kataoka *et al*.[11] and Capitan *et al*.,[5] and converted GSH and hGSH to their respective *N*,*S*-ethoxycarbonyl methyl (NSECM) esters. After the pH was adjusted to 7.5, 200 µl of ethyl chloroformate were added, and the mixture was shaken for 10 min at room temperature. The pH was then adjusted to 1.5 with 1 mol l^{-1} HCl, the mixture transferred into a 10 ml glass tube containing 0.5 g of NaCl, and extracted twice with 4 ml of peroxide-free diethyl ether. The organic phase extract was evaporated to dryness under nitrogen flux at 50 °C. 250 µl of 1 mol l^{-1} HCl in methanol (prepared freshly by mixing 250 µl of 36% HCl into 7.5 ml methanol) were added, and the mixture was incubated for 10 min at 80 °C. After cooling, the mixture was evaporated under nitrogen flux and the residue subsequently dissolved in 400 µl of ethyl acetate for injection into the gas chromatography–mass spectrometer.

GCMS parameters

Analysis was carried out on an electron impact ionization gas chromatograph–mass selective detector (5890 series II® gas chromatograph coupled with a 5971® mass selective detector, Hewlett-Packard, Palo Alto, CA, USA) using an HP-5MS® capillary column (10 m × 0.25 mm internal diameter (id), 0.1 µm film thickness, Hewlett-Packard) operated in the split mode with a 20 : 1 split. Injector and detector temperatures

were 270 °C and 280 °C respectively; the column head pressure was 3 psi, and the helium flux was set at 1 ml min^{-1}. The volume injected was 1–2 µl. The oven temperature was initially set to 150 °C, and maintained at 150 °C for 30 s after injection, then raised to 275 °C at a rate of 15 °C min^{-1}, and finally ramped up at the rate of 40 °C min^{-1} to 300 °C, where it was maintained for 3 min. The acquisition time was 12 min. These settings allow a sufficient separation of the GSH and hGSH. Using the selective ion monitoring (SIM) mode of the mass detector, with a dwell time set at 100 ms, ions at mass-to-charge ratio $m/z = 363$ and 364, representing natural and ^{15}N-GSH respectively, were selectively monitored for the GSH peak, and the ion at $m/z = 363$ for the hGSH peak.

Glutamate and glutamine analysis

To determine red blood cell GSH fractional synthesis rate over the course of an infusion of L-[^{15}N] glutamic acid, the ^{15}N-enrichment in intracellular red blood cell glutamate, the precursor pool for erythrocyte GSH enrichment, must be measured accurately. Most biological samples, however, contain both glutamine and glutamate. As glutamine's amide nitrogen is spontaneously lost during sample storage and processing *in vitro*, a fraction of the glutamine present in the original sample can degrade, and result in the ''contamination'' of ''true'' glutamate with glutamate derived from degraded glutamine. Although our previously published method[12] quantified the ''contamination'' of glutamate by deamidated glutamine, the separation of glutamine from glutamate was tedious, and the glutamine and glutamate fractions extracted from biological samples had to be treated and analyzed separately. We therefore developed a method that allowed for the concomitant assessment of glutamate and glutamine enrichments and concentrations, while still allowing for quantitation of glutamine degradation in the same sample. Homoglutamate, a six-carbon analog of glutamate, was used as internal standard for glutamate, and [U-^{13}C] glutamine as an internal standard for glutamine. The derivatization procedure used was previously described by Reeds *et al*.[9]

Preparation of red blood cells

Red blood cells were hemolyzed as described above for GSH analysis. 500 µl of hemolyzed cells were spiked with 20 nmol [U-^{13}C] glutamine as an internal standard to quantify glutamine degradation to glutamate, and 75 nmol homoglutamate to quantify the sum (glx) of ''true'' glutamate plus glutamine-derived glutamate. Protein was precipitated with 300 µl of 25% (w/v) SSA, and samples were centrifuged for 20 min at 3000*g* at 4 °C. The supernatant was transferred, adjusted to pH 3, poured on top of a disposable plastic column containing 1.5 ml of AG50 ion exchange resin, and washed with 10 ml distilled water. After applying the sample, the column was washed a second time with 10 ml cold distilled water, and the eluate discarded. 2 ml of 6 mol l^{-1} ammonium hydroxide were added to each column to elute both glutamate and glutamine, and the eluate collected, and evaporated to dryness under vacuum before derivatization.

Preparation of standard solutions

Three sets of standards are required with this method. The first set of standards was designed to assess glutamine concentration and its degradation to glutamate, and was prepared by mixing graded amounts of [U-^{13}C] glutamine and natural glutamine in order to obtain [U-^{13}C] glutamine/glutamine mole ratios ranging between 0 and 15%. The second set of standards was designed to measure the sum of ''true'' glutamate plus glutamate derived from glutamine degradation, and was obtained by adding graded amounts of natural glutamate to homoglutamate, in order to obtain mole ratios in the 0–6% range. The third set of standards was designed to assess ^{15}N-glutamate enrichment and was obtained by preparing L-[^{15}N] glutamic acid/natural glutamic acid mixtures with mole ratios ranging between 0 and 20%. A 200 µl aliquot of each standard was evaporated to dryness under vacuum before derivatization.

Derivatization

The derivation procedure converted glutamine and glutamate to their heptafluorobutyramide propyl (PHFBA) ester derivatives. The procedure we used produces the same derivative for glutamate as described by Knapp[13] and applied by Matthews and Campbell,[14] but the use of a lower temperature prevents complete glutamine degradation to glutamate as described by Reeds *et al*.[9]

A 600 µl amount of 5/1 (v/v) propan-1-ol to acetylchloride mixture was added to dry samples or standard solutions. The mixture was incubated 2 h at room temperature,[9] then evaporated to dryness under a nitrogen stream. A 50 µl amount of heptafluorobutyric anhydride (HFBA) was added, tubes were shaken, capped, incubated 20 min at 60 °C in heating block, and evaporated under nitrogen stream. The residue was then dissolved in either 2 ml or 10 ml of ethyl acetate for samples or standard solutions respectively.

GCMS parameters

Analyses were carried out using electron impact ionization GCMS with a DB-1® capillary column (30 m × 0.25 mm id, 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) in the splitless mode. Injector and detector temperatures were 250 and 280 °C respectively; the column head pressure was 8 psi, and the helium flux was set at 1 ml min^{-1}. The oven temperature was initially set at 80 °C, and then ramped to 250 °C at the rate of 15 °C min^{-1}. The acquisition time was 13 min. The volume injected was 1 µl. These settings allowed a sufficient separation of glutamine, glutamate and homoglutamate (at 7.3, 8.9 and 9.7 min, respectively). Ions at $m/z = 252$ (natural glutamate, glutamine-derived glutamate and homoglutamate), 279 (natural glutamine), 280 (natural glutamate and ^{15}N-glutamine), 281 (^{15}N-glutamate), 283 ([U-^{13}C] glutamine) and 284 ([U-^{13}C] glutamate derived from [U-^{13}C] glutamine degradation) were monitored using the SIM mode, with a dwell time of 30 ms.

Calculations

GSH concentration ([GSH]/µmol l^{-1}) was determined from the peak area ratio of the ion at $m/z = 363$ in the GSH peak

to the ion at the same $m/z = 363$ in the hGSH peak:

$$[\text{GSH}] = n\text{hGSH} \times [(363\text{GSH}/363\text{hGSH}) - b_1]/(a_1 \times \text{Hcrit}/100)$$

where Hcrit is the hematocrit, defined as the fraction of whole blood volume accounted for by red blood cells (and expressed as a percentage); *n*hGSH the number of nanomoles of hGSH added as internal standard per milliliter of sample; 363GSH/363hGSH is the peak area ratio of GSH/hGSH at *m/z* 363; a_1 and b_1 are the slope and the intercept respectively of the standard curve obtained by adding graded amounts of natural GSH in hGSH aqueous solutions; and 100 converts the hematocrit into a fraction of unity.

Glutamine concentration ([gln]/μmol l^{-1}) was calculated using:

$$[\text{gln}] = a_2 \times [\text{U-}^{13}\text{C-gln}]/[(283\text{gln}/279\text{gln} - b_2) \times \text{Hcrit}/100]$$

where a_2 and b_2 are the slope and the intercept respectively of the standard curve obtained by adding increasing amounts of [U-^{13}C]glutamine in natural glutamine, [U-^{13}C-gln] is the concentration of [U-^{13}C] glutamine (added internal standard) in the sample, expressed in micromoles per liter, and 283gln/279gln is the ratio of $(m + 4)/m$ in the glutamine peak.

The ^{15}N-enrichment in erythrocyte glutamine (E ^{15}Ngln, expressed in mole percent excess; MPE) was calculated as follows. First, the ^{15}N-glutamine/natural glutamine mole ratio at time t (MR ^{15}Ngln) was calculated as:

$$\text{MR } ^{15}\text{Ngln} = (280\text{gln}/279\text{gln}_t - b_3)/a_3$$

where $280\text{gln}/279\text{gln}_t$ is the 280/279 peak area ratio measured by GCMS in erythrocyte glutamine peak at time t, and a_3 and b_3 are the slope and the intercept respectively of the standard curve obtained by adding increasing amounts of ^{15}N-glutamate in natural glutamate. The ^{15}N-enrichment in erythrocyte glutamine at time t was then calculated using:

$$E\ ^{15}\text{Ngln} = (\text{MR } ^{15}\text{Ngln}_t - \text{MR } ^{15}\text{Ngln}_{t0})/(\text{MR } ^{15}\text{Ngln}_t - \text{MR } ^{15}\text{Ngln}_{t0} + 1) \times 100$$

where MR $^{15}\text{Ngln}_t$ and MR $^{15}\text{Ngln}_{t0}$ are the molar ratios of ^{15}N in glutamine at isotopic plateau and before L-[^{15}N] glutamic acid infusion (i.e. at baseline) respectively.

The concentration of the sum of ''true'' glutamate plus glutamate arising from glutamine degradation to glutamate ([glx]/μmol l^{-1}) was calculated thus.

$$[\text{glx}] = 252\text{glx}/252\text{hglu} \times n\text{hglu}/(a_4 \times \text{Hcrit}/100)$$

where 252glx/252hglu is the peak area ratio of ion 252 in the glutamate plus glutamine-derived glutamate peak (''glx peak'') to ion 252 in the homoglutamate peak, *n*hglu is the number of nanomoles of homoglutamate added as internal standard per milliliter of sample, and a_4 is the slope of the standard curve obtained by adding increasing amounts of natural glutamate in homoglutamate.

The fraction of glx that is glutamate derived from glutamine degradation (fdegr), was calculated using:

$$\text{fdegr} = (284\text{glx}/280\text{glx})/a_2$$

where 284glx/280glx is the ratio of $(m + 4)/m$ peak area assessed by GCMS in the glx fraction.

Glutamate concentration ([glu]/μmol l^{-1}) was calculated using:

$$[\text{glu}] = [\text{glx}] \times [1 - \text{fdegr}]$$

The ^{15}N-enrichment in the glutamate peak (glx, a mixture of true glutamate plus degraded glutamine), E ^{15}Nglx, expressed in MPE, was calculated as follows. The ^{15}Nglx/natural glx mole ratio (MR ^{15}Nglx) was first calculated as:

$$\text{MR } ^{15}\text{Nglx} = (281\text{glx}/280\text{glx} - b_3)/a_3$$

where 281glx/280glx is the peak area ratio of ion 281 to 280 in the glutamate plus glutamine-derived glutamate fraction assessed by GCMS, and b_3 and a_3 are the intercept and slope of the corresponding standard curve respectively.

Erythrocyte ^{15}N-glx enrichment (E ^{15}Nglx) was then calculated as:

$$E\ ^{15}\text{Nglx} = \frac{\text{MR } ^{15}\text{Nglx}_t - \text{MR } ^{15}\text{Nglx}_{t0}}{\text{MR } ^{15}\text{Nglx}_t - \text{MR } ^{15}\text{Nglx}_{t0} + 1} \times 100$$

where MR $^{15}\text{Nglx}_t$ and MR $^{15}\text{Nglx}_{t0}$ are the ^{15}Nglx/natural glx mole ratios measured before the tracer infusion (at baseline) and at isotopic plateau respectively.

^{15}N-glutamate enrichment (E ^{15}Nglu/MPE) was calculated using:

$$E\ ^{15}\text{Nglu} = \frac{[\text{glx}] \times E\ ^{15}\text{Nglx} - \text{fdegr} \times [\text{glx}] \times E\ ^{15}\text{Ngln}}{[\text{glu}]}$$

where E ^{15}Nglx and E ^{15}Ngln were the ^{15}N enrichments in the glutamate plus glutamine-derived glutamate fraction and in the glutamine fraction, respectively, expressed in MPE.

The *GSH fractional synthesis rate* (FSR/%d^{-1}), is calculated as follows. The ^{15}N-GSH/natural GSH mole ratio (MR ^{15}Ngsh) was calculated using:

$$\text{MR } ^{15}\text{Ngsh} = (364\text{gsh}/363\text{gsh} - b_5)/a_5$$

where 364gsh/363gsh is the peak ratio of ion 364 to 363 in the GSH peak, and a_5 and b_5 are the slope and intercept respectively of the standard curve obtained by adding increasing amounts of ^{15}N-GSH in natural GSH. The ^{15}N-enrichment in plasma GSH at time t (E ^{15}Ngsh) was then calculated using:

$$E\ ^{15}\text{Ngsh} = \frac{\text{MR } ^{15}\text{Ngsh}_t - \text{MR } ^{15}\text{Ngsh}_{t0}}{\text{MR } ^{15}\text{Ngsh}_t - \text{MR } ^{15}\text{Ngsh}_{t0} + 1} \times 100$$

where MR $^{15}\text{Ngsh}_t$ and MR $^{15}\text{Ngsh}_{t0}$ are the molar ratios of ^{15}N in glutathione at time t and before ^{15}N-glutamate infusion respectively.

The GSH FSR was then defined as:

$$FSR = 100 \times 24 \times \Delta E\ ^{15}Ngsh/(\Delta t \times E\ ^{15}Nglu)$$

where $\Delta E\ ^{15}Ngsh$ (MPE h^{-1}) is the rise in erythrocyte ^{15}N-gsh enrichment during the time interval Δt (expressed in hours), when erythrocyte-free ^{15}N-glutamate enrichment ($E\ ^{15}Nglu$/MPE) was at steady state, e.g. between the end of the second and fifth hours of isotope infusion; 24 converts FSR from h^{-1} to day^{-1}, and 100 converts a fraction of unity to a percentage.

In vivo experiment

A single dog, from the kennel of the National Veterinary School of Nantes, was studied after approval of the experimental procedure by the ethical committee of the National Veterinary School.

The synthesis rate of erythrocyte GSH was measured *in vivo* in a single Beagle dog on two separate occasions: (i) after an overnight physiological fast; and (ii) after a 72 h fast. On the day of isotope infusion at 8:30 am the dog was weighed, and two short intravenous catheters (Vasocan® 20 gauge, BBraun Medical, Emmenbrücke, Germany) were placed using an aseptic technique: one in the cephalic vein of the forelimb for isotope infusion, and the other in the contralateral forelimb for blood sampling. At 8:45 am, a baseline blood sample was obtained to determine background isotope enrichment and concentration of erythrocyte GSH and free glutamate. Starting at 9:00 am, the dog received a priming dose of L-[^{15}N] glutamic acid (45 μmol kg^{-1}), immediately followed by a continuous 5.5 h intravenous infusion of L-[^{15}N] glutamic acid (45 μmol kg^{-1} h^{-1}) delivered by means of a calibrated Bioblock (Fisher Scientific, Illkirch, France) syringe-pump. 5 ml venous blood samples were obtained in EDTA tubes at 90, 120, 150, 180, 210, 240, 270, 300, and 330 min after the start of isotope infusion. Tubes were kept on ice until centrifugation at 4 °C at 5000*g* for 10 min. Plasma was immediately removed and an equivalent volume of cold distilled water was added. The mixture was shaken, and immediately frozen at −80 °C. Hematocrit was determined in triplicate in baseline samples for further calculations, by centrifuging blood aliquots in capillary tubes at 5000 *g*, for 5 min. Hematocrit (expressed in percent) was calculated as the length of the red blood cell fraction in the capillary tube, divided by the total length (plasma + red blood cells), as measured after the end of the centrifugation.

RESULTS

GSH concentration

As shown in Figs 1 and 2, the GSH and hGSH peaks were free of interfering peaks on the chromatogram. Under the chromatographic conditions used, GSH eluted just before hGSH, but their separation was sufficient to measure their respective ion intensities separately without any cross-contamination (Fig. 2). Standard curves were linear over the range of GSH/hGSH molar ratios chosen (Fig. 2). The within-day and day-to-day coefficients of variation were less than 5% (Tables 1 and 2). When hemolyzed red blood cell samples were spiked with known amounts of natural GSH, the recovery of added GSH reached 103.3 ± 5.4%, which did not differ significantly from 100% (Table 3).

GSH enrichment

The ^{15}N-enrichment in erythrocyte GSH from both *in vivo* tracer studies and *in vitro* synthesized labeled GSH was measured with within-day and day-to-day coefficients of variation of less than 3% (Tables 1 and 2). A linear standard curve was obtained when increasing amounts of ^{15}N-glutathione were added to natural glutathione (Table 4). The intercept value represents the $m + 1/m$ peak area ratio in unlabeled GSH. This value can be predicted and cannot be expected to be zero, due to the presence of ^{13}C, ^{2}H, ^{17}O, ^{15}N and ^{33}S at their natural abundance in natural GSH. For instance, for the ion at $m/z = 363$ in the GSH derivative, the chemical formula is: $C_{13}H_{23}O_7N_2S$. We calculated the predicted percent natural abundance of ion at $m/z = 364$ in GSH as follows:

$$13 \times 1.11 + 23 \times 0.015 + 7 \times 0.037$$
$$+2 \times 0.37 + 1 \times 0.76 = 17.6\%$$

i.e. 0.176 when expressed as a fraction, where 13, 23, 7, 2 and 1 are the numbers of atoms of carbon, hydrogen, oxygen, nitrogen, and sulfur respectively in the compound, and 1.11, 0.015, 0.037, 0.37, and 0.76 are the percentage natural abundances of ^{13}C, ^{2}H, ^{17}O, ^{15}N and ^{33}S respectively. Therefore, the experimental value we measured (0.171) is not different from this predicted value.

Glutamate concentration

Figure 3 shows the typical chromatogram and spectrum of erythrocyte glutamate and homoglutamate. Linear standard curves were obtained when homoglutamate was used to assay glutamate concentration (Table 4). We verified that the degradation of glutamine to glutamate during storage and *in vitro* sample processing was not negligible, as degraded glutamine accounted for as much as 22% of the erythrocyte ''glx'' peak (i.e. ''true glutamate'' + glutamate arising from degraded glutamine) in some instances.

With the appropriate correction for glutamine degradation—allowed by the use of [U-^{13}C] glutamine internal standard—however, the precision of the ''true'' glutamate assay, as defined by its within-day coefficient of variation, was 0.4% (Table 1). When hemolyzed red blood cell samples were spiked with known amounts of natural glutamate, the recovery of added glutamate was not different from 100% (Table 3).

Glutamate enrichment

The ^{15}N-enrichment in erythrocyte glutamate was measured with a within-day coefficient of variation of less than 4% (Table 1).

Effects of fasting on GSH synthesis in red blood cells in one dog

A 72 h fast was associated with a 39% decrease in red blood cell GSH concentration without any alteration in glutamine and glutamate concentrations (Table 5). Over the course of

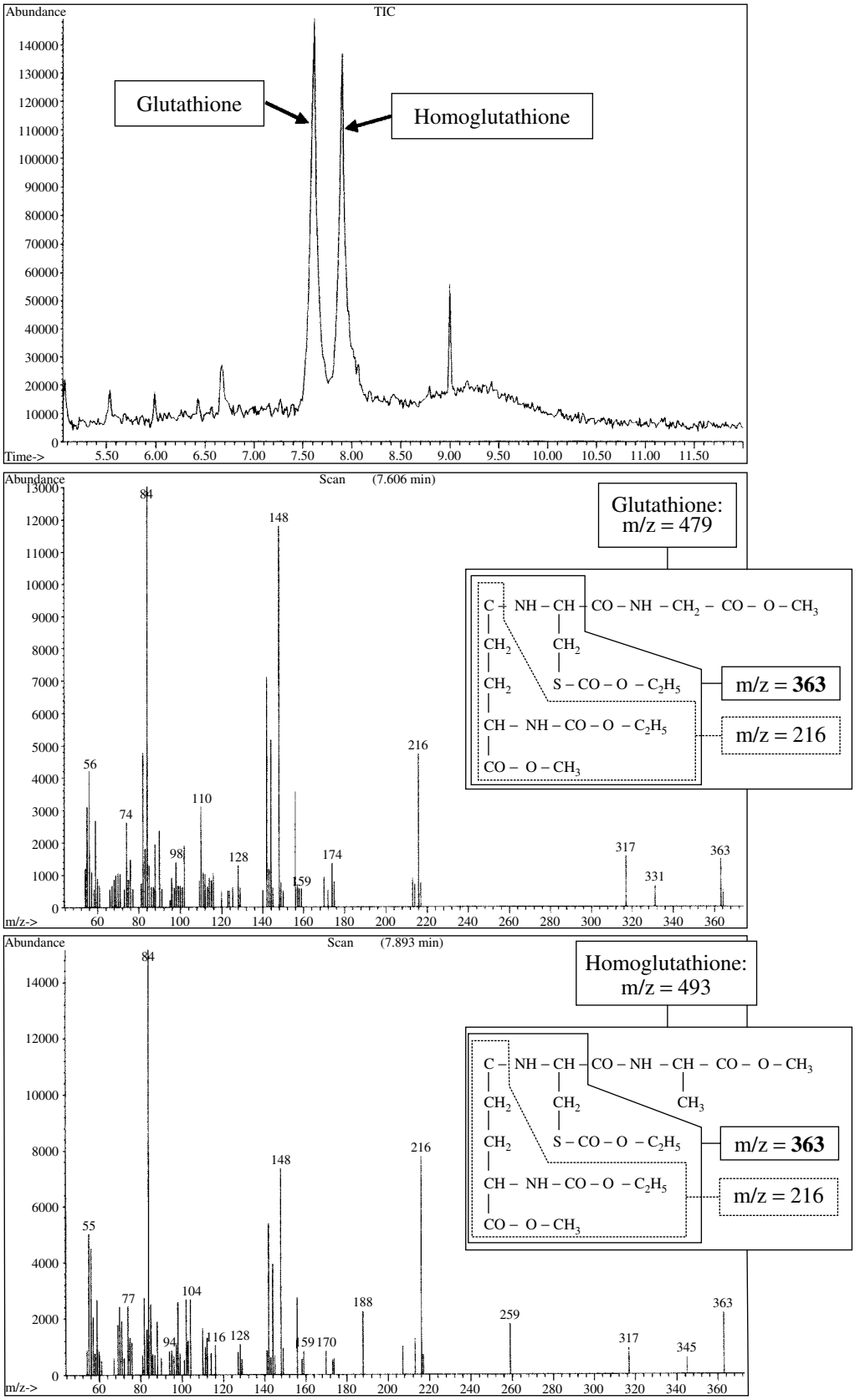

Figure 1. Typical chromatogram (upper panel) and mass spectra of *N*,*S*-ethoxycarbonyl methyl ester of GSH (middle panel) and hGSH (bottom panel) in red blood cell sample analyzed by electron impact ionization GCMS operating in the scan mode.

the L-[^{15}N] glutamic acid infusion, erythrocyte ^{15}N-glutamate enrichment reached a plateau by the end of the second hour of tracer infusion, and ^{15}N-GSH enrichment increased linearly after the ^{15}N-glutamate enrichment plateau had been reached (Fig. 4). The slopes of the lines describing the rise in ^{15}N-GSH enrichment did not differ between the two experimental days

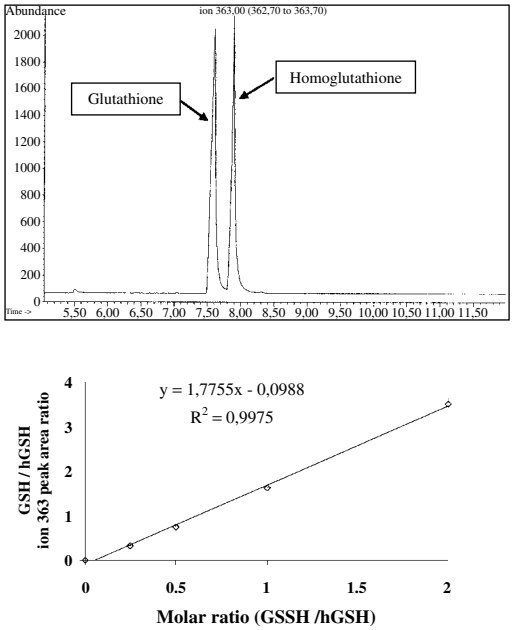

Figure 2. *Upper panel*: typical chromatogram of *N,S*-ethoxycarbonyl methyl esters of GSH and hGSH in red blood cell sample analyzed by electron impact ionization GCMS in the SIM mode. *Bottom panel*: typical standard curve obtained by adding graded amounts of GSH in hGSH in a molar ratio in the range of 0–2. The ion selected was *m/z* 363 in both GSH and hGSH peaks.

Table 1. Typical precision of the assays: the within-day (intra-assay) coefficient of variation (CV) is given for the assay of erythrocyte GSH and glutamate concentrations ([GSH] and [glutamate] respectively), and for ^{15}N-enrichment (Enr) determination in GSH and in glutamate. Data are expressed as mean ± standard error

Assay	Mean ± SE	CV (%)	Number of samples	Number of injections per sample
[GSH] (μmol l^{-1})	1982 ± 18	1.8	4	3
Enr ^{15}N-GSH (MPE)	64.5 ± 0.7	2.2	4	2
[glutamate] (μmol l^{-1})	351 ± 1	0.4	4	3
Enr ^{15}N-glutamate (MPE)	8.68 ± 0.17	3.4	3	3

(0.41 MPE h^{-1} versus 0.37 MPE h^{-1}, fasting versus control respectively), and fasting failed to affect red blood cell GSH

Table 2. Day-to-day inter-assay coefficient of variation (CV) for GSH and glutamate concentration, and for GSH enrichment in red blood cell hemolyzates. Data are expressed as mean ± SE and coefficients of variation between different days of assessment of the same sample

	Mean ± SE	CV (%)	Number of assays	Number of injections
[GSH] (μmol l^{-1})	743 ± 13	4.7	7	2
Enr ^{15}N-GSH (MPE)	65.1 ± 0.4	1.8	8	2
[glutamate] (μmol l^{-1})	310 ± 2	0.8	2	6

Table 3. Recovery of added GSH and glutamate in red blood cell mixture. Recovery is defined as the number of micromoles in excess in the sample divided by the number of micromoles added to the original sample, and expressed as a percentage. Values are means ± SE. ($n = 7$ for each added amount of GSH tested, and $n = 2$ for each added amount of glutamate tested)

Glutathione		Glutamate	
GSH added (μmol)	Recovery (%)	Glutamate added (μmol)	Recovery (%)
0.5	98.0 ± 3.1	0.125	99.9
1	108.7 ± 9.5	0.25	98.1
		0.5	106.5
Mean	103.3 ± 5.4	Mean	101.6 ± 2.4

FSR (Table 5). For this calculation, we hypothesized that the slope of the standard curve used for the measurement of ^{15}N-GSH-to-natural GSH mole ratio was not different from unity and that this slope did not differ between the different analysis days. We verified the second hypothesis through repeated measurements on aliquots of the ^{15}N-enriched GSH mixture synthesized in the laboratory (Tables 2 and 4).

DISCUSSION

The current report describes a method to assess both GSH isotope enrichment and concentration simultaneously in the same biological sample, and in the same run, using GCMS. In addition, we describe an approach to assess glutamate enrichment and concentration. Finally, we describe a simple procedure for the preparation of highly enriched stable-isotope-labeled GSH to produce calibration standards for

Table 4. Slope and intercept of the standard curves used for determination of GSH and glutamate concentrations and enrichments. Results are expressed as mean ± SE

Standard curve	Slope	Intercept	Correlation coefficient	Number of analyses
[GSH] (a_1, b_1)	1.724 ± 0.055	−0.090 ± 0.019	0.998 ± 0.000	9
[U-^{13}C] gln (a_2, b_2)	0.933 ± 0.038	0.000 ± 0.001	0.997 ± 0.001	4
^{15}N-glx (a_3, b_3)	1.069 ± 0.020	0.113 ± 0.008	0.999 ± 0.000	3
[glx] (a_4)	0.639 ± 0.088	0.000 ± 0.000	0.992 ± 0.006	3
^{15}N-GSH (a_5, b_5)	0.977 ± 0.019	0.171 ± 0.002	1.000 ± 0.000	2

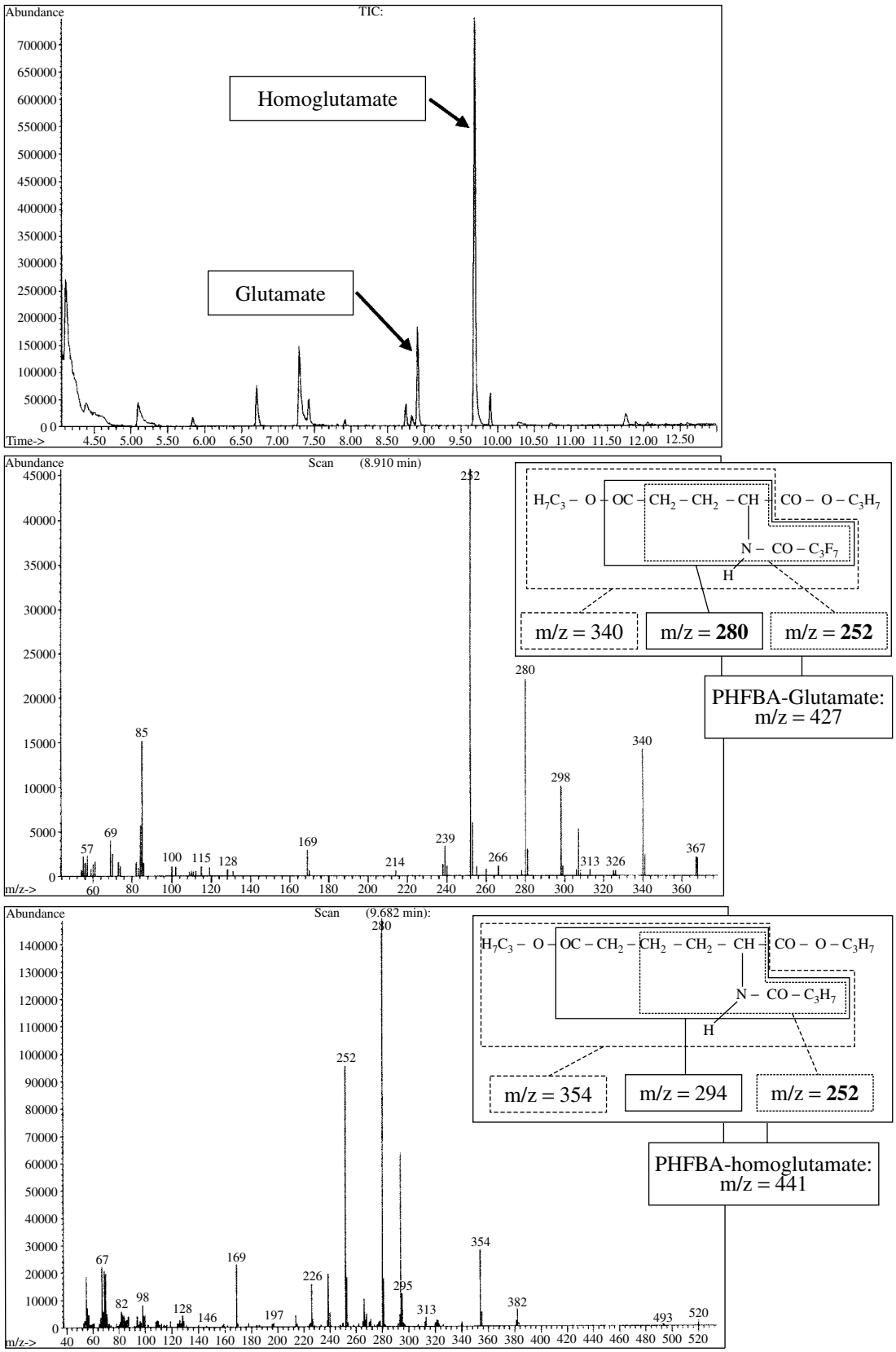

Figure 3. Typical chromatogram and mass spectra of glutamate (upper scan) and homoglutamate (bottom scan) heptafluorobutyramide propyl ester derivatives, as analyzed by electron impact ionization GCMS operating in the scan mode.

the assay of labeled GSH enrichment. Taken together, these methods allow determination of GSH concentration and synthesis rate in red blood cells or other tissues, using a 5.5 h L-[^{15}N] glutamic acid tracer infusion *in vivo*.

When we applied the current assay to the measurement of erythrocyte GSH in one dog, we found a concentration of 4.7 mmol l^{-1}. Although this value is higher than the 2.6 mmol l^{-1} values previously measured in Beagle dogs

Table 5. Concentrations and stable isotope enrichments of GSH, glutamate (glu), glutamine (gln), and glutamate + glutamine-derived fraction (glx), and GSH FSR in red blood cells, during an infusion of L-[^{15}N] glutamic acid in a single dog after overnight fast (control), and after 72 h of fasting

	Control	Fasting
L-[^{15}N] glutamic acid infusion rate (μmol kg^{-1} h^{-1})	51.3	44.0
[U-^{13}C] gln/natural gln molar ratio	0.132 ± 0.003	0.125 ± 0.003
[U-^{13}C] glx/natural glx molar ratio	0.024 ± 0.001	0.035 ± 0.001
^{15}N-glx enrichment (MPE)	10.4 ± 0.3	11.0 ± 0.3
^{15}N-gln enrichment (MPE)	1.85 ± 0.33	1.55 ± 0.30
[glx] (μmol l^{-1})	194 ± 7	192 ± 14
[glu] (μmol l^{-1})	159 ± 7	138 ± 9
^{15}N-glu enrichment (MPE)	12.2 ± 0.3	14.5 ± 0.3
^{15}N-GSH/natural GSH molar ratio at time 0	0.0059	−0.0028
^{15}N-GSH/natural GSH molar ratio at time *t*	0.0252	0.0176
Δ*t* (h)	5.5	4.5
GSH fractional synthesis rate (% day^{-1})	71.3	67.4
[GSH] (mmol l^{-1})	4.66 ± 0.52	2.87 ± 0.40
[glutamine] (μmol l^{-1})	670 ± 16	653 ± 14

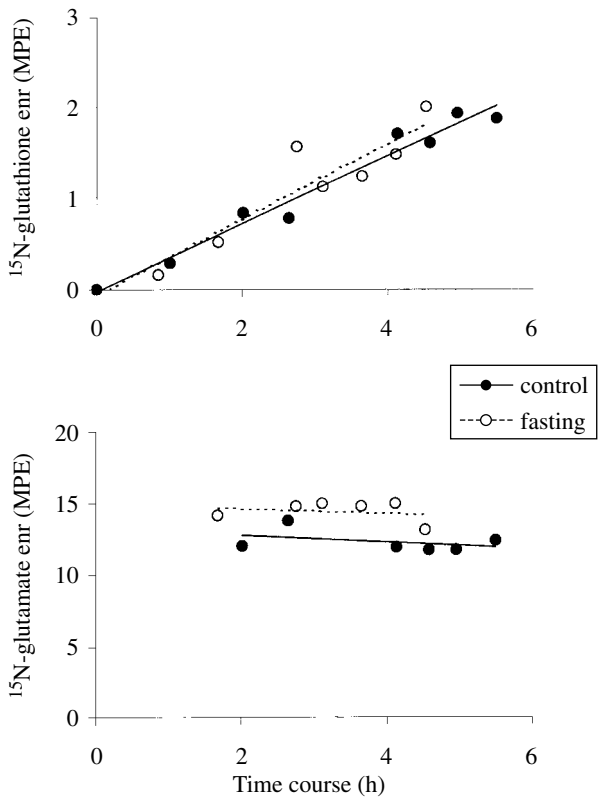

Figure 4. Time course of erythrocyte ^{15}N-GSH (upper panel) and ^{15}N-glutamate (bottom panel) enrichments (enr) during an infusion of L-[^{15}N] glutamic acid after overnight physiological fasting (control), and after a 72 h fasting period (fasting) in the same dog.

by Vajdovich *et al*.[15] using an enzymatic assay, there are many differences in experimental design between that study and the current report. For instance, in that earlier study the dogs received a diet containing 75% protein, versus 25% in the current study. In addition, Vajdovich *et al*. documented higher glutathione levels in female dogs, compared with male dogs, and the single dog used in the current report was female.

To our knowledge, the current study is the first to report on the fractional synthesis rate of erythrocyte GSH in dogs. Although the 67–71% day^{-1} GSH FSR values obtained in the current study in dogs differ from the value (23% day^{-1}) reported in one study carried out in a single human using [$^{2}H_2$] cysteine,[5] this discrepancy is unlikely to result from either interspecies or methodological differences. As a matter of fact, two other studies carried out with [$^{2}H_2$] glycine,[4] and L-[1-^{13}C] cysteine[6] report erythrocyte GSH FSR of 71 or 65% day^{-1} in humans, i.e. very close to the findings of the current study.

The current report also describes a simple and rapid technique to synthesize *in vitro* an inexpensive ^{15}N-GSH-enriched mixture, useful for calculation of ^{15}N-GSH enrichments and normalization of the results independently of the day of the measurement.

Although fasting has been shown to decrease GSH levels in several tissues, such as intestine,[16,17] liver, heart, and muscle[18] in rodents, the effect of food deprivation had not, to our knowledge, been tested on GSH synthesis in canine red blood cells. Using the current method, we found that a 72 h fast was associated with a 39% drop in red blood cell GSH content, without any alteration in GSH fractional synthesis rate. This result is consistent with the view that (a) fasting may have transiently increased GSH breakdown, resulting in lower erythrocyte level, and (b) the failure of a rise in GSH synthesis prevented the repletion of the erythrocyte GSH pool, presumably due to the discontinuation of dietary protein and energy intake. Although circulating amino acid levels were not determined in the current study, lack of protein intake may indeed limit the availability of the precursor amino acids glycine, cysteine and glutamate during the 72 h fast. As the current results are, however, based on a single dog, they should only be construed as very preliminary data, and the effect of fasting on dog erythrocyte GSH synthesis would clearly warrant further study.

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