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Journal of Chromatography B, 732 (1999) 127–135

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Gas chromatographic–mass spectrometric analysis of stable isotopes of cysteine and glutathione in biological samples

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Received 15 February 1999; received in revised form 25 April 1999; accepted 10 June 1999

Abstract

A gas chromatographic–mass spectrometric (GC–MS) procedure for the determination of stable isotope labelled glutathione has been applied to animal and human samples. The method, based on preparation of the *N*,*S*-ethoxycarbonyl methyl ester derivative of the intact peptide, is rapid and requires little or minor tissue treatment. The same method was applied to cysteine. The method was found to be reliable in terms of within-day and between-day precision, accuracy and linearity. The procedure was applied in humans and animals to determine in vivo the glutathione fractional synthesis rate using labelled cysteine infusion. The glutathione fractional synthesis rate was found to be 22.5%/day in blood from a healthy volunteer and 337±29%/day in rat liver.

Keywords: Derivatization, GC; Cysteine; Glutathione

1. Introduction

Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) is a physiologically important tripeptide, present in most mammalian cells. It plays an important role in the antioxidant status and detoxification of reactive oxygen species, free radicals, and xenobiotic compounds [1]. By affecting cellular thiol redox status, GSH can regulate cellular processes, such as signal transduction and gene transcription, and metabolism. It is also involved in the immune response to injury and infection [2,3]. As might be expected from its functions, the glutathione level is altered in many inflammatory conditions. GSH deficiency has been reported in human diseases such as HIV infection and trauma [4,5]. In the first days following an inflammatory challenge, tissue glutathione levels were elevated in treated animals compared with controls [6,7]. However, depletion of tissue GSH levels were observed later on [7,8].

Variations of glutathione levels under oxidative conditions may result from modification of synthesis and/or loss. To determine the relative importance of these two potential mechanisms, it is necessary to measure glutathione kinetics in vivo. The method of choice consists in measuring the incorporation of an isotopically labelled precursor amino acid into glutathione, but few data have been reported [9–11]. Moreover, radioactive isotopes are not suitable for human studies. Jahoor et al. [11] have developed a stable isotope tracer method to measure GSH syn-

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00273-X

thesis rates in vivo. The method requires the isolation of GSH by HPLC, since the peptide is further hydrolyzed before measuring the enrichment in the labelled amino acid used as precursor. In this case, sample preparation is time consuming and great care is needed to avoid contamination and fractionation.

The aim of this study was to develop a selective and rapid procedure to determine enrichment of the peptide as a whole. Glutathione was analyzed as its *N*,*S*-ethoxycarbonyl methyl ester by gas chromatography–mass spectrometry. This method was adapted from that of Kataoka et al. [12] and Takegi et al. [13] for measurement of glutathione levels in blood and tissues. The same derivative was used for cysteine enrichment measurement. GSH synthesis rates in human blood and animal tissues with continuous infusion of cysteine were measured using the procedure described here.

2. Experimental

2.1. Reagents

L-[^{15}N] and [3,3 $^{2}H_2$] cysteine (further on referred as [^{15}N] and [D_2] cysteine) were purchased from Cambridge Isotope Laboratories (99 and 98% isotopic purity respectively; Andover, MA USA). Ethyl chloroformate was obtained from Acros Organics (Geel, Belgium), peroxide-free ethyl ether from Prolabo (Fontenay Sous Bois, France). L-cysteine, reduced glutathione and all other reagents were obtained from Sigma Chemical Co (L'Isle d'Abeau Chesnes, France).

2.2. Preparation of samples

Plasma cysteine. Two ml of plasma were mixed with 0.2 ml of 0.2 *M* phosphate buffer, 5 m*M* EDTA, pH 8.5 containing 0.08 mmol of dithiotreitol (DTT). The reduction reaction was allowed to proceed at room temperature for 15 min, in order to obtain total cysteine [14], and then stopped by addition of 0.2 ml of 50% (w/v) sulfosalicylic acid (SSA). The precipitate was removed by centrifugation at 10 000 *g* for 15 min and 1 ml of supernatant was used for derivatization.

Blood glutathione. Two ml of blood were sonicated. Then 1 ml of 0.2 *M* phosphate buffer, 5 m*M* EDTA, pH 8.5 containing 0.08 mmol of DTT was added and the mixture was left for 15 min at room temperature. Then 0.2 ml of 50% SSA were added and the total volume was made up to 4 ml with phosphate buffer. After centrifugation (10 000 *g*, 15 min) the supernatant was removed and 1 ml was used for derivatization.

Tissue cysteine and glutathione. Frozen powdered aliquots of tissue (0.2–0.4 g) were homogenized by sonication in 2 ml of 0.2 *M* phosphate buffer, 5 m*M* EDTA, 80 m*M* DTT, pH 8.5 and left for 15 min at room temperature. Then the homogenate was centrifuged (10 000 *g*, 15 min) and the supernatant was used for derivatization.

2.3. Standard solutions

[^{15}N] or [D_2] cysteine was mixed with natural cysteine to make serial standard dilutions of [^{15}N] or [D_2] cysteine enrichment in the range 0–10 moles per cent excess and at a concentration of about 2.5 m*M*. Small portions of these standard solutions (200 μl) made up in water and containing 500 nmol were stored at −80°C. They were stored for 1 year without any degradation.

Labelled glutathione was produced in vivo by infusing 300 g rats with either [^{15}N] or [D_2] cysteine (25 μmol/h during 6 h). Blood from rats that had received [D_2] cysteine was collected from the abdominal aorta at the end of the infusion and treated as described above. The supernatant obtained was used as a source of labelled glutathione. The glutathione concentration of this solution was determined by the standard enzymatic recycling procedure using 5,5′-dithio-bis 2-nitrobenzoic acid [15]. This labelled [D_2] glutathione solution (3.763±0.014 moles per cent excess, MPE) was then mixed with natural glutathione to make standard dilutions of [D_2] glutathione in the range 0–2 MPE. Small portions of these standard solutions (100–500 μl), containing 500 nmol, were stored at −80°C and thawed just before use. They were stored for 1 year without any damage. The same procedure was used for [^{15}N] glutathione, except that the source of labelled glutathione used was liver, in order to obtain higher enrichments. Enrichment of the labelled [^{15}N] gluta-

thione standard solution was 5.944±0068 atoms per cent excess (APE).

2.4. Derivatization

The pH of the supernatants obtained from plasma, blood or tissue homogenates was adjusted to 7–7.5 with 2 *M* NaOH or 2 *M* HCl. For standard solutions, 0.8 ml of 0.2 *M* phosphate buffer, 5 m*M* EDTA, 80 m*M* DTT, pH 7.5 was added to the volumes mentioned above. After addition of 100 μl ethyl chloroformate, the mixture was shaken on a tube rotator (Stuart Scientific, Poly Labo, Strasbourg, France) at 33 rpm for 15 min at room temperature. The pH of the mixture was then adjusted to 1.5 with 2 *M* HCl. The mixture was saturated with NaCl (0.3 g per ml) and extracted three times with 3 ml of peroxide-free diethyl ether. The ethereal extracts were evaporated to dryness at 80°C, then 200 μl of 1 *M* HCl in methanol was added, the vial capped and incubated at 80°C for 10 min in a heating block. The solvent was then evaporated to dryness at 80°C under a stream of air and the residue dissolved in 50–100 μl of ethyl acetate.

2.5. Gas chromatography–mass spectrometry

GC–MS analyses were carried out on a Hewlett Packard gas chromatograph/mass selective detector (Model 5890/5972). The temperature of the GC injector was 250°C for cysteine and 270°C for glutathione. The column used was a 0.25 mm I.D.× 0.25 μm film thickness, crosslinked 5% diphenyl 95% dimethyl siloxane capillary column (HP 5 MS, Hewlett Packard, Les Ulis, France). The length of the column was 30 m and 10 m for cysteine and glutathione analyses, respectively. Injections (1–2 μl) were made in the split mode with a 20:1 split. For cysteine, the helium carrier gas was at a head pressure of 114 kPa and the gas flow 1 ml/min. The GC oven temperatures were: 200°C for 1 min, then increased to 210°C at a rate of 3°C/min, 210°C for 1 min, increased to 280°C at a rate of 40°C/min and 280°C for 1 min. For glutathione, the helium carrier gas was at a head pressure of 27 kPa and the gas flow 1.15 ml/min. The GC oven temperatures were: 200°C for 0.5 min, then increased to 280°C at a rate of 30°C/min, 280°C for 0.1 min, increased to 295°C at a rate of 5°C/min and 295°C for 2 min.

The mass spectrometer was operated under electron impact ionization conditions (electron energy 70 eV, source temperature 175°C). The ion fragments of masses 220 and 221 or 222 for *N*,*S*-ethoxycarbonyl methyl ester derivative of cysteine, and 363 and 364 or 365 for *N*,*S*-ethoxycarbonyl methyl ester derivative of glutathione were monitored using selective ion monitoring.

2.6. Measurement of glutathione synthesis rate in vivo

The glutathione method was applied to rat liver and human blood. Male Sprague Dawley rats ($n=8$; 300 g body weight) (IFFA CREDO, Saint Germain sur l'Arbresle, France) were catheterized in the external jugular vein 7 days before the study. They received a primed constant intravenous infusion of [^{15}N] cysteine (priming dose: 5 μmoles; infusion rate: 7.4 μmol/h) for 6 h. After anesthesia, rats were bled and the liver was rapidly excised, frozen in liquid nitrogen and stored at −80°C until analysis.

A human subject was given a primed constant intravenous infusion of [D_2] cysteine (priming dose: 2 μmol/kg; infusion rate: 2 μmol/kg.h) for 10 h beginning at 8 A.M. after an overnight fast. During the infusion, the subject drank every 20 min 21 ml of an enteral formula (Sondalis HC, Nestlé Clinical Nutrition, Creully, France). Blood samples were collected at intervals during the study for measurement of plasma cysteine and blood glutathione enrichment. Samples were frozen in liquid nitrogen and stored at −80°C until analysis.

3. Results and discussion

3.1. Method

Glutathione, cysteine and other aminothiols such as cysteinylglycine (CysGly) and γ-glutamylcysteine (γ-GluCys) in biological samples can be easily converted into their *N*,*S*-ethoxycarbonyl methyl ester derivatives by a modification of the method described by Kataoka et al. [12] and Takagi et al. [13]. The first step is the *N*,*S*-ethoxycarbonylation of

aminothiol compounds with ethylchloroformate in an alkaline medium. Since the pH decreased during the reaction, we used a buffered medium. Then, the *N,S*-ethoxy carbonyl derivatives were extracted into diethyl ether in acidic media. In order to obtain clean chromatograms, especially stable baselines, we found that the pH was required to be below 2 (ideally 1.2–1.5). In contrast to the methods described previously [12,13], we found that the derivatization steps can be applied directly to powdered tissue, but not to blood. In this case, the ether extraction does not proceed efficiently and deproteinization of the sample was required.

3.2. GC–MS analysis

As underlined by Kataoka et al. [12], GSH derivatives could not be eluted with columns commonly used for amino acids. By shortening the column to about 10 m and employing our chromatographic conditions, the GSH derivative eluted at about 4.5 min (Fig. 1) (this time can vary depending on the age and length of the column). Under the same conditions, the dipeptide L-γ-glutamyl-L-cysteine eluted at about 3 min and the dipeptide cysteinylglycine at about 2 min. The retention time of cysteine on the 30 m column was about 3.7 min.

The electron impact ionization mass spectra of *N,S*-ethoxycarbonyl methyl esters of cysteine and glutathione are shown in Fig. 2. The largest suitable fragments for selected ion monitoring of cysteine and glutathione are the isotope clusters at *m/z* 220 and 363, respectively. The stable isotopes [^{15}N] or [D_2] cysteine are present in the fragment used for determination of glutathione enrichment, but this fragment does not contain the glycine residue.

Fig. 3 shows the mass chromatograms from derivatized [D_2] cysteine at *m/z* 220 and 222, and [D_2] glutathione at *m/z* 363 and 365. For glutathione, the asymetric chromatographic peak is perhaps due to column overload, but our apparatus does not allow to use column with diameter larger than 0.25 mm as suggested by Kataoka et al. [12]. Moreover, this peak form has no adverse effect on the quantification of peak area. Standard curves are linear as indicated in Table 1.

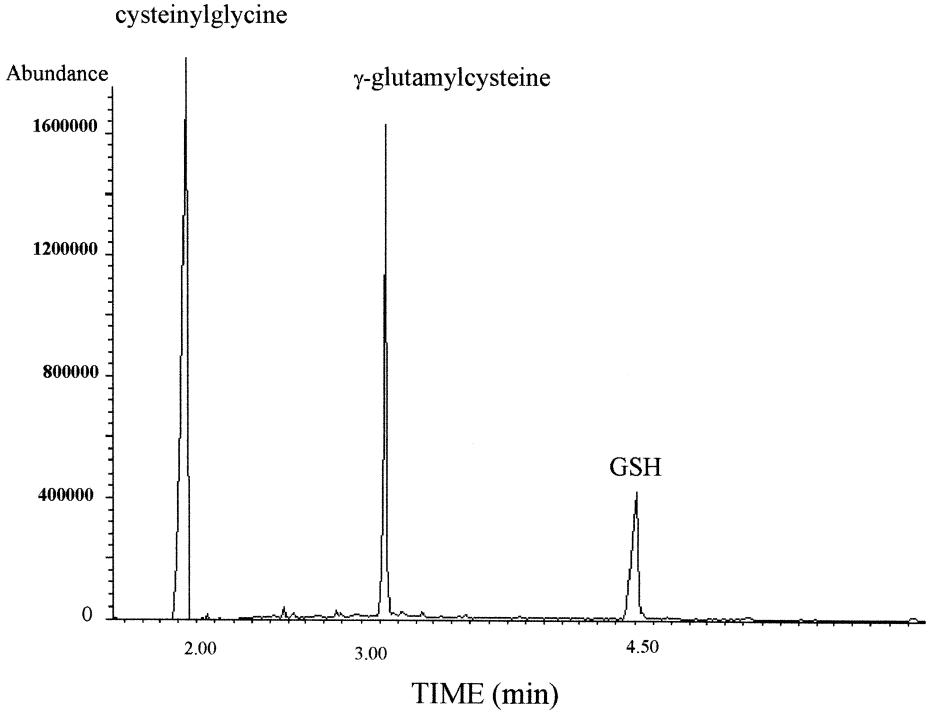

Fig. 1. Typical total ion chromatograms of the *N,S*-ethoxycarbonyl methyl ester of glutathione.

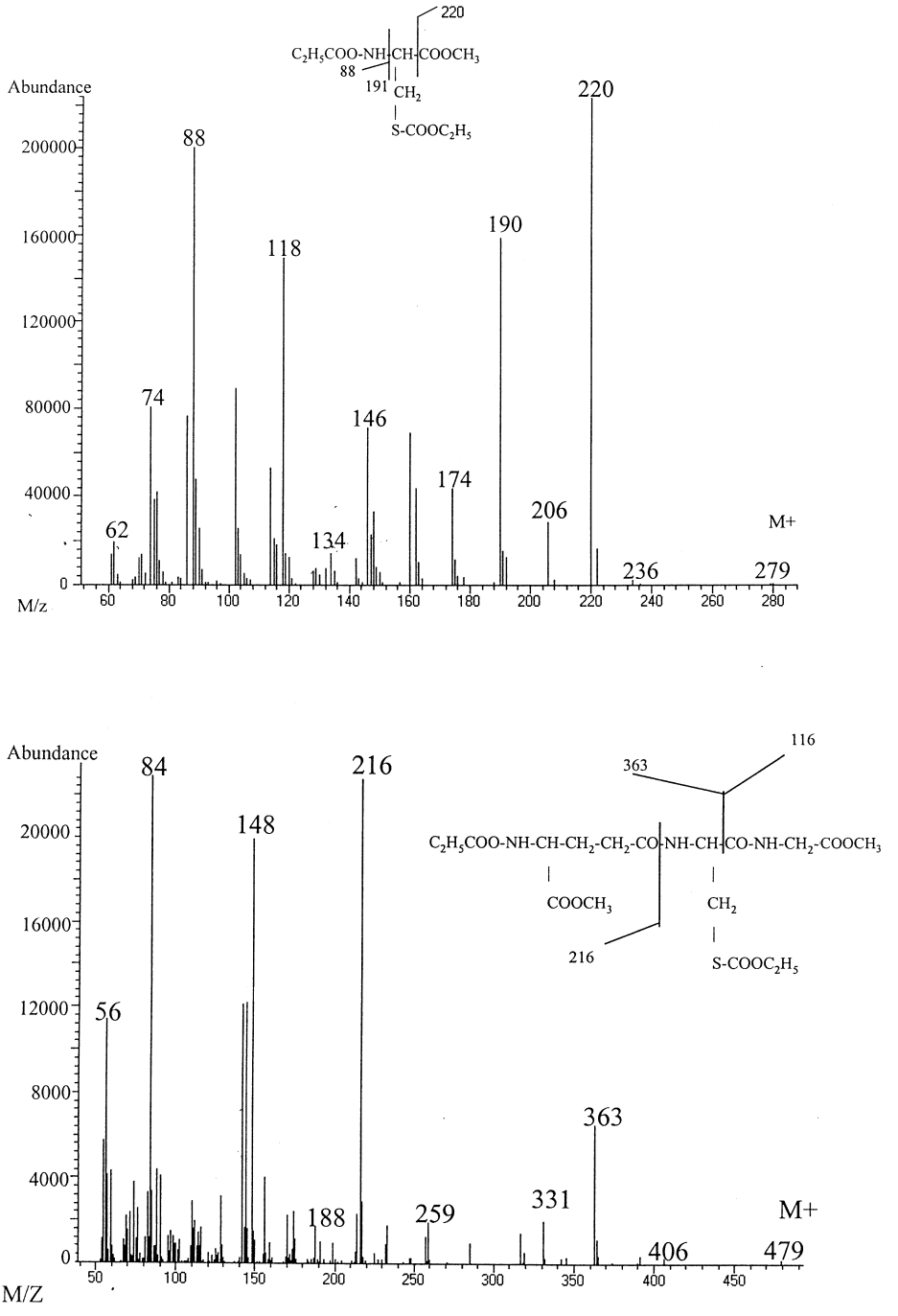

Fig. 2. Electron impact mass spectra of cysteine and glutathione as their *N*,*S*-ethoxycarbonyl methyl esters.

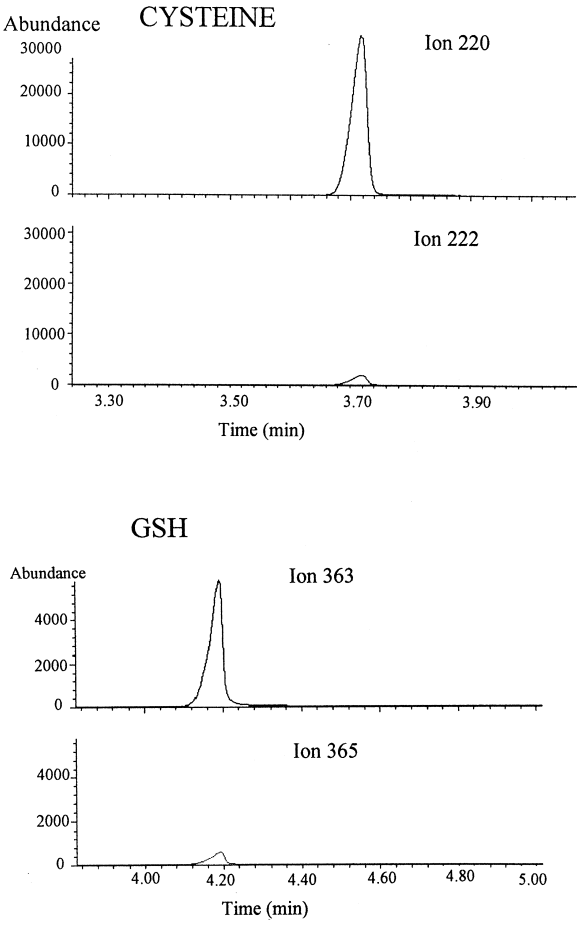

Fig. 3. Electron impact GC–MS selected ion chromatograms from samples of cysteine (ion 220, *M*), [3,3 $^{2}H_{2}$] cysteine (ion 222, M+2), glutathione (ion 363, *M*) and [D_2] glutathione (ion 365, M+2).

The repeatability is indicated in Table 2. The peak ratios of the *N*,*S*-ethoxycarbonyl methyl esters of cysteine and glutathione were determined with a coefficient of variation <1.5%. Measurements of standard solutions repeated on different days showed that the enrichment can be determined with a coefficient of variation <2%, Table 3.

As a measure of the accuracy of our method, we determined the difference between the enrichment obtained from the GC–MS measurements using calibration data (measured values) and the enrichment calculated from weighed amounts of non enriched and enriched compounds (theoretical values). Table 4 indicates that the difference between the two values was generally less than 2%.

3.3. Applications

The rate of blood glutathione synthesis was measured in an healthy human volunteer, using [D_2] cysteine infusion. As presented in Fig. 4, cysteine enrichment reached a plateau after about 4 h, giving a total plasma cysteine production rate of 39.8 μmol/kg.h in this subject. This value is in agreement with those obtained by Hiramatsu et al. [16] using a different derivatization procedure. Blood glutathione enrichment increased linearly during the infusion (Fig. 4). The fractional synthesis rate calculated from the slope of the time course of glutathione enrichment and the plasma cysteine enrichment at the plateau value was 22.5%/day. This is the first in vivo measurement of the blood glutathione synthesis rate in humans. Our result is consistent with the

Table 1
Calibration data for labelled *N*,*S*-ethoxycarbonyl methyl esters of cysteine and glutathione measured by GC–MS[a]

Compound	Slope	Intercept	Residual SE	Range (molar ratio ×100)	*n*
[^{15}N] Cysteine	1.023±0.002	10.213±0.017	0.021	0–15	4
[D_2] Cysteine	1.109±0.003	5.936±0.016	0.026	0–10	8
[^{15}N] Glutathione	0.978±0.032	17.39±0.12	0.162	0–6	7
[D_2] Glutathione	0.971±0.005	7.426±0.006	0.009	0–2	7

[a] Slope and intercept ±SD for one calibration curve for each compound; *y* axis: ratio of ion abundance (×100), 221/220 for [^{15}N] Cysteine, 222/220 for [D_2] Cysteine, 364/363 for [^{15}N] Glutathione and 365/363 for [D_2] Glutathione; *x* axis: theoretical molar ratio of labelled/natural compounds (×100) in the range indicated; *n*: number of different standards each analyzed in duplicate.

Table 2
Repeatability for labelled *N,S*-ethoxycarbonyl methyl esters of cysteine and glutathione in biological samples measured by GC–MS[a]

Compound	Peak ratio		Coefficient of variation (%)	Number of injections
	Selected ions	(%)		
[^{15}N] Cysteine	221/220	18.11±0.22	1.2	4
		25.06±0.32	0.6	3
[D_2] Cysteine	222/220	11.58±0.03	0.3	4
		13.95±0.04	0.3	4
[^{15}N] Glutathione	364/363	19.71±0.19	1.0	8
		22.20±0.20	0.9	5
[D_2] Glutathione	365/363	8.018±0.035	0.4	5
		8.754±0.036	0.4	3

[a] Values are means±SD; for each compound, the results on 2 independent samples from the studies described in Section 2 are indicated; [^{15}N] Cysteine in rat plasma; [D_2] Cysteine in human plasma; [^{15}N] Glutathione in rat liver; [D_2] Glutathione in human blood; coefficient of variation: SD as percentage of the mean.

value of 17%/day found by incubating human erythrocytes with ^{14}C glutamate [9]. In piglets, the glutathione synthesis rate measured with an infusion of ^{15}N glycine has been found to be 60%/day in blood erythrocytes [11].

Glutathione fractional synthesis rate was also determined in rat liver using [^{15}N] cysteine infusion. The plateau value for plasma cysteine enrichment was reached within 3 h (data not shown). The results of cysteine and glutathione enrichments in liver obtained after 6 h of infusion in a group of rats are shown in Table 5. From these data, we calculated that the glutathione fractional synthesis rate was 337±29%/day in this group of animals. Little work has been performed in vivo on glutathione synthesis in animal tissues, but all studies indicate that glutathione turnover rate is very rapid in the liver. Using ^{15}N glycine infusion, Jahoor et al. [11] reported a fractional synthesis rate of 175%/day in piglet liver two days after injection of turpentine. The apparent GSH turnover in liver, determined from the decrease of GSH specific radioactivity after injection of ^{35}S cysteine, is about 350%/day in young rats [17]. By inhibiting GSH synthesis using buthionine sulphox-

Table 3
Day to day precision for labelled *N,S*-ethoxycarbonyl methyl esters of cysteine and glutathione in standards at different levels of enrichment measured by GC–MS[a]

	MPE	Coefficient of variation (%)	Number of different days
[^{15}N] Cysteine	4.974±0.052	1.1	4
	10.513±0.059	0.6	3
[D_2] Cysteine	2.058±0.039	1.9	6
	4.277±0.037	0.9	6
[^{15}N] Glutathione	2.285±0.012	0.5	3
	4.649±0.086	1.9	3
[D_2] Glutathione	0.784±0.015	2.0	3
	1.404±0.010	0.7	3

[a] Values are mean ±SD; each standard was analyzed three times on different days distributed over 4 to 10 months; coefficient of variation: SD as percentage of the mean.

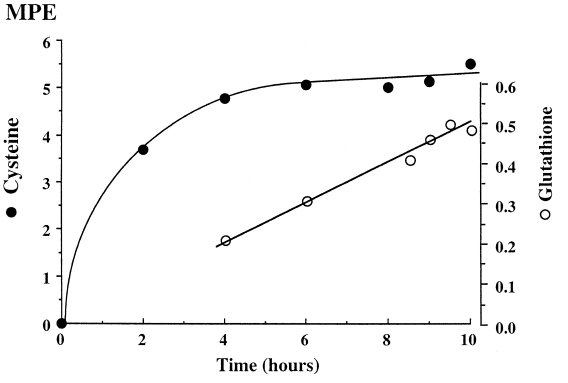

Fig. 4. Enrichment values of plasma cysteine and blood glutathione of a human subject given a continuous infusion of [3,3 $^2H_2$] cysteine. Glutathione fractional synthesis rate was calculated as follows: glutathione enrichment slope (%/h)×2400/cysteine enrichment plateau value (%). The value for this subject is 22.5%/day.

Table 4
Comparison of theoretical and measured enrichments of *N,S*-ethoxycarbonyl methyl esters of cysteine and glutathione in standards (MPE)[a]

	Calculated	Measured[b]	Difference[c]
[^{15}N] Cysteine	4.941	4.973±0.069 (3)	0.032 (0.6)
	10.430	10.474±0.054 (3)	0.044 (0.4)
[D_2] Cysteine	2.427	2.387±0.047 (4)	0.040 (1.7)
	5.980	6.028±0.014 (3)	0.048 (0.8)
[^{15}N] Glutathione	2.231	2.329±0.201 (8)	0.091 (4.0)
	4.631	4.697±0.194 (5)	0.066 (1.4)
[D_2] Glutathione	0.786	0.771±0.038 (3)	0.015 (1.9)
	1.687	1.714±0.012 (3)	0.027 (1.6)

[a] Values are means ±SD.
[b] Numbers between brackets: number of injections.
[c] Numbers between brackets: difference as percentage.

Table 5
Typical enrichment values of liver cysteine and glutathione at the end of a 6 h continuous infusion of [^{15}N] cysteine and glutathione fractional synthesis rates in rats

Animal	Cysteine (APE)	Glutathione (APE)	Glutathione synthesis (%/day)[a]
1	4.827	3.909	324
2	4.540	3.509	309
3	1.787	1.436	321
4	4.039	3.892	385
5	6.185	4.965	321
6	4.661	3.717	319
7	5.341	4.481	336
8	5.692	5.404	380
Mean ±SD			337±29

[a] Glutathione fractional synthesis rate was calculated as follows: glutathione enrichment×400/cysteine enrichment.

imine and assuming that the rate of GSH disappearance reflects GSH synthesis, Hunter and Grimble [6] indirectly estimated glutathione synthesis in rat liver to be 440%/day.

4. Conclusion

In conclusion, we propose an adaptation of an existing GC method for the determination of glutathione enrichment by GC–MS, using direct analysis of the peptide rather than measurement of the constitutive amino acids after hydrolysis of the molecule. This approach is a convenient and reliable tool to determine GSH synthesis rate in vivo and avoids time consuming and tedious treatment of samples, such as purification of the peptide, which can be critical with such an unstable molecule. The derivatization, also applied to cysteine, is fairly rapid, allowing sample derivatization and analysis by GC–MS on the same day. The results obtained show that the measurement of either [D_2] or [^{15}N] glutathione is accurate, precise and reproducible. Moreover, we have successfully determined the glutathione synthesis rate in human and animal tissues, showing that this method is useful for application in biochemical and biomedical research.

Acknowledgements

We wish to thank C. Bouteloup-Demange and B. Beaufrère for performing the human protocol, and P. Denis and C. Buffière for the animal study.

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