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Liquid chromatography–tandem mass spectrometry assay of reduced and oxidized glutathione and main precursors in mice liver

Jérôme Bouligand [a], Alain Deroussent [b], Angelo Paci [a,c,*], Jackie Morizet [a], Gilles Vassal [a]

[a] *UPRES EA3535 Pharmacology and New Cancer Treatments, Institute Gustave Roussy and Paris XI University, Villejuif, France*
[b] *Mass Spectrometry Platform, IFR 54, Institute Gustave Roussy, Villejuif, France*
[c] *Department of Clinical Pharmacy, Institute Gustave Roussy, Villejuif, France*

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Abstract

A liquid chromatography/tandem mass spectrometry assay of glutathione (GSH), glutathione disulfide (GSSG) and of precursors (γ-glutamyl-cysteine, cysteinyl-glycine, cysteine, cystine, homocysteine and homocystine) was developed to study glutathione synthesis in mice liver. After iodoacetic acid derivatization, the analytes were analyzed using reversed-phase gradient HPLC and detected using multiple reaction monitoring. Linear calibrations were performed over the concentrations range of 100–10,000 ng/mL for the thiol-containing precursors and extended up to 100,000 ng/mL for GSH and GSSG. The method was validated for each compound with inter-day accuracy below 11.9% and with precision below 15%. The method showed low limits of quantitation of 100 ng/mL for each thiol-containing compound and GSSG and of 200 ng/mL for other disulfides.

Keywords: HPLC–MS/MS; Glutathione; Disulfide glutathione; Glutathione precursors; Liver; Mice

1. Introduction

Endogenous glutathione (GSH) is a ubiquitous tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), present in both prokaryotes and eukaryotes. GSH and other intracellular low molecular mass thiols play a crucial role to protect cells against reactive oxygen species produced in mammalians during respiration, metabolism or inflammation. Glutathione peroxidase and glutathione reductase are essential enzymes allowing a fine regulation of glutathione redox status, i.e. ratio reduced/disulfide forms, one of the key point of glutathione antioxidant properties. Furthermore, GSH plays a key role in protecting cells from various electrophile xenobiotics which are eliminated through their GSH conjugation by various glutathione *S*-transferases [1]. Many studies have pointed out the importance of glutathione homeostasis during human diseases or intoxication by toxins, particularly in the liver [2–5], in the kidney [6] and in the central nervous system [7]. A progressive alteration of glutathione status was shown during aging [8]. There is increased evidence that toxicity of chemicals interacting with GSH may be due either to GSH depletion [4] or to the activation of different metabolic pathways enabling GSH synthesis such as the *trans*sulfuration pathway with homocysteine accumulation [9]. The simultaneous quantification of GSH and its precursors [10] through the metabolic pathways (Fig. 1) can be useful to interpret more precisely the role of GSH during intoxication. Furthermore, recent experiments in mice have shown the possible enhancement of GSH synthesis during low oxidative stress conditions through activation of the Nrf2/Keap1 pathway [11,12] with activation of the antioxidant responsive element and induction of the expression of several phase-2 genes, glutathione synthetase being a case in point. Accurate and simultaneous determination of GSH metabolome and redox status for several of these thiols is thus necessary to establish correlation between genes transcription, enzymes expression and the flow of metabolites produced in vivo or in vitro by these enzymes, under various stress conditions. Such an approach has already been proposed recently in vitro on yeasts injured with cadmium [13]. We chose to develop another method to study fine regulation of glutathione synthesis in the liver during treatment of mice with high doses of drugs known as liver

* Corresponding author. Tel.: +33 1 42 11 47 30; fax: +33 1 42 11 53 08.
E-mail address: apaci@igr.fr (A. Paci).

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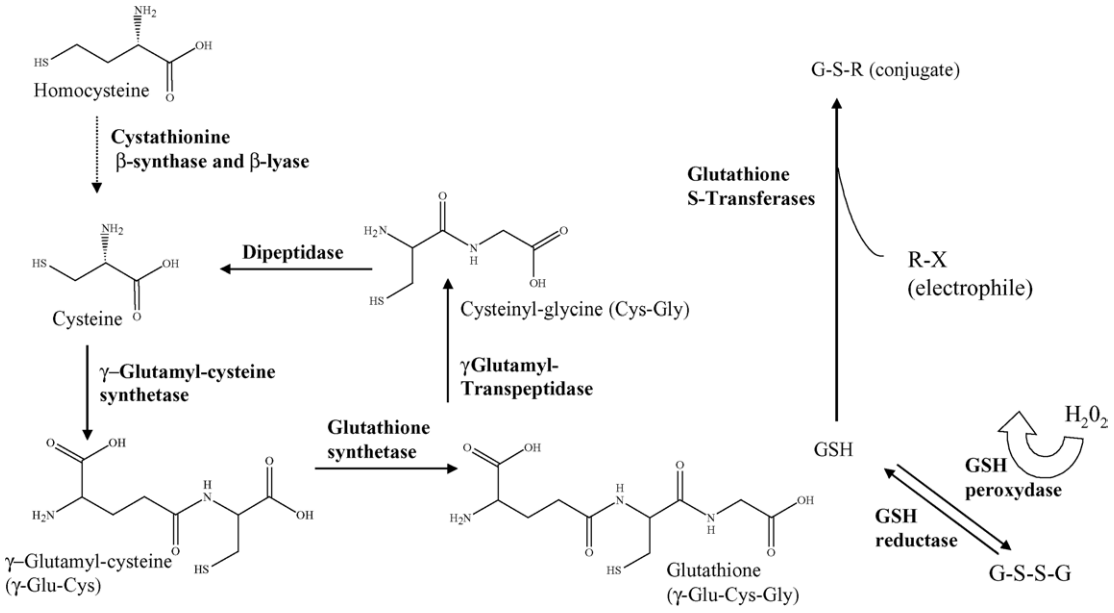

Fig. 1. Glutathione metabolic pathways.

toxins, such as acetaminophen [14] and various anticancer drugs [4].

Several previous analytical methods claimed to quantify thiols content in tissues, such as in various organ homogenates [15–18] or in blood plasma [19,20]. These methods used gas chromatography [19], capillary electrophoresis [21,22] or high performance liquid chromatography [10,16–18,23–25]. Gas chromatography/mass spectrometry was specially applied to homocysteine determination in human plasma [19]. HPLC methods were used on several applications and were developed with various detection techniques such as ultra-violet absorbance [26], fluorescence [16,23], electrochemical detection [27–29] and mass spectrometry [17,18,20,24,25]. HPLC methods using fluorescence [16,23] or tandem mass spectrometry detection [18] demonstrated better sensitivity in tissues (LLOQ of 50 ng/mL for GSH) than those using selected ion monitoring mass spectrometry [17].

For determination of low mass thiols content in organs, pieces of tissue were most frequently homogenized at +4 °C with an acidic solution (pH ≤ 2) to prevent the oxidation of the thiol moiety to the disulfide counterpart. For this purpose, perchloric acid (10% v/v) is most often used leading to whole proteins precipitation [16,26]. In these acidic conditions, the assay results are thus given relative to tissue wet weight, because protein content was not available. Other strategies proposed less acidic homogenization conditions [17] enabling subsequent protein determination with similar results in term of sample quality and allowed taking into account some variations due to homogenization procedure. After sample homogenization, few methods determined underivatized thiols content [24], whereas most strategies proposed a derivatization step useful for thiols quenching [26] and for providing stable derivatives more easily detectable [15,16,23]. Santori et al. [26] had demonstrated that iodoacetic acid was one of the most efficient thiol quencher to obtain carboxymethyl (CM) derivatives. However, an additional derivatization step, with 1-fluoro-2,4-dinitrobenzene, was required for UV detection. Nevertheless, HPLC–MS methods allowed faster preparation and direct detection of carboxymethyl derivatives and disulfides. High performance liquid chromatography and tandem mass spectrometry (HPLC–MS/MS) has shown to be a selective and sensitive method to perform determination of glutathione [18,24,25].

Thus, after thiols derivatization with iodoacetic acid and subsequent precipitation of proteins with sulfosalicylic acid, a rapid and simultaneous HPLC–MS/MS method was developed to quantify reduced (GSH) and oxidized glutathione (GSSG), γ-glutamyl-cysteine (γ-Glu-Cys) and cysteinyl-glycine (Cys-Gly), cysteine, cystine, homocysteine and homocystine, using glutathione ethyl ester (GSHee) as a single internal standard. This method for determining the relative content of thiols in tissue homogenates was validated over the range of concentrations from 100 to 10,000 ng/mL for thiols precursors and extended up to 100,000 ng/mL for GSH and GSSG. These ranges of concentrations were shown to be suitable for the assay of these endogenous compounds in the liver of mice.

2. Experimental

2.1. Chemicals and reagents

Reduced glutathione, oxidized glutathione, γ-glutamyl-cysteine trifluroacetate salt, cysteine hydrochloride salt, homocysteine, homocystine, cysteinyl-glycine and glutathione ethyl ester, cystine were supplied with high purity from Sigma (St. Quentin Fallavier, France). HPLC grade acetonitrile was provided by Carlo Erba (Rodano, Italy), formic acid by Merck (Darmstadt, Germany), potassium chloride by Prolabo (Paris, France), ethylene diamine-tetracetic acid, disodium salt, dihydrate (EDTA) by Labosi (Elancourt,

France). Iodoacetic acid (IAA), ammonium bicarbonate and *batho*phenanthrolinedisulfonic acid disodium salt (BPDS) were from Aldrich (Steinheim, Germany), bovine albumin from Euromedex (Mundosheim, France). Sterile water was provided from Fresenius (Sèvres, France) and deionized water was prepared using a Milli-Q™ system (Millipore, St Quentin-en-Yvelines, France).

2.2. Stock solutions and standards

Independent standard and quality control stock solutions were prepared and stored at −20 °C. Each thiol (GSH, γ-Glu-Cys, Cys-Gly, cysteine and homocysteine) was dissolved in water to obtain a 5 mg/mL stock solution. For cysteine hydrochloride and γ-glutamyl-cysteine trifluoroacetate, we took into account the concentration of the base form with the conversion factor (base MW/salt MW). Each disulfide (GSSG, cystine and homocystine) was dissolved in HCl 1N to obtain a 5 mg/mL stock solution. These stock solutions were mixed to prepare a working solution containing reduced and oxidized glutathione at 0.5 mg/mL and another working solution containing all the eight analytes set at 0.05 mg/mL. The internal standard, glutathione ethyl ester (GSHee), was prepared in water to a 1000 ng/mL concentration. Quality control working solutions were set at 0.4 and 0.04 mg/mL. Calibration standards and quality controls samples were prepared by adding 100 μL of an appropriate working solution containing the eight analytes to 400 μL of solution of bovine serum albumin solution (10 g/L in water and formic acid, 0.1% v/v). Thus, six standard concentrations containing the eight analytes (100, 200, 500, 1000, 5000 and 10,000 ng/mL) and two other standards with GSH and GSSG (50,000 and 100,000 ng/mL) were prepared. Three quality control (QC) samples containing all compounds were prepared: 400, 800 and 8000 ng/mL. Another QC (80,000 ng/mL) with GSH and GSSG only were prepared. Standard and QC samples were treated with the steps of derivatization and of protein precipitation before analysis by HPLC–MS/MS.

2.3. Liver sample homogenization

Livers were excised from male C57BL6 mice of 26 ± 2 g. The samples were frozen immediately in liquid nitrogen and stored at −80 °C until analysis. Hepatic samples (50 mg of frozen tissue) were thawed at +4 °C with 800 μL of homogenization solution (pH 2) composed of formic acid (0.1% v/v), potassium chloride (1.15% w/v), EDTA 1mM, BPDS 2 mM. A homogenizer PowerGen125 (Fischer Scientific, France) was used during 15 s per sample. The mixtures were centrifuged at 16,000 × *g* during 15 min at +4 °C. The supernatant (homogenates) were retrieved and processed to determine glutathione and related thiols. After dilution (1/20) of homogenates, proteins content were determined in liver homogenates using the micro BCA™ protein reagent assay (Pierce, Rockford, IL, USA). Experiments with animals were carried out in compliance with the conditions established by the European Union (Directive no. 86/609/CEE).

2.4. Sample preparation

Fifty-microlitre aliquot of sample (standard, QC or tissue homogenate) was mixed with 50 μL of internal standard solution (GSHee). It was then treated with 100 μL of 10 mM IAA in 10 mM aqueous ammonium bicarbonate and ammoniac (0.5% v/v) derivatization solution (pH 9.5). This mixture was stored at room temperature for 15 min. The reaction was stopped and the proteins were precipitated by addition of 50 μL of cold sulfosalicylic acid solution (10% w/v). The mixture was then centrifuged at 16,000 × *g* at +4 °C for 15 min. The supernatant (200 μL) was transferred to glass snap-ring clipped vials and stored at −20 °C until analysis. Twenty microlitres were injected into the HPLC–MS/MS system.

2.5. High performance liquid chromatography and tandem mass spectrometry

Samples were analyzed with an 1100 series HPLC system (Agilent Technologies, Massy, France) including an autosampler, a binary pump and a Uptisphere® C18 column 3 μm, 2 mm i.d. × 100 mm length (Interchim, Montluçon, France). The flow-rate of 0.25 mL/min was achieved with a elution gradient composed of solvent A (0.1% formic acid in water) and of solvent B (acetonitrile/water 20:80, v/v with 0.1% formic acid). The gradient was as follows: 100% solvent A for 2 min; 2-min linear increase up to 100% solvent B; 100% B step for 3 min; 100% solvent A from 7.1 to 15 min. The total analysis time was 15 min. The autosampler syringe was washed with solvent A before each injection.

Detection was performed on a Quattro-LCZ triple quadrupole mass spectrometer equipped with the orthogonal electrospray source (Micromass, Manchester, UK). The HPLC flow was diverted from 0 to 2.0 min to waste avoiding contamination of the detector with sulfosalicylic acid. Analytes were detected in the positive ion mode using tandem mass spectrometry with multiple reaction monitoring (MRM). The dwell time was set at 0.5 ms. The capillary voltage was set at 3500 V. The source temperature and the nebulization gas temperature were set at 100 and 350 °C, respectively. Collision gas (argon) pressure was set at 1.3 mbar. The cone voltages were set at 30 V, except at 40 V for GSSG. Collision energies and transitions ion pairs were optimized for each analyte on reference compounds (Table 1). Data were processed using MassLynx™ software (Micromass, Manchester, UK).

2.6. Validation procedure of the HPLC–MS/MS assay

The quantitative HPLC–MS/MS assay was validated according to International Conference of Harmonization (ICH) guidelines [30,31] in terms of selectivity, calibration, accuracy and precision.

2.6.1. Selectivity

The selectivity was studied by preparing and analyzing a LLOQ standard compared to blank samples and blank bovine

Table 1
MRM transitions for the detection of carboxymethyl thiols and disulfides by HPLC–MS/MS

Analyte	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	Collision energy (eV)
Cystine	241.1	152.1	15
CM-cysteine	180.1	89.2	18
Homocystine	269.2	136.1	15
CM-homocysteine	194.1	56.2	15
CM-Cys-Gly	237.2	174.1	15
γ-Glu-(CM)Cys	309.2	180.1	15
CM-GSH	366.2	237.1	15
GSSG	613.2	355.1	20
CM-GSHee	394.2	265.1	15

CM = carboxymethyl.

albumin samples spiked with the internal standard. Underivatized liver homogenate with ammonium bicarbonate 10 mM were also analyzed. The selectivity was thus warranted through specific thiol derivatization procedure, chromatographic separation and tandem mass spectrometry detection.

2.6.2. Calibration curves and LLOQ

Calibration curves were obtained with Masslynx™ software by plotting the peak area ratio of each analyte and the internal standard against the actual concentration of analyte using regression and $1/x^2$ weighting over the range from 100 to 10,000 ng/mL and extended up to 100,000 ng/mL for GSH and GSSG. Each standard was prepared in duplicate over 3 days.

The low limit of quantitation (LLOQ) was defined as the lowest concentration of each analyte that can be determined with accuracy and precision [31].

2.6.3. Accuracy and precision

Accuracy was measured by the deviation or bias (%) of the mean found concentration from the actual concentration on standards and on quality controls (QC).

Repeatability and intermediate precision were studied. Intra-day precision, expressed as the coefficient of variation of repeatability (CVr), was performed for the four levels of QC (five replicates). Inter-day precision, expressed as the coefficient of variation of intermediate precision (CVi), was evaluated for each level of QC over 3 days (fifteen replicates).

2.6.4. Derivatization and extraction yields and recovery study

We checked for each compound that derivatization yield was complete by analyzing both underivatized and derivatized thiols. Extraction yield was calculated for each analyte by the comparison of concentrations determined for standards prepared with or without albumin (10 g/L). The recovery study was conducted through the preparation of two biological samples. The first sample was a reference liver homogenate and the second sample was the same sample spiked with 5000 ng/mL of the different compounds. The value determined from subtraction of the analyte concentration determined in the reference sample from that determined in the spiked liver sample was used to determine recovery.

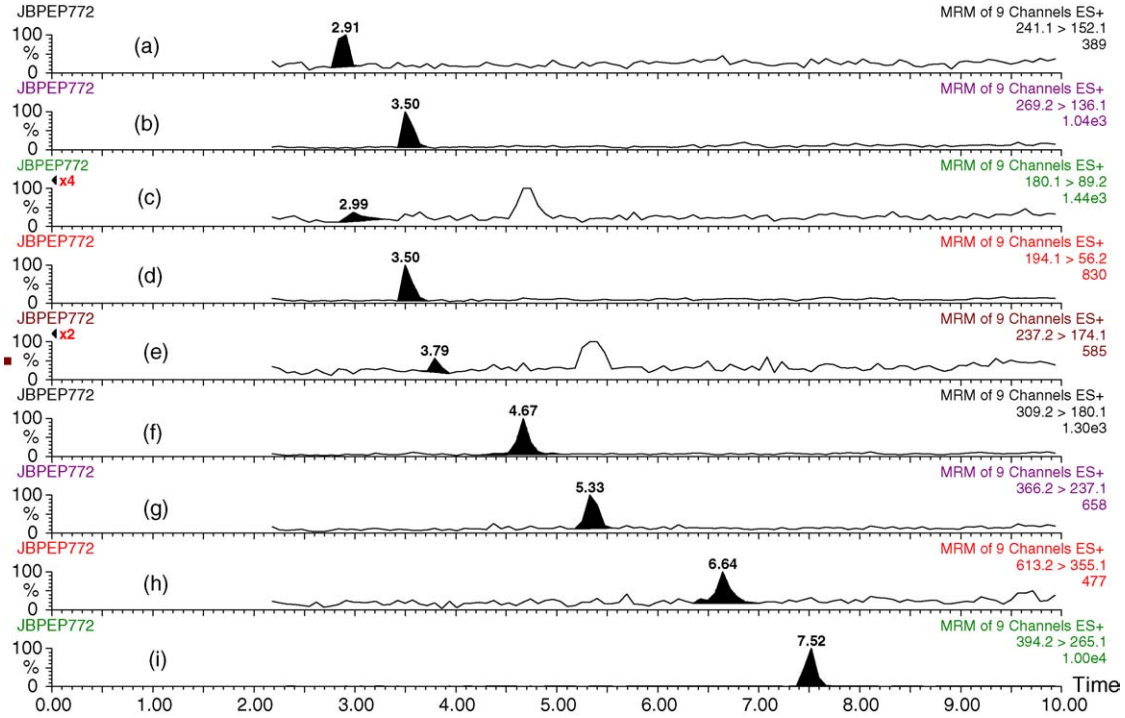

Fig. 2. Mass chromatogram of a LLOQ standard sample spiked with 100 ng/mL of (a) cystine, (b) homocystine, (c) CM-cysteine, (d) CM-homocysteine, (e) CM-Cys-Gly, (f) γ-Glu-(CM)Cys, (g) CM-GSH, (h) oxidized glutathione GSSG and with 1000 ng/mL of (i) internal standard CM-GSHee. Concentrations are expressed in underivatized compounds.

3. Results and discussion

3.1. HPLC–MS/MS analysis

For each analyte, ion scan (MS) and daughter scan (MS/MS) analyses have been previously achieved by direct infusion of each standard diluted in acetonitrile/water (20:80 v/v) with 0.1% formic acid. These data allowed optimizing the MS/MS parameters in order to obtain the best sensitivity for each compound in MRM mode of the quantitation method. Thus, selected MS/MS transitions were presented for each analyte in Table 1. Derivatized carboxymethyl thiols and their corresponding disulfides could be resolved by a linear gradient reversed-phase HPLC and by tandem mass spectrometry with positive electrospray ionization (ESI-MS/MS) under acidic conditions (pH 2.5) compatible with the reverse phase C18 column and for positive ESI detection. The HPLC–MS/MS run allowed adequate separation of cysteine, homocysteine, Cys-Gly, γ-Glu-Cys, GSH, GSSG and GSHee (IS) with retention times of 3.0, 3.5, 3.8, 4.7, 5.4, 6.6, and 7.5 min, respectively (Fig. 2). Nevertheless, this method could only resolve cysteine from cystine by means of their specific MRM transitions (m/z 180 → 89, m/z 241 → 152, respectively) and homocysteine from homocystine transitions (m/z 194 → 56, m/z 269 → 136, respectively).

3.2. Validation of the HPLC–MS/MS method

3.2.1. Stability

Stability of carboxymethyl derivatives in standards and in sample extracts was at least 2 months at −20 °C. Furthermore, stability of the analytes in extracted samples kept at room temperature under experimental conditions was at least 48 h.

3.2.2. Selectivity and sensitivity

The method was selective using specific MRM transitions and HPLC separation for each analyte. The selectivity was demonstrated by comparing analyses of a standard mixture sample at 100 ng/mL (Fig. 2) and an extracted blank sample.

Carboxymethyl-glutathione (CM-GSH) was detected on the mass chromatogram with the main transition of m/z 366 → 237 corresponding to the loss of pyroglutamic acid (129 a.m.u.) and with the second transition of m/z 237 → 174 corresponding to detection of a CM-Cys-Gly moiety. This was free of consequence, because the two analytes were separated with HPLC (t_R = 5.4 and 3.8 min). A similar observation was made between γ-Glu-(CM)Cys (m/z 309 → 180, t_R = 4.7 min) and cysteine (m/z 180 → 89, t_R = 3.0 min). These observations confirmed the choice of performing a 7-min linear gradient HPLC combined with the specific MS/MS detection (Figs. 2 and 3).

3.2.3. Calibration curves and LLOQ

The calibration curves were determined over the range 100–10,000 ng/mL for the compounds (γ-Glu-Cys, Cys-Gly, cysteine and homocysteine) and over the range 200–10,000 ng/mL for cystine and homocystine, and up to 100,000 ng/mL for reduced glutathione (GSH) and for GSSG. Linear regressions with $1/x^2$ weighting were performed for the five thiol-containing compounds, while quadratic fits were applied for the three disulfides. Mean calibration equations were obtained with regression coefficients (r^2) from 0.961 (γ-Glu-Cys) to 0.996 (GSH) and are shown in Tables 2 and 3. According to criteria of accuracy and precision lower than 20%, LLOQ were found at 100 ng/mL for GSH, GSSG, γ-Glu-Cys, Cys-Gly, homocysteine and cysteine, while LLOQ were found at 200 ng/mL for homocystine and cystine.

3.2.4. Accuracy and precision

The results of the accuracy study performed for each quality control over 3 days are summarized in Table 4. Mean inter-day accuracies were lower than 15% for all compounds since all bias values were within −11.9% and +10.3%. Studies of intra-day precision (repeatability or CVr) and of inter-day intermediate

Table 2
Calibration and LLOQ for thiols compounds

Compound	r^2	b	a	LLOQ (ng/mL)	CV (%)
GSH	0.996	1.61×10^{-3}	3.48×10^{-1}	100	5
γ-Glu-Cys	0.960	4.05×10^{-3}	-3.68×10^{-2}	100	6
Cys-Gly	0.978	4.94×10^{-4}	-1.28×10^{-2}	100	20
Cysteine	0.981	3.05×10^{-4}	-4.05×10^{-3}	100	9
Homocysteine	0.961	1.56×10^{-3}	-1.16×10^{-2}	100	12

Typical equation is $y = bx + a$, where x is the analyte concentration and y is the ratio of analyte area and internal standard area. The slopes, intercepts and regression coefficient r^2 were obtained from three calibration curves fitted with least-square linear regression with a $1/x^2$ weighting factor.

Table 3
Calibration curves and LLOQ for disulfides compounds

Compound	r^2	c	b	a	LLOQ (ng/mL)	CV (%)
GSSG	0.987	6.41×10^{-3}	5.41×10^{-4}	7.31×10^{-2}	100	10
Cystine	0.973	-4.43×10^{-3}	2.13×10^{-4}	6.00×10^{-3}	200	16
Homocystine	0.969	-3.35×10^{-8}	1.70×10^{-3}	5.32×10^{-2}	200	13

Typical equation is $y = cx^2 + bx + a$, where x is the analyte concentration and y is the the ratio of analyte area and internal standard area. Mean equation and the regression coefficient r^2 were obtained from three calibrations curves fitted with least-square quadratic regression with a $1/x^2$ weighting factor.

Table 4
Inter-day accuracy of glutathione and its precursors in quality control (QC) samples

Compound	QC 400 ng/mL (%)	QC 800 ng/mL (%)	QC 8000 ng/mL (%)	QC 80000 ng/mL (%)
GSH	−0.6	+0.2	+0.2	−11.9
γ-Glu-Cys	−4.4	−7.3	−3.9	
Cys-Gly	+4.2	−0.7	+9.7	
Cysteine	−3.9	+6.1	+0.3	
Homocysteine	−4.1	+10.3	+5.1	
GSSG	+0.8	−5.6	−3.7	+0.1
Cystine	+1.8	+5.8	−0.5	
Homocystine	+5.3	+5.5	−6.5	

Mean values are calculated for each QC level. Inter-day accuracy is expressed as bias (%).

Table 5
Repeatability (CVi) of glutathione and its precursors in quality control (QC) samples

Compound	QC 400 ng/mL (%)	QC 800 ng/mL (%)	QC 8000 ng/mL (%)	QC 80000 ng/mL (%)
GSH	11.8	13.6	4.2	4.7
γ-Glu-Cys	9.6	11.4	8.6	
Cys-Gly	4.9	7.3	3.5	
Cysteine	5.2	6.7	9.7	
Homocysteine	6.4	9.7	6.2	
GSSG	11.9	10.7	11.0	10.1
Cystine	10.0	6.8	15.0	
Homocystine	7.0	8.4	6.9	

Values were calculated for five different preparations over 1 day.

Table 6
Intermediate precision (CVi) of glutathione and its precursors in quality control (QC) samples

Compound	QC 400 ng/mL (%)	QC 800 ng/mL (%)	QC 8000 ng/mL (%)	QC 80000 ng/mL (%)
GSH	9.3	12.0	6.5	5.7
γ-Glu-Cys	14.3	13.3	12.5	
Cys-Gly	4.2	6.9	8.0	
Cysteine	13.2	13.9	7.7	
Homocysteine	6.2	7.5	6.3	
GSSG	12.2	14.9	13.9	10.1
Cystine	14.8	11.7	13.3	
Homocystine	11.2	6.6	7.9	

Values were calculated for fifteen different preparations over 3 days.

precision (CVi) were performed on each quality control for the eight analytes. The method was repeatable for all compounds with CVr below 15% (Table 5). CVi results varied from 4.2 to 14.9% as shown in Table 6 and were in agreement with CVi lower than 15% for all analytes (thiols and disulfides).

3.2.5. Derivatization and extraction yields and recovery study

We checked that thiols derivatization after 15 min was quantitative for standards and for extracted samples with yields >99%. Extraction yields for the different compounds and for the internal standard were between 85 and 104%. The mean recovery yields ($n = 3$) in liver samples (spiked with $c = 5$ μg/mL) for the main analytes, GSH, GSSG, γ-Glu-Cys, Cys-Gly, cysteine and homocysteine, were 89 ± 13, 113 ± 12, 96 ± 6, 111 ± 9, 84 ± 9 and 106 ± 9%, respectively. The recovery results were lower for cystine and homocystine with 58 ± 18 and 62 ± 13%, respectively.

3.3. Determination of glutathione and precursors in liver of mice

This validated HPLC–MS/MS assay was applied to study the regulation of glutathione in the liver of mice. Mass chromatogram (Fig. 3) of a mouse liver sample showed GSH and its five precursors. The concentrations of the analytes in liver samples were expressed in nanomoles per mg of protein content as shown in Table 7. The value of total glutathione was in agreement with previous results obtained in the same strain of mice by Lee et al. [32]. The ratio of GSSG to total glutathione was found to be always below 12%. The content of cysteine, γ-Glu-Cys, Cys-Gly were also determined about 1–10% of total

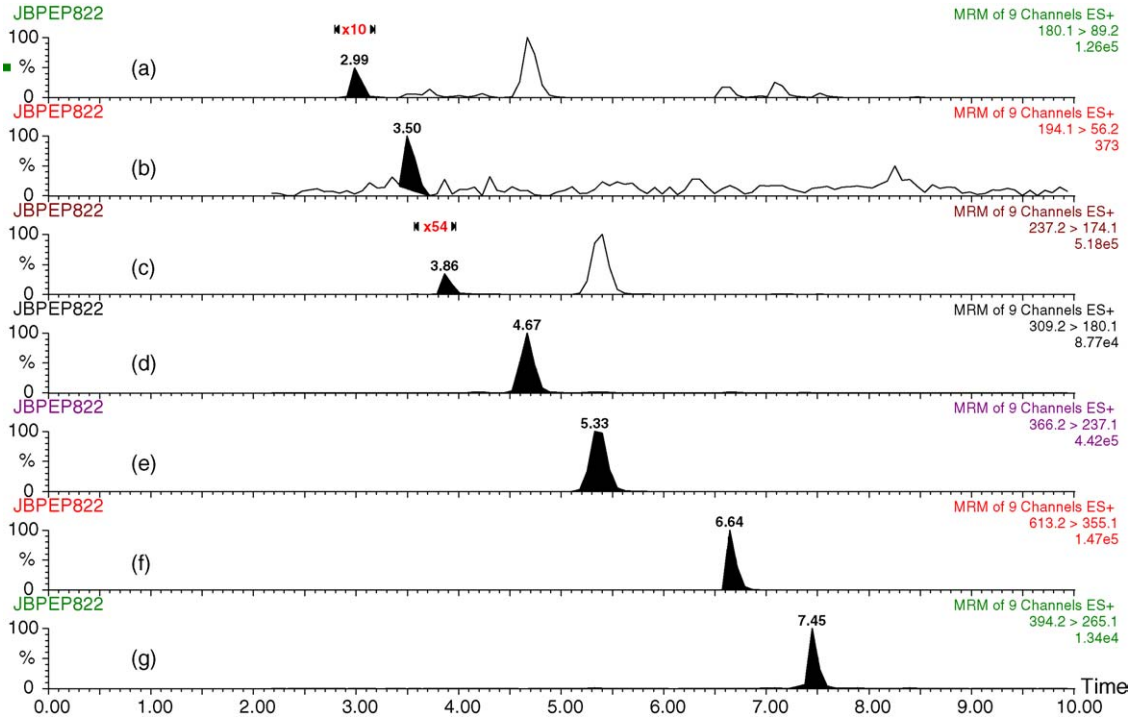

Fig. 3. Mass chromatogram of a mouse liver sample containing concentrations of (a) CM-cysteine at 3027 ng/mL, (b) CM-homocysteine at 51 ng/mL, (c) CM-Cys-Gly at 1121 ng/mL, (d) γ-Glu-(CM)Cys at 5173 ng/mL, (e) CM-GSH at 73,908 ng/mL, (f) oxidized glutathione GSSG at 24,159 ng/mL and (g) internal standard CM-GSHee at 1000 ng/mL. Concentrations are expressed in underivatized compounds.

Table 7
Determination of glutathione and its precursors in the liver of mice

Compound	Concentration ± S.D. (nmol/mg protein)[a]
Total glutathione	60 ± 17
Reduced glutathione GSH	53 ± 15
Oxidized glutathione GSSG	3.7 ± 2.8
γ-Glu-Cys	1.1 ± 0.3
Cys-Gly	0.60 ± 0.15
Cysteine	2.6 ± 0.9
Cystine	ND
Homocysteine	0.17 ± 0.03
Homocystine	ND

[a] Mean values are given from six different animals.; ND: not detected.

glutathione. Homocysteine was detected at a concentration close to the LLOQ. Its content was estimated about 0.2–0.5% of total glutathione. Homocystine and cystine could not be detected in the studied mice liver.

4. Conclusion

This HPLC–MS/MS assay was applied to study glutathione synthesis in mice liver. This method was selective, accurate and precise in agreement with the validation guidelines. Its sensitivity and the concentration range were suitable for glutathione and its main precursors. The LLOQ (100 ng/mL) for the main six compounds (Tables 2 and 3) was similar to previous results in biological samples obtained by other HPLC–MS/MS methods [17,18,25]. However, due to the use of a single IS, determination of the thiol content is not "absolute" but "relative" to the sample preparations. Deuterated internal standards or ^{15}N metabolic labelling [24] have been used and could be applied for determinations of GSH metabolome. We would like to emphasize that the variation of analytes results in tissue homogenates was necessary "relative" to the experimental conditions due to the pre-analytical procedure, i.e. the homogenization of the tissue piece. Furthermore, it means that standardization of the homogenization procedure was useful and essential to be in order to compare the content of compounds in the different biological samples. To achieve this purpose, samples should be homogenized and prepared the same day under standardized conditions. Determination of protein content in the homogenate may help reflecting partially the extraction of the studied analytes.

The present work has shown the development and the validation of a convenient HPLC–MS/MS method to quantify reduced and oxidized glutathione and main precursors (γ-Glu-Cys, Cys-Gly, cysteine, homocysteine, cystine and homocystine) with good sensitivity, accuracy and precision. After a fast sample preparation, a simple assay allowed the determination of GSH and thiol-containing compounds in the liver of mice and can be further applied to study the regulation of glutathione synthesis in this mice strain under various anticancer treatments.

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References

[1] C.M. Paumi, B.G. Ledford, P.K. Smitherman, A.J. Townsend, C.S. Morrow, J. Biol. Chem. 276 (2001) 7952.
[2] P. Zhao, J.T. Slattery, Drug Metab. Dispos. 30 (2002) 1413.
[3] P. Zhao, T.F. Kalhorn, J.T. Slattery, Hepatology 36 (2002) 326.
[4] L.D. DeLeve, Hepatology 24 (1996) 830.
[5] C.S. Lieber, Alcohol 34 (2004) 9.
[6] F. Santangelo, V. Witko-Sarsat, T. Drueke, B. Descamps-Latscha, Nephrol. Dial. Transplant. 19 (2004) 1951.
[7] M. Hsu, B. Srinivas, J. Kumar, R. Subramanian, J. Andersen, J. Neurochem. 92 (2005) 1091.
[8] A. Hernanz, E. Fernandez-Vivancos, C. Montiel, J.J. Vazquez, F. Arnalich, Life Sci. 67 (2000) 1317.
[9] L.E. Adinolfi, D. Ingrosso, G. Cesaro, A. Cimmino, M. D'Anto, R. Capasso, V. Zappia, G. Ruggiero, Hepatology 41 (2005) 995.
[10] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[11] S.A. Chanas, Q. Jiang, M. McMahon, G.K. McWalter, L.I. McLellan, C.R. Elcombe, C.J. Henderson, C.R. Wolf, G.J. Moffat, K. Itoh, M. Yamamoto, J.D. Hayes, Biochem. J. 365 (2002) 405.
[12] H. Motohashi, M. Yamamoto, Trends Mol. Med. 10 (2004) 549.
[13] A. Lafaye, C. Junot, Y. Pereira, G. Lagniel, J.C. Tabet, E. Ezan, J. Labarre, J. Biol. Chem. (2005), doi:10.1074/jbc.M502285200.
[14] N.L. Kobrinsky, D.E. Sjolander, J.A. Goldenberg, T.C. Ortmeier, Pediatr. Blood Cancer 45 (2005) 222.
[15] A.I. Minchinton, Int. J. Radiat. Oncol. Biol. Phys. 10 (1984) 1503.
[16] J. Martin, I.N. White, J. Chromatogr. 568 (1991) 219.
[17] X. Guan, B. Hoffman, C. Dwivedi, D.P. Matthees, J. Pharm. Biomed. Anal. 31 (2003) 251.
[18] A.F. Loughlin, G.L. Skiles, D.W. Alberts, W.H. Schaefer, J. Pharm. Biomed. Anal. 26 (2001) 131.
[19] J.O. Sass, W. Endres, J. Chromatogr. A 776 (1997) 342.
[20] K. Tuschl, O.A. Bodamer, W. Erwa, A. Muhl, Clin. Chim. Acta 351 (2005) 139.
[21] N. Maeso, D. Garcia-Martinez, F.J. Ruperez, A. Cifuentes, C. Barbas, J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 822 (2005) 61.
[22] W. Jin, Q. Dong, X. Ye, D. Yu, Anal. Biochem. 285 (2000) 255.
[23] B.A. Neuschwander-Tetri, F.J. Roll, Anal. Biochem. 179 (1989) 236.
[24] A. Lafaye, J. Labarre, J.C. Tabet, E. Ezan, C. Junot, Anal. Chem. 77 (2005) 2026.
[25] R.L. Norris, G.K. Eaglesham, G.R. Shaw, M.J. Smith, R.K. Chiswell, A.A. Seawright, M.R. Moore, J. Chromatogr. B. Biomed. Sci. Appl. 762 (2001) 17.
[26] G. Santori, C. Domenicotti, A. Bellocchio, M.A. Pronzato, U.M. Marinari, D. Cottalasso, J. Chromatogr. B. Biomed. Sci. Appl. 695 (1997) 427.
[27] G. Carro-Ciampi, P.G. Hunt, C.J. Turner, P.G. Wells, J. Pharmacol. Methods 19 (1988) 75.
[28] Y. Hiraku, M. Murata, S. Kawanishi, Biochim. Biophys. Acta 1570 (2002) 47.
[29] P. Houze, S. Gamra, I. Madelaine, B. Bousquet, B. Gourmel, J. Clin. Lab. Anal. 15 (2001) 144.
[30] ICH-Q2A, International Conference on Harmonization, 1995.
[31] ICH-Q2B, International Conference on Harmonization, 1996.
[32] F.Y. Lee, M.J. Allalunis-Turner, D.W. Siemann, Br. J. Cancer 56 (1987) 33.