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Fast liquid chromatography–mass spectrometry glutathione measurement in whole blood: micromolar GSSG is a sample preparation artifact

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Abstract

We describe a new, fast (6 min) and reliable method to measure reduced or oxidized glutathione (GSH) or (GSSG) in whole blood. The method is based on a LC/MS measurement in positive electrospray ionization mode after a chromatographic separation on a specific column which does not need any counter-ion in the mobile phase, improving the sensitivity of detection. A 50 μl sample of whole blood is sufficient for analysis. We demonstrate that the lack of an alkylating agent during the sample preparation brings out an underestimation of GSH and an artefactual production of GSSG, corresponding to 2–3% of GSH. The simultaneous use of *N*-ethyl-maleimide and a strong deproteinising acid prevents these two drawbacks. This efficient and new method of preparation and analysis lets us show that, unexpectedly, GSH is stable in whole blood for some hours and that deproteinised samples can be stored without GSH loss for at least three weeks at −20 or −80 °C. The reference interval, measured on 22 volunteers, on blood samples collected either with heparin or with EDTA, is 1310 ± 118 μM for GSH and 0.62 μM for GSSG. The within-run precision of this method, with γ glutamyl-glutamic acid as an internal standard, evaluated in three successive series ($n = 30$), lies between 2.1 and 4.8% for a GSH level at 580 or 1150 μM. The one step sample preparation we propose seems well suited for GSH routine measurements in hospital laboratories and avoids any underestimation of GSH, a now well accepted biomarker of oxidative stress.
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1. Introduction

Interest in methods for measuring reduced and oxidized glutathione, two interconvertible forms (Fig. 1) has been heightened by awareness of the key role of these compounds in protective mechanisms against oxidative stress. The majority of assays involved the separation of these compounds or their derivatives from a complex biological matrix by HPLC followed by UV absorption, fluorescent or electrochemical detection [1–4]. Some authors have reported the quantification of GSH by spectrometry derivative [5], of GSSG by bioluminescence [6] and, more recently, by capillary zone electrophoresis [7] or liquid chromatography–electrospray ionisation-mass spectrometry (LC/ESI/MS) [8,9]. Many factors can explain the varying values obtained for blood GSH and GSSG: preanalytical processing, sample preparation conditions with, for example, the type of acid used and the presence or not of an alkylating agent, and the analytical methods [2,10–15]. Furthermore, recent data suggested that, in fact, GSSG could be only a sample preparation artifact [2,4,16].

In this study, we described a simple, rapid, reliable and specific method for the determination of GSH in whole blood based on an HPLC–ESI–MS technique which combined both the specificity and selectivity of chromatographic separation and mass spectrometric detection. The interest in measuring GSH in whole blood was that the sample preparation was easy and quick in comparison with the preparation of washed red blood cells (RBC), the standard procedure. Furthermore, this quick one-step sample preparation avoided artifactual GSSG production and was well suited for hospital laboratories because samples could be processed easily, 24 h a day, before storage for further analysis.

2. Materials and methods

2.1. Chemicals and reagents

GSH, GSSG, γ glutamyl-glutamic acid (γ glu–glu) and EDTA were obtained from Sigma (St. Louis, MO, USA),

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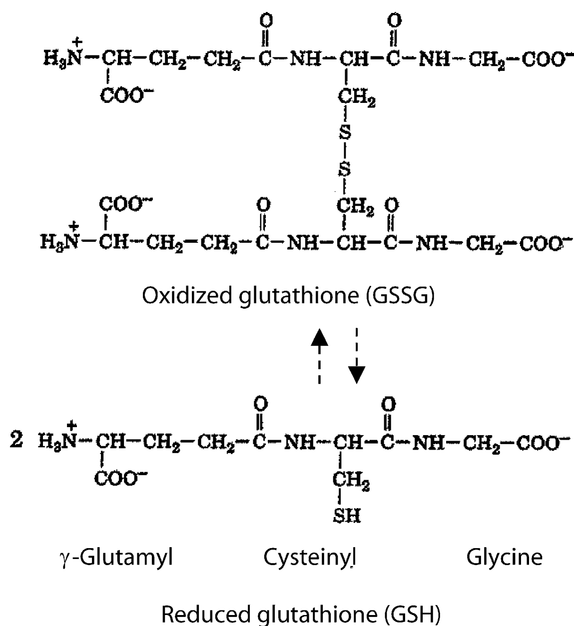

Fig. 1. The structure of the two interconvertible forms of glutathione: the thiol-reduced (GSH) and the disulfide-oxidized (GSSG) forms. In the two forms, the peptide bond linking the amino-terminal glutamate and the cysteine residue is through the γ-carboxyl group of glutamate and makes it resistant to current proteases.

ammonium acetate (molecular biology grade) from Calbiochem (San Diego, CA, USA), sulfo salicylic acid (SSA) dihydrate and *N*-ethylmaleimid (NEM) from Aldrich. All other chemicals and solvents were of analytical grade. Aqueous solutions were made with pure water.

2.2. Preparation of calibration standards and reagents

GSH and GSSG calibrator solutions were prepared as follows: GSH (20 mM) in 8.5 mM acetic acid and GSSG (2 mM) in water. Both were stored at −80 °C. Working standard solutions of GSH (250, 500, 1000 and 2000 μM) and GSSG (6.25, 12.5, 25, 50 and 100 μM) were prepared daily by diluting the calibrator solutions with a K_2HPO_4 buffer, 500 mM adjusted at pH 8.9 with NaOH.

The precipitating solution was made by mixing 150 μl of a solution A containing NEM, EDTA and γ glu–glu (in water/methanol, 85/15 (v/v)) with 50 μl of SSA; the final concentrations in the precipitating solution were 20 mM, 2 mM, 250 μM and 2% (w/v) for NEM, EDTA, γ glu–glu and SSA, respectively. The solution A was aliquoted and stored at −20 °C for 2 months.

2.3. Sample collection

Blood was collected on an anticoagulant, heparin or EDTA. Reference intervals were determined from 22 healthy volunteers from our laboratory (14 women, 8 men, aged 21–51 years) who had given their informed consent.

2.4. HPLC–MS analysis

Analytical HPLC–MS separations were performed with a Thermo Finnigan liquid chromatograph (pump P 4000, thermostated autoinjector AS 3000 and a Navigator Aqua mass spectrometer detector (equipped with a diverting valve between the column and the detector) on a 150 mm × 2 mm Stability BSC 17 (5 μm particle size) column (Cluzeau Info Labo, Sainte Foy la Grande, 33220 France). The flow rate was 0.2 ml/min with the cartridge kept at 45 °C in a column oven. The Stability BSC 17 stationary phase was a new patented silica-based mixed mode (anionic and reverse phase) support (Fig. 2) which enabled ionic species like GSH and GSSG to be separated without any counter-ion in the mobile phase.

The mobile phase was ammonium acetate 7.5 mM (final concentration) adjusted to pH 2.4 with acetic acid, and 50% (v/v) of methanol. The wash phase was 1000 mM ammonium acetate in water/methanol/acetonitrile (20/40/40, v/v/v). The standards and samples were kept at 15 °C in the autosampler, and the injection volume was 5 μl. Detection was carried out with a single quadrupole mass spectrometer in ESI+. The probe was set at 225 °C and 2.5 kV with an entrance cone voltage at 15 V for GSH, γ glu–glu and GSNEM and 30 V for GSSG. These parameters were defined by infusion then checked by LC/MS. Chromatograms were recorded in single ion monitoring mode $(M + H)^+$ at $m/z = 277.3$ (γ glu–glu), 308.3 (GSH), 433.7 (GSNEM) and 614.1 (GSSG) for 6 min and integrated with Xcalibur software (version 2.51).

3. Results

3.1. LC/MS separation

Optimisation of the LC/MS conditions was obtained in two steps: first, optimisation of the mobile phase by defining the right equilibrium between the pH and the ionic content of the phase (Table 1) (bearing in mind that infusion experiments had shown that a pH at around 2.5 was the most sensitive for MS detection) and, second, increase in the

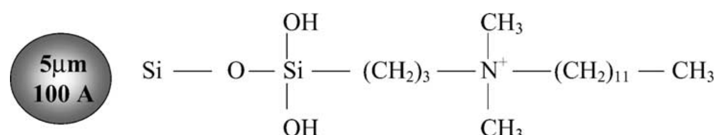

Fig. 2. Diagram depicting the silica-based mixed mode anion exchanger of the BSC 17 stationary phase.

Table 1
Influence of the composition of the mobile phase on the retention times (RT) of the reduced glutathione (GSNEM), the internal standard (γ glu–glu) and the oxidized glutathione (GSSG) on a BSC17 column

Mobile phase	RT (min)		
	GSNEM	γ glu–glu	GSSG
pH(1) 2.9, NH_4(2) = 10 mM, M(3) = 50%	3.5	3.8	6.9
pH(1) 2.8, NH_4(2) = 5 mM, M(3) = 50%	4.5	5.7	11.5
pH(1) 2.6, NH_4(2) = 5 mM, M(3) = 50%	3.3	3.7	5.7
pH(1) 2.6, NH_4(2) = 5 mM, M(3) = 45%	3.2	nd(4)	5.2
pH(1) 2.6, NH_4(2) = 5 mM, M(3) = 40%	3.2	nd(4)	5.0
pH(1) 2.6, NH_4(2) = 2.5 mM, M(3) = 50%	3.8	4.3	6.4

(1): the pH is adjusted with pure acetic acid; (2): NH_4, final ammonium acetate concentration; (3): M, methanol content (v/v); (4): nd, not determined.

methanol content to improve volatility without disturbing the chromatographic resolution. In the conditions described above, GSNEM, γ glu–glu and GSSG were eluted at 2.9, 3.4 and 4.4 min, respectively (Fig. 3). On the lowest calibration point, the mean signal-to-noise ratio, on two successive measurements, was 280 for GSH (250 picomoles injected) and 80 for GSSG (6.25 pmol injected). It decreased to 12 at GSSG = 1.25 μM, not far from the limit of detection. All the S/N ratio were calculated with a smoothing filter at 3.

3.2. Choice of conditions for sample preparation

Preliminary tests on remaining blood samples (after assessing acid–base status), deproteinised either with

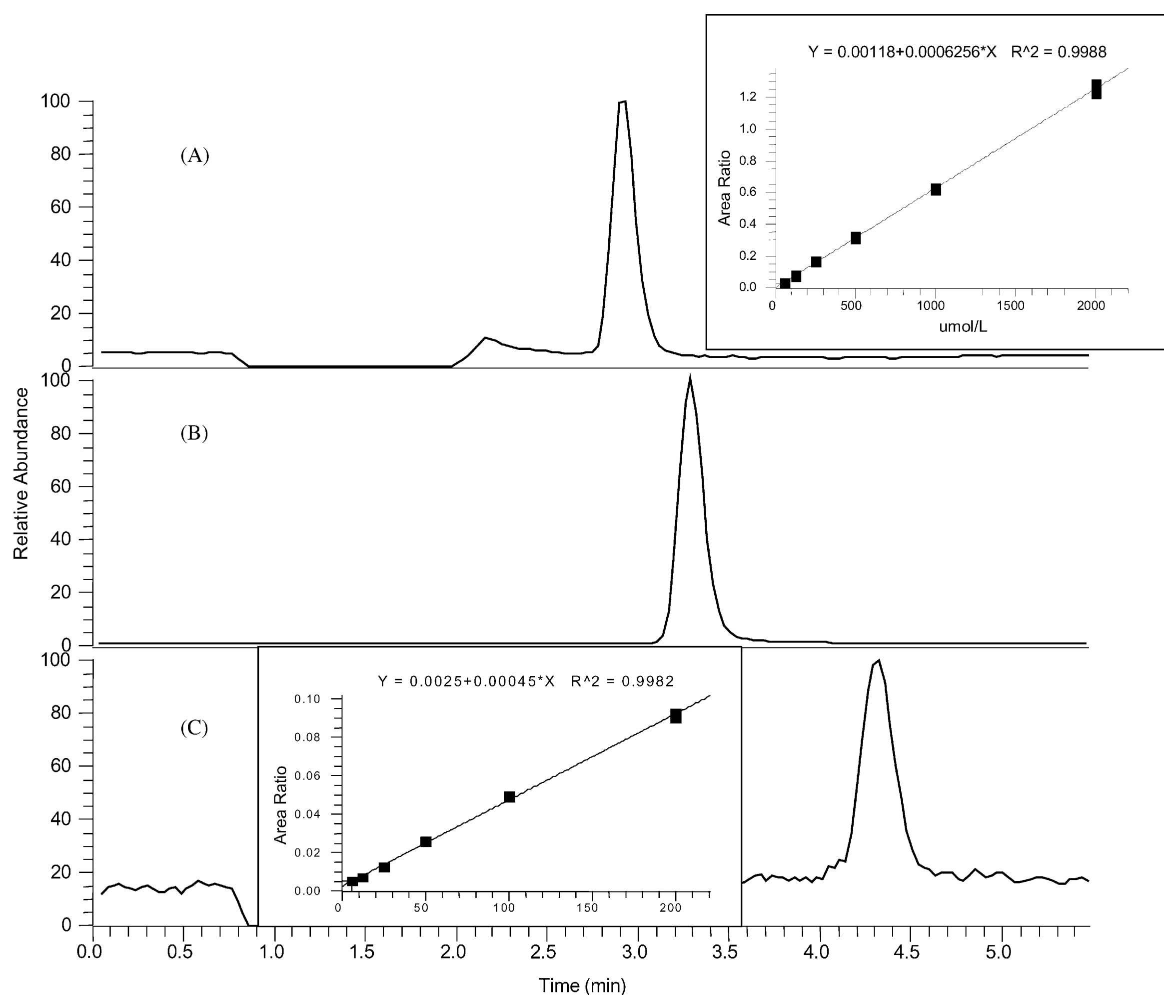

Fig. 3. A typical LC/MS chromatogram, recorded in the SIM mode, of the lowest calibration point showing: in A, the upper trace, the GSNEM peak detected at m/z = 433.7 and retention time 2.9 min and, in the inset, the corresponding calibration curve (with each calibration point injected in duplicate, even if the symbols are not visible), in the middle trace (B), the internal standard ((γ glu–glu) at a retention time of 3.4 min and $m/z = 277.3$, in C (the lower trace), the chromatogram of GSSG at a RT = 4.4 min and $m/z = 614.1$, with its calibration curve in the inset (each calibration point in duplicate).

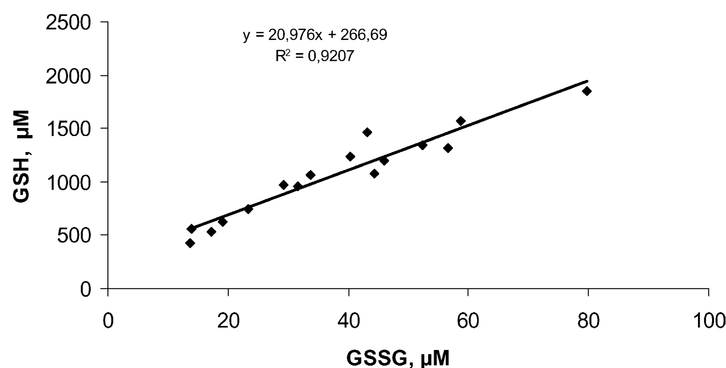

Fig. 4. An example of the correlation between GSH and artefactual GSSG contents measured in 16 blood samples, deproteinised with MPA 5% and in the absence of NEM.

metaphosphoric acid (MPA, 5%) or with MPA + NEM, showed that the mean value ($n = 30$ in six series) for GSH was 1219 μM with NEM and 761 μM without NEM. Interestingly, GSSG was barely detectable in the presence of NEM whereas its mean value was 28 μM without NEM. Moreover, in the absence of NEM, GSSG was correlated with the GSH content (16 samples in two series) (Fig. 4), as described previously by many authors who did not use any alkylating agent for sample preparation [2,4,14,17]. These results strongly suggested that a portion of the thiols was oxidized during deproteinisation, and MPA alone was not sufficient to prevent GSH oxidation. This was confirmed by another experiment: the recovery of GSH added to blood samples treated with MPA and heated 1 min at 100 °C (to inactivate enzymes involved in GSH metabolism) was lower than expected. Thus, even heating in an acid medium was not able to hinder GSH oxidation.

In a second phase, as MPA needed to be prepared daily and as it has been shown that the protein content of samples deproteinised with MPA was higher than with other acids [11], we tried to change it for SSA (1 or 2% final concentration). We compared GSH and GSSG values in six series of blood samples treated with each acid. Values for GSH and GSSG were highly correlated (data not shown, linear regression y (SSA) = 1.0484 × (MPA) − 38.173, $r^2 = 0.96$).

On account of these data, the sample preparation, now in use, was obtained by adding 50 μl of blood to 200 μl of precipitating solution. The sample was vortex-mixed for 15 s, derivatised for 30 min at room temperature and centrifuged (14,000 × g, 3 min). The supernatant was diluted 5-fold in water before injection. The calibrator solutions were treated under the same conditions but with a derivatisation time extended to 60 min, as their reaction rate was slower than that of blood samples. It was easy to check the time necessary for full conversion of GSH into GSNEM in standards or samples by recording the decrease in the GSH peak at $m/z = 308.3$ at regular time intervals.

In order to confirm the dramatic efficiency of NEM combined with an acid to prevent any GSH oxidation in whole blood, two series of 10 samples were spiked with 6.25 μM of GSSG, derivatised and diluted as described above. The mean GSSG content measured in the spiked samples was 6.81 ± 0.55 μM. The mean value of GSSG detected in native samples was 0.64 ± 0.22 μM, a value not far from the limit of detection of the system, as the mean S/N ratio at $m/z = 614.1$ was between 5 and 6 for the 20 samples.

3.3. Comparison of GSH and GSSG measured in whole blood and RBC

The optimised LC/MS method was applied to 20 samples, either as RBC or as whole blood.

RBC were washed three times in saline before hemolysis in pure water, and the hemoglobin (Hb) concentration was measured with a hemocytometer. Then 50 μl of each hemolysed preparation was used as sample instead of whole blood, as described above, for GSH and GSSG measurement.

In whole blood, mean GSSG was 0.012 μM/g Hb, whereas in RBC it was 0.08 μM/g Hb.

3.4. Within-run imprecision and recovery

Blood samples ($n = 3$) were prepared, diluted and injected 10 times, in three different series. The within-run CV for each series was between 2.1 and 4.8%.

To evaluate the recovery, aliquots of blood were spiked with known amounts of GSH or GSSG and measured along with the spiking amount as described above. Results were given in Table 2.

Table 2
Recovery of added GSH and GSSG to blood samples: *N*, number of samples, S.D., standard deviation of the mean amount recovered

N	Expected (μM)	Recovered (μM)	Recovery (%)	S.D. (μM)
GSH addition				
7	316	324	102	67
7	391	417	106	89
10	283	288	101	33
GSSG addition				
16	49	47	96	0.7
10	77	80	103	3.5

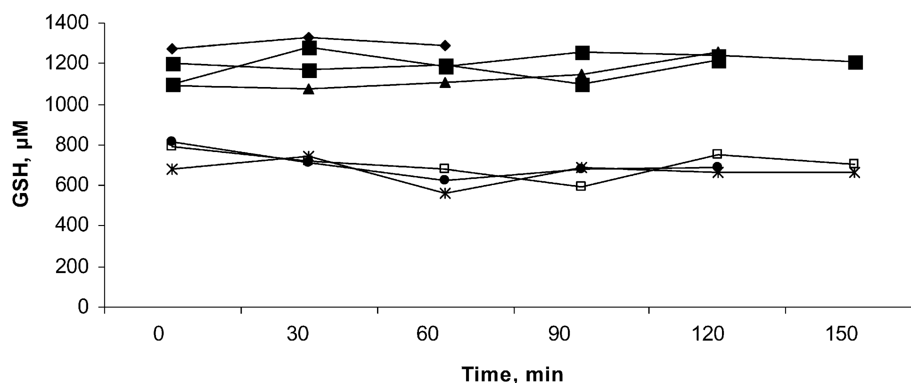

Fig. 5. An example of the stability of the GSH content in seven whole blood samples, kept at room temperature and under agitation for 150 min (every 30 min an aliquot is drawn and processed as described in the text).

The mean recovery was 103% for GSH and 100% for GSSG but the standard deviation of the recovered amount of GSH was higher than that of GSSG. As the amount of GSSG added was much lower than that of GSH, the variability induced by GSH spiking was not due to analytical imprecision but seemed more likely to be related to thiol exchange between GSH and proteins in the sample. This exchange explained why a high NEM (>20 mM, data not shown) content was necessary during the sample preparation.

3.5. GSH stability in whole blood before processing

GSH was assayed on seven blood samples from healthy volunteers, processed immediately after drawing or kept at room temperature, under agitation, and reprocessed at time-intervals of 30 min for 2 h. GSH concentrations were stable (Fig. 5) for at least 2 h. This contrasted with former data which have shown decreased concentrations of GSH within minutes of collection [12,17–19], which could be prevented either by blood collection with reducing agents and/or by transporting samples from the ward on ice.

Therefore, in our conditions, such caution seemed unnecessary and a simpler procedure was sufficient to fulfil correct conditions for GSH measurement.

3.6. GSH stability in deproteinised samples stored at −20 or −80 °C

Blood samples from 22 healthy volunteers were collected into two Vacutainer® tubes containing either heparin or EDTA as anticoagulant. They were deproteinised as already described and stored at −20 or −80 °C for 1 month.

An aliquot was periodically removed to determine the GSH concentration.

The stability was similar at −20 and −80 °C, whatever the anticoagulant used, and samples could be stored for at least four weeks without significant loss (Fig. 6).

3.7. Reference intervals

These were measured on 22 samples from healthy volunteers from the laboratory staff (women, $n = 14$ and men, $n = 8$) with a mean age of 35 (21–51 years).

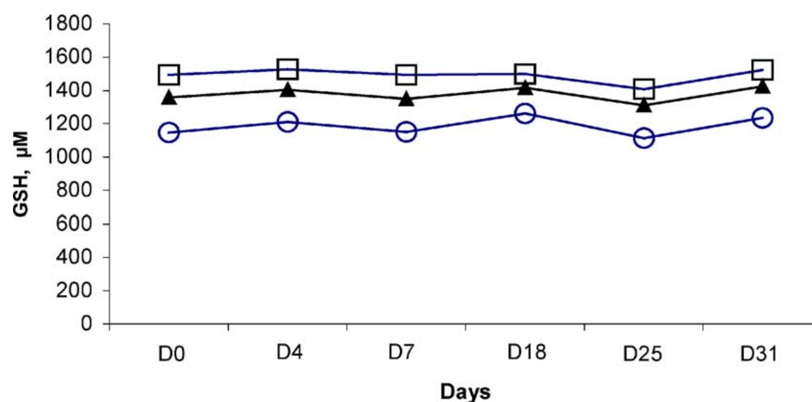

Fig. 6. GSH stability in multiple aliquots of 22 samples processed and stored at −80 °C for 1 month (follow-up of the highest sample (□), the mean content of the 22 samples (▲), and of the lowest sample (○)).

The mean value was 1310 ± 118 μM, in agreement with recent data [14,20,21] but higher than many other authors [3,10,22].

4. Discussion and conclusion

The new method we describe is fast, easy to handle and benefits from the advantages of a highly specific detection method. To obtain the best results, care must be taken first to obtain a whole resolution between GSNEM and the internal standard to ensure a correct quantification of the IS. Secondly, after each series of samples, the column must be washed for some hours (more easily, overnight) at room temperature with the wash phase at a flow rate of 50 μl/min. Under these conditions, more than 2500 samples can be analyzed on the same column and, as the chromatographic separation is fast and reproducible, it is easy to multiply tests to find out the right conditions for the sample preparation.

In the absence of an alkylating agent, we were able to reproduce the results often described previously, i.e. a mean GSH content in whole blood of around 1000 μM with an oxidized GSSG corresponding to 2–3% of the GSH content. However, we demonstrated that the use of an alkylating agent played a central role in avoiding loss of GSH, though its efficiency was highly dependent on the pH of the derivatisation. The mean value of the GSH content we measured on 22 "normal" samples was around 30% higher than that proposed by some authors. The main difference came from the sample pretreatment we described: we used NEM in a strong acidic condition (pH = 2–2.3, in the presence of SSA), which could explain this apparent discrepancy. Our results showed that, with a pH between 6 and 8.5 as previously recommended [2,10,11,13], the use of NEM alone was insufficient to protect GSH. In the same way, we checked that heating to 100 °C in the presence of NEM (at a neutral pH) was insufficient too, and it seemed obvious that both an acidic medium (for blocking enzymes) and an alkylating agent (as a reduced thiol preservative) were necessary to prevent any GSH loss. Unlike with fluorescent methods (the most current ones), it was possible with mass spectrometric detection to record GSH either in its native form or as a derivative, and this could explain why the synergy between an acid and NEM was not assessed before. The sample preparation we described has two advantages: it is a one step process and it avoids any artefactual GSH oxidation.

From a biological point of view, a direct consequence was that, in whole blood, the amount of GSSG, if present, appeared to be very low, in a nanomolar range. This is in complete agreement with very recent data obtained also by LC/MS and presented by Camera et al. [8] who measured GSH and GSSG in lymphocytes isolated from 5 ml of blood. According to these authors, GSSG was not far from the limit of detection of their method and it was not surprising that we could not quantify but only detect GSSG on 50 μl of whole blood, even under our LC/MS conditions. However, GSSG was easily quantified when it was actually present, for example, in hepatic microsomes (data to be published elsewhere).

Regarding GSSG, its content (μM/g Hb) was around seven-fold higher in RBC than in whole blood; as the Hb content was around 60 g/l for hemolysates instead of 100 g/l for whole blood, the GSSG amount was around 15-fold higher in hemolysates than in whole blood. That is why this higher GSSG in RBC seems to be due to the washing steps.

The reference interval was similar with blood collected on heparin or EDTA and the stability was not different when the derivatised samples were stored at −20 or −80 °C.

As LC/MS is not yet current equipment in many hospital laboratories, it could be interesting to change NEM for a fluorescent equivalent and to ensure the transferability of our method with fluorescent detection although, of course, for GSH alone.

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