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LC–MS determination of oxidized and reduced glutathione in human dermis: A microdialysis study

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ABSTRACT

A simple, highly selective, sensitive and reproducible liquid chromatography-electrospray ionization mass spectrometry method has been developed for the direct and simultaneous determination of reduced (GSH) and oxidized (GSSG) glutathione in microdialysis samples from human dermis. Chromatographic separation was carried out on an MODULO CART QS KROMASIL 5C18 (250 mm × 2 mm × 5 μm) analytical column at a flow rate of 0.25 ml/min. An isocratic mode was used and consisted of acidified water and acetonitrile (50/50, v/v). To improve the sensitivity, silver nitrate was added as post-column reagent. A trap mass spectrum was used equipped with an ESI interface. The limits of detection and quantification were respectively 0.12 and 0.4 ng/ml for GSH and 0.2 and 0.5 ng/ml for GSSG. Intra-day and inter-day accuracy and precision were determined and the variability was less than 6.2% (R.S.D.).

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1. Introduction

Glutathione is a thiol found intracellularly at high concentrations and is also present in small amounts in extracellular fluid [1]. Glutathione exists as reduced glutathione (GSH) and in oxidized form as glutathione disulfide (GSSG). GSH is a tripeptide of glycine, glutamate, and cysteine and GSSG is a dimer of GSH, where two GSH molecules are linked through a disulfide bond. A deficiency in glutathione is thought to be associated with a variety of diseases, such as cancer, neuro-degenerative disorders, cystic fibrosis, lung diseases, HIV, liver diseases and dystrophic skin fibroblasts [1–3]. One of the important functions of GSH in biological systems is antioxidant activity where GSH is oxidized to GSSG as it scavenges reactive oxygen species [2,4]. Therefore, the simultaneous determination of GSH and GSSG in biological fluids, such as microdialysates, is important since the ratio of GSH to GSSG concentration may be used as a biomarker of oxidative stress [2,5].

Methodologies for the determination of both GSH and GSSG have been reviewed recently [6,7]. Selectivity and sensitivity were achieved using chromatographic techniques such as thin layer chromatography and high-performance liquid chromatography (HPLC). Recent methods have used HPLC coupled to a variety of detection techniques, including UV–vis and diode array detection, fluorimetry, electrochemical detection and mass spectrometry. To prevent the thiol oxidation and consequently to further enhance sensitivity, common procedures include derivatization of the free thiol group in the GSH molecule with chromophores, fluorophores or with idioacetamide and isopropylchlorformate [8]. In general, GSSG is measured by reducing it to GSH with the enzyme glutathione reductase. The difference between the total (after reduction) and initial GSH values is used to estimate the GSSG concentration in the sample. Using this procedure, the GSSG concentration found in a given sample often could be similar to the standard deviation between replicates [9].

HPLC–MS techniques have been a major step toward the determination of GSH and GSSG during recent years [8–12]. These techniques improve selectivity and avoid the GSSG reduction step. LC/MS has become increasingly popular and the method of choice in the pharmaceutical industry for the analysis of biological samples. We describe in this paper an LC/MS method with nitrate silver as post-column reagent to simultaneously quantify GSH and GSSG in microdialysates from human skin. The method requires minimal sample treatment and offers high selectivity and sensitivity.

2. Materials and methods

2.1. Materials

Glutathione, glutathione disulfide and glutathione ethyl ester (internal standard) were obtained from Sigma Chemical (St Louis, MO). HPLC grade acetonitrile was purchased from Carlo Erba

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(Milan, Italy). Metal salts (LiI, $CoCl_2$, $ZnCl_2$, NaCl, KCl, $AgNO_3$) were commercially available at 99.9% (Sigma, St Louis, MO, USA).

2.2. Standard solutions

Ringer's lactate solution, which was used as perfusate for the microdialysis probes (Cl^-: 111 mmol/l; K^+: 5.3 mmol/l; Ca^{2+}: 1.8 mmol/l; Na^+: 130 mmol/l; lactate: 27.6 mmol/l; pH 6; Maco Pharma, Paris, France). A stock solution of GSH, GSSG and internal standard (IS) were prepared in acetonitrile (ACN) at 1000 μg/ml, 1500 μg/ml and 1000 μg/ml, respectively and stored at 4 °C. Calibration standards and quality control samples were prepared by adding appropriate volume of the stock solution of GSH and GSSG, 50 μl of IS and an appropriate volume of Ringer's solution to get a final volume of 500 μl. Thus eight standard concentrations containing the two analytes (GSH and GSSG) and the IS were prepared in the range 0.2–200 ng/ml. Three quality control (QC) samples containing all compounds were prepared at 5, 100 and 200 ng/ml.

2.3. Microdialysis' samples collection

The microdialysis system, used in this investigation, consisted of a CMA/20® syringe pump (Phymep, Paris, France) and a Univentor 820® microfraction collector which collected sample at 4 °C. The microdialysis probes (CMA/20®), used in this work, had a 20 kDa cut off with a polyarylethersulfone membrane (length 10 mm) and they were perfused with Ringer lactate solution at a flow rate of 3 μl/min. The collected microdialysis samples were frozen at −20 °C until their analysis. The microdialysis samples were analyzed without any pre-treatment.

2.4. Determination of the relative recovery

2.4.1. Relative recovery (RR)

This method consists in continuously perfusing a physiological fluid, i.e. Ringer solution or ultrapure water, which creates a concentration gradient along the dialysis membrane. The compounds diffuse through the membrane from the interstitial fluid to the perfusate or from the perfusate to the interstitial fluid. The outlet perfusate is collected in microtubes. The principal diffusion parameter is expressed by an RR which is the ratio between the concentration in the dialysate (C_{out}) and the concentration in the medium surrounding the probe (C_{in}). This relation is represented in the following equation

$$RR = \frac{C_{in} - C_{out}}{C_{in}}$$

In our investigation, *in vitro* recovery experiments were conducted with probes placed in a vial containing either GSH or GSSG at 1 mM dissolved in Ringer' solution and perfused with Ringer lactate' solution at a flow rate of 3 μl/min. This flow rate was considered as optimal by Mary et al. [13]. Samples were collected every hour for a period of 6 h.

3. Instruments and method

3.1. High-performance liquid chromatographic system

A Spectra system (Thermo Fisher, Boston, USA) composed of a SCM1000 degasser, a SN4000 solvent delivery system; a P1000XR injector (20 μl injection loop) was used. An MODULO CART QS KROMASIL 5C18 (INTERCHIM, France) (250 mm × 2 mm × 5 μm) was used. An isocratic solvent program was used as followed: acetonitrile/water acidified with 0.1% of formic acid (50/50, v/v). The flow rate of the mobile phase was 0.25 ml/min. Metal salt solutions (100 μmol/l) were introduced into the LC effluent after the column at a flow rate of 0.05 ml/min by using a T connector (Interchim, Montlucon, France) and an Hewlett Packard 1050 pump (Agilent Technologie, Santa Clara, USA).

3.2. ESI-MS

The column described above was connected to a Thermo-Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Fisher, Boston, USA) equipped with an ESI ion source. The entire volume of the column effluent was directed to the mass spectrometer. Operating conditions for the ESI source, used in positive ionization mode, were optimized in the infusion mode. Typical optimized values for the source parameters were source voltage 4.5 kV, capillary temperature 250 °C, capillary voltage 20 V and tube lens offset 5 V. Nitrogen was used both as sheath gas and as an auxiliary gas at a flow rate of 75 and 50 (arbitrary units), respectively. In the infusion mode, sample concentrations were typically 10 μg/ml in ACN/water (0.1% formic acid) (50/50, v/v). All solutions were continuously infused by means of a syringe pump at a typical flow-rate of 5 μl/min into the electrospray probe. Mass spectrometry analysis was conducted in positive ion mode and set-up in selected ion monitoring (SIM) mode. Data processing was performed on Xcalibur data system (Woburn, MA, USA).

3.3. Validation procedure of the LC–MS assay

3.3.1. Selectivity

The selectivity was studied by preparing and analyzing a QC standard (2 ng/ml) compared to blank microdialysis sample spiked with the internal standard. This test was done with three QC samples at 2 ng/ml and three microdialysis blank samples.

3.3.2. Calibration curve, LLOD and LLOQ

Calibration curves were obtained by plotting the peak area ratio of each analyte and the internal standard against the actual concentration of the analyte using regression over a range from 0.2 to 200 ng/ml. Each standard was prepared in triplicate over 3 days.

3.3.3. Accuracy and precision

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

Repeatability and intermediate precision were studied. Intraday precision, expressed as the coefficient of the variation of repeatability (CV), was performed for the three levels of the QC (five replicates). Inter-day precision, expressed as the coefficient of variation of intermediate precision (CV) was evaluated for each level of QC over 5 days (five replicates).

3.3.4. Stability

The stability of GSH and GSSG in Ringer's lactate solution was evaluated under different temperature and storage conditions. Samples of GSH and GSSG were subjected to room temperature, 4 °C and −20 °C. All stability studies were conducted at three concentrations of GSH and GSSG (5, 100 and 200 ng/ml) with three determinations each.

4. Results and discussion

4.1. Method development

The purpose of the present study was to develop a method to determine simultaneously GSH and GSSG concentrations in human skin. A simple sample preparation, short separation time and a low quantification limit were considered when the study started.

Microdialysis offered the advantages to be less invasive than other technique such as suction bubbles and also to collect protein free samples. Therefore, less sample treatments were necessary. However, the main disadvantages of this technique are the sample size (100 μL) and also the relative recovery. Therefore, a very sensitive method is necessary. To improve the limit of quantification several metal ions have been investigated. Co^{2+}, Zn^{2+}, Li^{+}, Na^{+}, K^{+} and Ag^{+} were tested. These metals were added as post-column reagent and the results obtained showed that the best sensitivity was obtained with silver nitrate as post-column reagent. The sensitivity was improved by a factor of 5 comparing the intensity of $[M+H]^{+}$ (obtained with a solution of 100 ng/ml of GSH, without silver nitrate addition as post-column reagent) and $[M+Ag]^{+}$ ions obtained with GSH solution at 100 ng/ml with $AgNO_3$ as post-column reagent (Fig. 1a and b). Similar results were get with GSSG. Therefore, the following steps were done by using silver nitrate (100 μM in acetonitrile/water (50/50, v/v)) as post-column reagent with a flow rate of 0.05 ml/min. The ions selected for GSH were

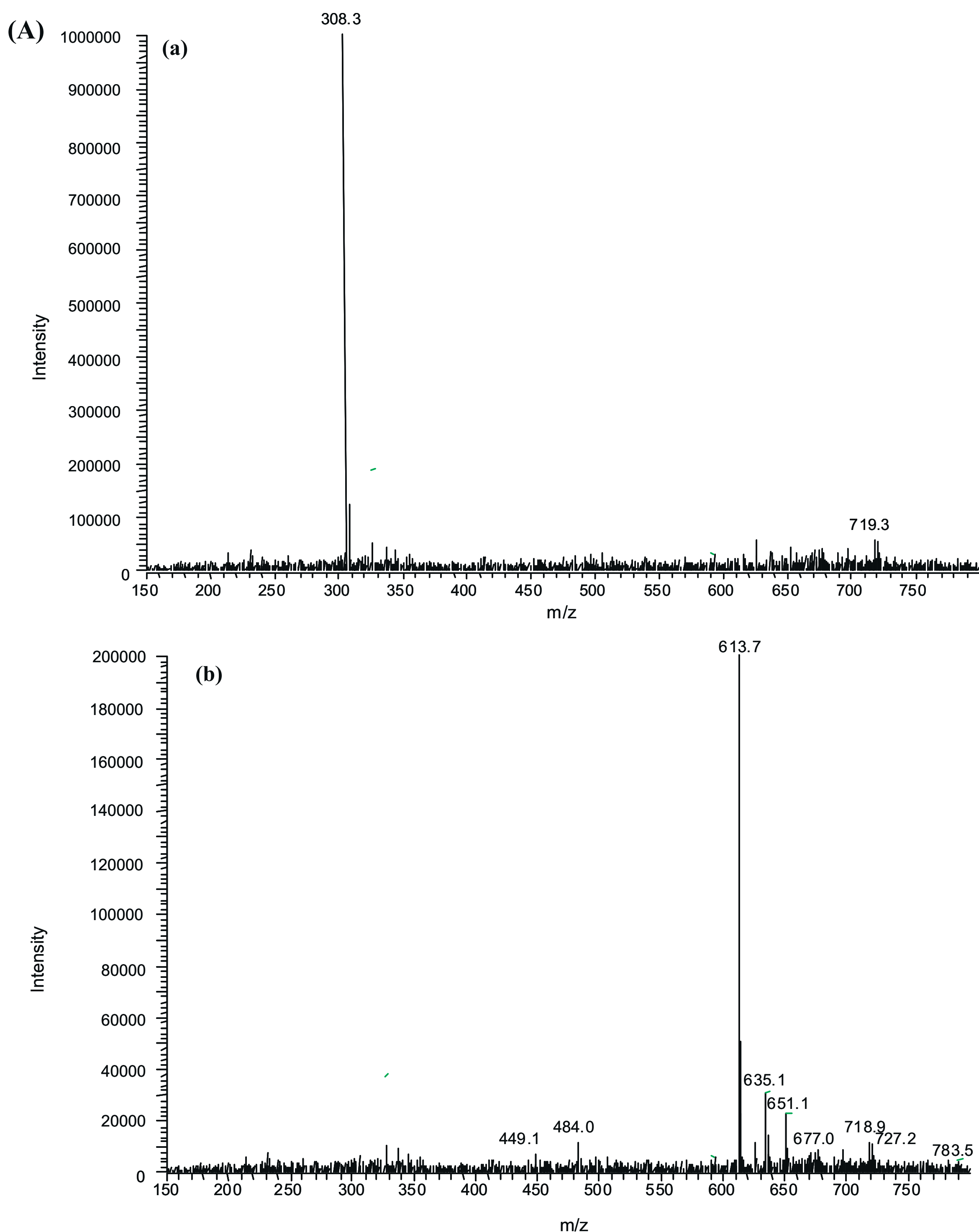

Fig. 1. (A) ESI mass spectra of (a) GSH and (b) GSSG standards in positive ion mode without $AgNO_3$ as post-column reagent. (B) ESI mass spectra of (a) GSH and (b) GSSG standards in positive ion mode with $AgNO_3$ as post-column reagent.

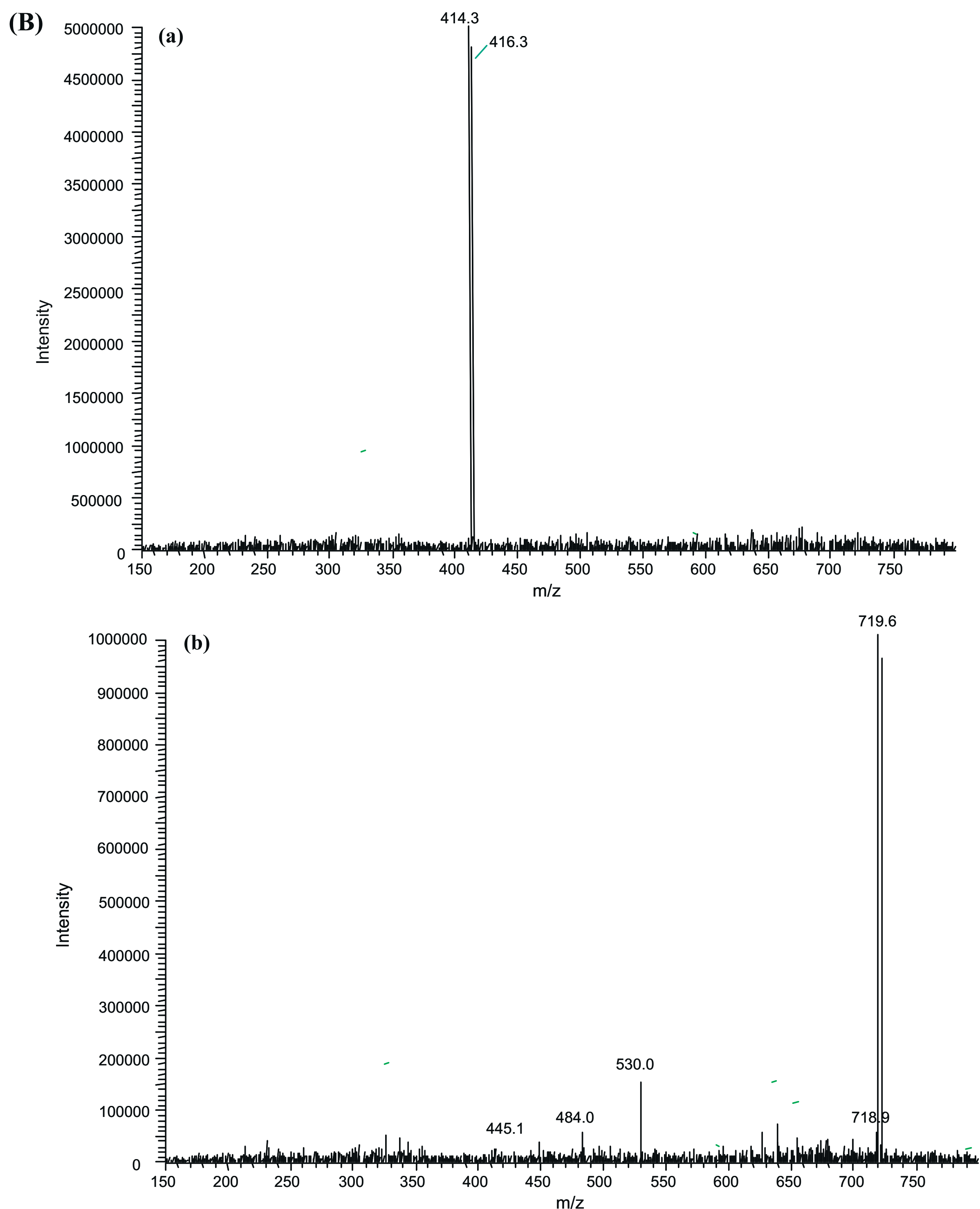

Fig. 1. *(Continued.)*

414.3–416.3 and for GSSG 719.6–721.6. The doublets obtained were due to silver as it has two isotopes ^{107}Ag and ^{109}Ag with similar abundance.

4.2. Validation of the method

4.2.1. Selectivity

The selectivity was demonstrated by comparing analyses of standard mixture sample at 100 ng/ml (Fig. 2) and a microdialysis blank sample. No significant interfering peaks were detected at retention times of the studied solutes when running blank microdialysis samples ($n = 3$) (Fig. 2). Different programs were investigated for exclusion of matrix interference. At the end of this investigation, the best program for a good resolution was selected and thus all experiments used the program described above.

4.2.2. Stability

GSH and GSSG were determined to be stable under different temperature and storage conditions. The analytes samples made in Ringer' solution were subjected to short term temperature

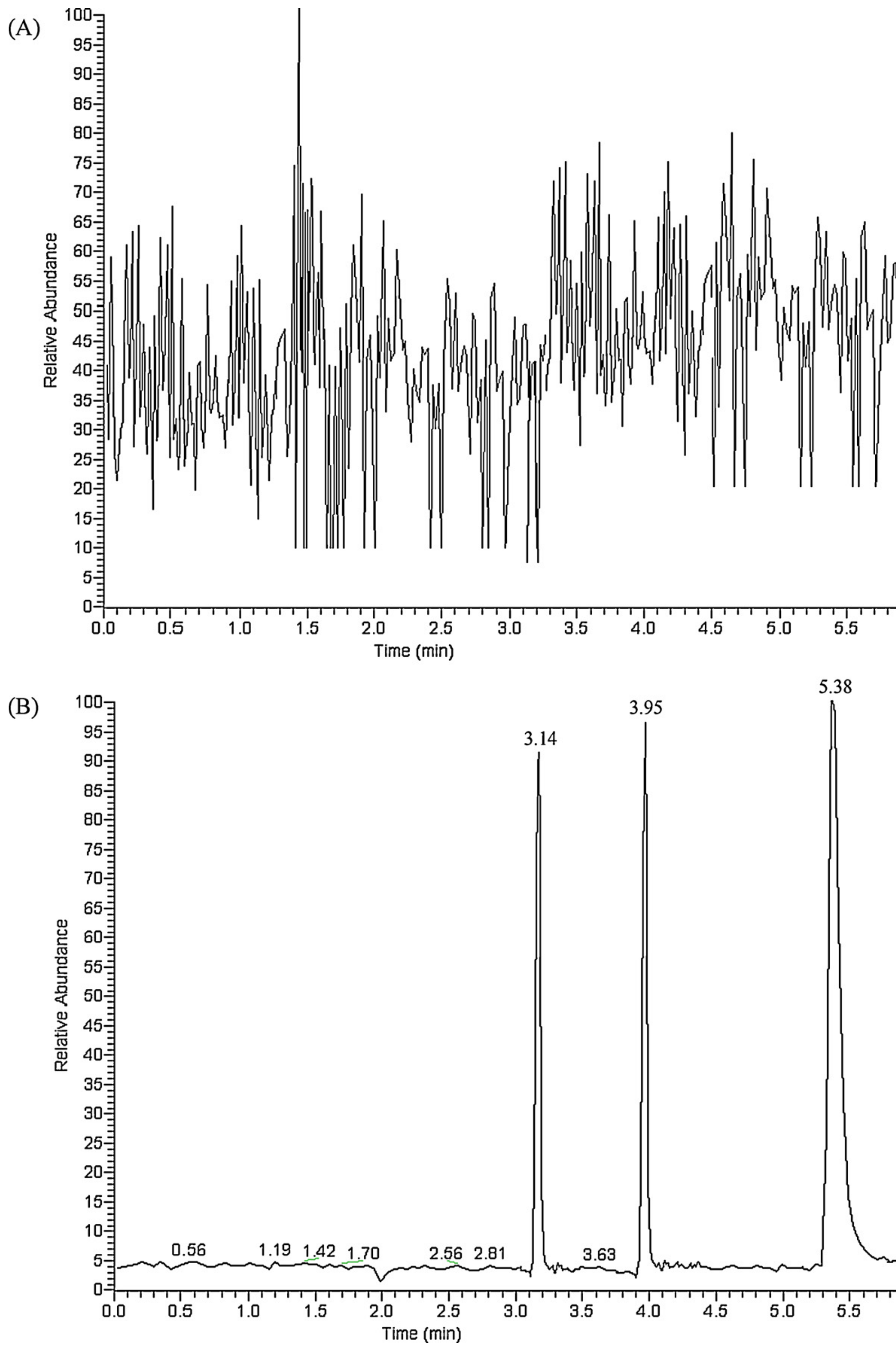

Fig. 2. LC–MS detection of GSH and GSSG and IS from *ex vivo* sampling (A) and blank sample (B) QC sample (2 ng/ml for each analyte).

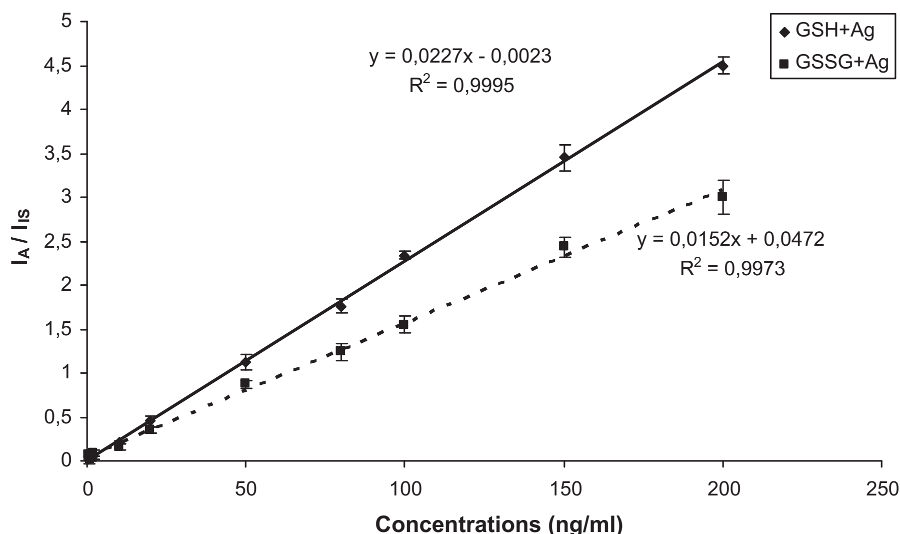

Fig. 3. Calibration curves of GSH and GSSG with silver nitrate as post-column reagent ($n = 9$).

Table 1
Stability of GSH and GSSG under various conditions ($n = 3$).

Storage conditions	Concentration (ng/ml)		R.S.D. (%)
	Nominal	Mean measured	
Stability at room temperature (1 h)	5	5.07	6.0
	100	100.98	3.4
	200	199.78	5.5
Stability at room temperature (4 h)	5	4.89	5.0
	100	98.98	5.4
	200	199.87	2.7
Stability at 4 °C (30 days)	5	5.34	5.4
	100	98.12	3.2
	200	198.34	1.9
Stability at −20 °C (30 days)	5	4.93	6.3
	100	101.23	3.2
	200	200.45	1.8

conditions for 1 and 4 h, long term storage conditions for 10, 20 and 30 days (at 4 °C and at −20 °C). Results reported in Table 1 corresponded to the stability at 30 days. The intermediate results (10 and 20 days) were not shown. All samples evaluated displayed variability less than 10% (R.S.D.) (Table 1).

4.2.3. Calibration curves and LLOQ

The calibration curves were determined over the range 0.2–200 ng/ml for the compounds (GSH and GSSG). The analytes calibration curves were constructed by plotting the peak area of GSH or GSSG to the IS versus the concentration of the analyte. The calibration curves revealed good linear correlation (Fig. 3).

The lowest limit of detection (LLOD) was estimated as the amount of GSH and GSSG that resulted in a signal three times the noise ($S/N \geq 3$). The LLOD were calculated to be 0.12 and 0.2 ng/ml for GSH and GSSG, respectively. The lowest limits of quantification (LLOQ) were found to be 0.4 ng/ml for GSH and 0.5 ng/ml for GSSG with acceptable accuracy and precision (<10%).

During the past decades, LC–MS has proven to be one of the most effective tools in biological research, particularly for the analysis of very low amounts of compounds in complex biological matrices. In Table 2 we have made a quick comparison between previous LC–MS methods developed to quantify GSH and GSSG (Table 2) that the addition of silver nitrate as post-column reagent improves the LOQ by a factor of 20 for GSH and 30 for GSSG. In addition, our method does not necessitate GSH and GSSG derivatization before their quantification.

Table 3
Intra-day ($n = 5$) and inter-day ($n = 5$) repeatability of the LC–MS method with nitrate silver as post-column reagent.

Compound	5 ng/ml		100 ng/ml		200 ng/ml	
	GSH	GSSG	GSH	GSSG	GSH	GSSG
Intra-day						
Mean assessed concentration	5.2	5.1	102.0	101.9	200.2	199.8
R.S.D. (%)	0.4	0.5	5.4	3.9	5.3	3.5
Bias (%)	4.0	2.0	2.0	1.9	0.1	−0.1
Inter-day						
Mean assessed concentration	4.9	5.0	99.1	100.8	201.7	199.6
R.S.D. (%)	0.4	0.7	3.9	3.9	6.2	5.1
Bias (%)	−2.0	0.0	−0.9	0.8	0.85	−0.2

4.2.4. Accuracy and precision

Accuracy was of this method was acceptable as all bias values were lower than 4% (Table 3).

Table 2
Comparison of LC–MS methods to quantify GSH and GSSG.

Reference	Derivatization	GSH (LOQ) (nM)	GSSG (LOQ) (nM)	Sample
[14]	Iodoacetic acid, dansyl choride	25	25	Hepatic cells
[15]	*n*-Ethylmaleimide	50	200	Human lymphocytes
[16]	5,5 dithiobis (2-nitrobenzoic acid)	500	500	Rat blood, lung, liver
[17]	*n*-Ethylmaleimide	100	1000	Saliva
[18]	Iodoacetic acid	163	82	Rat hepatocytes
Our work	Silver nitrate as post column reagent	1.3	0.8	Microdialysis samples from human skin

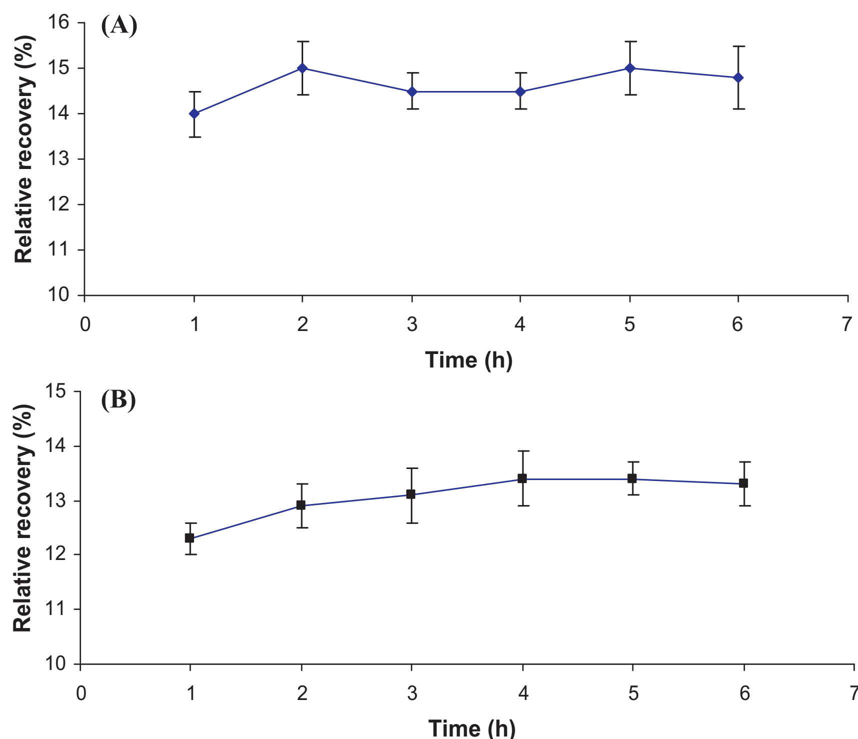

Fig. 4. *In vitro* relative recovery of (A) GSH and (B) GSSG ($n = 3$).

The intra-day repeatability of the LC–MS method with nitrate silver as post-column reagent was assessed from 5 consecutive chromatographic runs using a standard solution of GSG, GSSG and the IS at three concentration levels. The variation in peak area was tested for each analyte (Table 3).

The inter-day repeatability of the method was assessed by analyzing the same standard solutions for 5 consecutive days. The relative standard deviation (R.S.D.) for peak area was in the range 0.4–5.4% in the intra-day test and 0.4–6.2% in the case of the inter-day test (Table 3).

5. Determination of GSH and GSSG in microdialysis samples

5.1. Microdialysis probe relative recovery

The *in vitro* relative recovery (RR) from 1 mM solution of GSH and GSSG is represented in Fig. 4. Within 1 h after the beginning of the experiment, GSH and GSSG recovery values reached, respectively, a steady state, 14.7 ± 0.6% and 13.0 ± 0.5% (mean ± SD, $n = 3$) calculated by the previous equation. Thus the collect of microdialysates could be, for *ex vivo* experiment maintained at least 6 h. Our relative recovery results were lower than those obtained by Hoque et al. [18]. The differences could be explained by the materials as Hoque et al. [18] used CMA/100 probes while in this work we used CMA/20 microdialysis probes. As the pores of our probes were lowest than those of Hoque et al. [18] this could explained that our recoveries were smaller. In addition Hoque et al. [18] worked with a perfusion flow rate of 1 μl/min while we used a flow rate of 3 μl/min. Again, the lowest the flow rate was, the better was the RR as the membrane exchanges were favourable. However, if the flow rate was very low, then the sample quantity became very small and difficult to use for analysis. This is the reason why we decided to work with the flow rate of 3 μl/min which was considered as the best compromise between the relative recovery and the sample volume collected.

5.2. Basal dermal concentrations of GSH and GSSG

The basal GSH and GSSG concentrations were determined in the dermis of three human. Three probes were inserted in each skin fragment. Basal GSH and GSSG levels in the dermis, after correction with the relative recovery, are presented in Fig. 5. The mean average of the ratio GSSG/GSH during the 6 h experiments was 8.69 ± 2.80%.

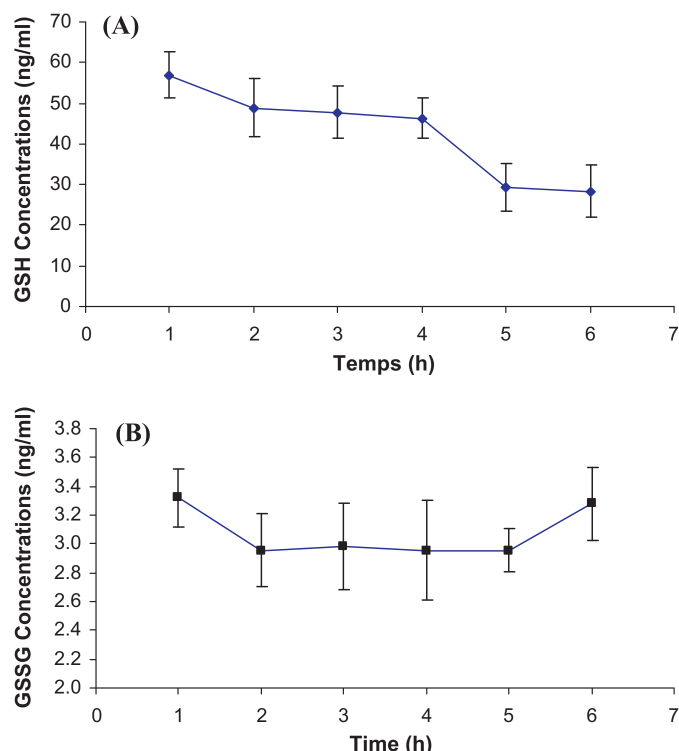

Fig. 5. Basal concentrations of (A) GSH and (B) GSSG in human dermis ($n = 9$).

This result is in a good accordance with the ratio get by Rhie et al. [19]. However, Rhie et al. [19] used biopsies which are an invasive method to determine this ratio while in this work we demonstrated that we get similar results with microdialysis which can be used *in vivo*.

6. Conclusion

A new LC–MS method with nitrate silver as post-column reagent has been developed to analyze simultaneously reduced and oxidized glutathione in microdialysis samples from human dermis. The method provided a simple and effect way for the detection of the analytes in a single run with an excellent sensitivity as it was increased by 500-fold relative to previous method developed for GSH and GSSG concentrations determination in microdialysis samples. The limits of detection for GSH and GSSG in microdialysates from human dermis were 0.4 and 0.5 ng/ml, respectively. The method was successfully used to determine GSH and GSSG basal concentrations in *ex vivo* human skin and could be employed in the future to monitor GSH and GSSG concentration changes with oxidative stress *in vivo*.

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