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Rapid determination of glutathione status in fish liver using high-performance liquid chromatography and electrochemical detection

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Abstract

A rapid and sensitive method for the detection of reduced (GSH), oxidised (GSSG) and protein-bound (PSSG) glutathione in fish liver, using reversed-phase HPLC with electrochemical detection has been developed. Separation was carried out isocratically at room temperature using 0.020 *M* sodium phosphate, pH 2.7 as mobile phase. A series dual-channel electrochemical detector was used for the simultaneous determination of GSH and GSSG. PSSG was determined after reduction by 1,4-dithiothreitol. The detection limits found for a 3:1 signal-to-noise ratio were 16.2 and 8.1 pmol for GSH and GSSG, respectively. The results obtained demonstrate that this method could be useful for measurement of the glutathione redox status in fish liver and are consistent with those reported for other fish. The method has been applied to follow the oxidative stress induced *in vivo* by copper(II) ions in the gilthead seabream fish (*Sparus aurata*). At longer times after copper(II) injection, the glutathione redox status of the exposed fish returned to a more reduced state, suggesting the existence of adaptive processes.

1. Introduction

The thiol tripeptide glutathione (GSH) and its oxidised dimeric form glutathione disulfide (GSSG) are metabolites involved in several important processes of living cells, such as maintenance of an adequate intracellular redox status, quenching of radicals and reactive oxygen species, conjugation with electrophilic xenobiotics to improve their excretion and amino acid transport. Glutathione can also act as a cofactor for various enzymes, *e.g.* as immediate electron donor for deoxyribonucleotide synthesis via glutaredoxin, and as a storage form of cysteine moieties [1]. The metabolic role, the physiological functions, and detection methods of glutathione have been extensively reviewed [2]. A wide variety of methods for the determination of glutathione have been proposed, ranging from photometric to chromatographic procedures [3].

In general, high-performance liquid chromatography (HPLC) provides the best results for the determination of thiols in complex biological samples. Most of the published HPLC methods are based on UV-Vis or fluorescence detection and require derivatization of the thiol group to enhance the sensitivity. Only in a few cases, simultaneous detection of thiols and disulfides

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has been reported [4–8]. Furthermore, gradient elution is frequently required in order to achieve adequate resolution, thus requiring long analysis times. Thiols have a low oxidation potential and thus are suitable substrates for electrochemical detection (ED). HPLC coupled with ED has proven to be a powerful analytical tool for the measurement of subnanomol amounts of GSH and GSSG [9–13]. Different electrode systems, based on graphite–epoxy resin [9], glassy carbon [13,14], or gold–mercury [11,12,15] have been used. In most cases, either pre-column derivatization [13] or post-column on-line reactions [10,16] have been used to achieve the required selectivity and detection limit. Addition of ion-pairing reagents to the mobile phases used was often needed to improve the resolution in most of the reversed-phase chromatographic methods [12,17]. However, HPLC–ED with dual-channel detectors provides the high sensitivity and specificity needed for thiol analysis in biological samples [18], and selection of the appropriate potentials in the flow cells eliminates many of the interferences caused by electroactive components present in complex samples.

Here we report a rapid, sensitive, selective and reproducible method for the simultaneous detection of GSH, GSSG and protein-bound GSH in fish liver samples at the subnanomole level, using reversed-phase HPLC coupled to a dual-channel multi-electrode electrochemical detection system. The samples were eluted isocratically without ion-pairing agent in the mobile phase. The method was also used for the analysis of protein-bound GSH and for estimation of the oxidative stress induced by copper(II) injection in the gilthead seabream fish *Sparus aurata*.

2. Experimental

2.1. Chemicals

N-Ethyl maleimide (NEM), glutathione reductase (EC 1.6.4.2) from baker's yeast, 1,4-dithiothreitol (DTT), sodium borohydride ($NaBH_4$), β-nicotinamide adenine dinucleotide phosphate (NADPH), disodium EDTA, reduced glutathione (GSH), and glutathione disulfide (GSSG) were obtained from Sigma (St. Louis, MO, USA). Perchloric acid, orthophosphoric acid and salts for the mobile phases were from E. Merck (Darmstadt, Germany). All other chemicals used were of analytical-reagent grade.

2.2. Equipment

HPLC was performed using a liquid chromatograph (Beckman, San Ramón, CA, USA), equipped with a programmable solvent delivery module 126, a type 502 autosampler with a 20-μl injection loop, and a type 406 analogical interface module for data capture from the electrochemical detector. Electrochemical detection was performed using an ESA Coulochem II (ESA, Bedford, MA, USA) detector, equipped with a guard cell (ESA 5020) and an analytical cell (ESA 5011). Chromatographic data were controlled by a Compaq Deskpro 386/20e computer (Houston, TX, USA) fitted with the System Gold version 6.0 software from Beckman. Centrifugations were carried out in J2-21 and L8-80M Beckman centrifuges. High-purity water was obtained from a Milli-Q reagent-water system (Millipore, Milford, MA, USA). The solvents were degassed under vacuum in an ultrasonic bath and filtered through 0.45-μm membrane filters (Millipore). An IKA Ultraturrax T-25 homogenizer (Janke and Kunkel, Staufen, Germany) and a Labsonic U cell disruptor (B. Braun Melsungen, Melsungen, Germany) were used for preparation of fish-liver samples.

2.3. Chromatographic procedure

Separation was achieved using a Supelcosil LC-18 (250 × 4.6 mm I.D., 5 μm particle size, 100 Å pore size) reversed-phase stainless-steel column. The analytical column was protected by a Supelguard (20 × 4.6 mm I.D.), pellicular reversed-phase cartridge precolumn, both purchased from Supelco (Supelco, Bellefonte, PA, USA). Elution was carried out isocratically with 20 m*M* sodium phosphate buffer, pH 2.7 as the mobile phase. Chromatographic separation was performed at ambient temperature and a flow-rate of 1.5 ml/min (column back-pressure approx. 16.5 MPa). Prior to use, the mobile phase

was degassed in an ultrasonic bath, and purged continuously with a slight helium stream during chromatography. The potential settings of the Coulochem II multidetector were: guard cell, +0.900 V; detector 1, +0.450 V; and detector 2, +0.800 V. A current of 5 μA full-scale suffices in most cases.

Unless otherwise stated, fish-liver extracts were diluted (1:20, v/v) in mobile phase buffer, and 20 μl were injected onto the column. In most cases this dilution yielded a concentration within the linear range of the standard calibration curve.

Peak identification was carried out as follows. GSH peak: to 10 μl of fish-liver extract, 10 μl of 100 m*M* NEM solution (omitted in the control) and 180 μl of 100 m*M* potassium phosphate buffer pH 7.5 containing 1 m*M* EDTA, were added; the mixture was incubated for 5 min at room temperature, and a 20-μl aliquot of the mixture was chromatographed. GSSG peak: 20 μl of liver cell-free extract was incubated for 10 min at room temperature with 10 μl glutathione reductase (100 U/ml), 10 μl NADPH (18.6 mg/ml; omitted in the control) and 160 μl of potassium phosphate buffer (same as above). The reaction was stopped by adding 200 μl 8.5% orthophosphoric acid solution; after centrifugation at 16 000 *g* for 5 min, a 20-μl aliquot of the reaction mixture (1:2 dilution in mobile phase) was injected onto the column.

Linearity of the detector response was verified by injecting pure GSH and GSSG standard solutions. Detector response for each compound was daily assessed from the standard curves. When a progressive decay in detector response was observed, a simple electrode cleaning with water, methanol and finally water, restored the efficiency of the system. Usually, a voltage pulse (−0.20 V) lasting 50 s for both electrode cells, was enough to restore the detector response, avoiding the complete polishing process.

2.4. Sample and standard preparations

Fish treatment

Sexually immature gilthead seabreams, *S. aurata*, weighing 150–170 g, were used for the experiments. Fish were maintained in 1800 l tanks at 15°C under constant aeration. Fresh water was pumped into the tank at a flow-rate of 225 l/h. Prior to the experiments, the fish were accommodated to this environment for at least two weeks. Fish were fed twice a day with Dibaq feed (Dibaq-Diproteg, Madrid, Spain) at an amount of 0.25% of the total weight of the animals in the tank. The experiment was started by netting a convenient number of animals and putting them in a 20 l container filled with a 10 mg/l quinaldine sulphate solution. After a few minutes, the anaesthetized fish were intraperitoneally injected with copper(II) chloride solution and immediately transferred back to the 1800 l tank. The fish were anaesthetized to avoid excessive damage to the fish during injection. Copper(II) chloride was dissolved in physiological saline solution (0.96% NaCl, w/v). The tested doses were 0.2 and 1.0 mg/kg of body weight. Controls were injected with saline solution. For each control and treatment, twelve animals were treated as described above, placed in separated tanks, and six animals were netted 2 and 7 days after the copper(II) treatment; their spinal cords were severed immediately and livers were excised, frozen in liquid nitrogen and kept at −80°C. Livers were ground in a mortar with liquid nitrogen and kept at −80°C until analysis. Assays were carried out on pools made from six livers of each copper(II) dose applied.

Fish liver extracts

A powdered liver sample (200 mg) was homogenised with 1.0 ml 1.0 *M* perchloric acid solution containing 2.0 m*M* EDTA. The preparation was centrifuged at 29 000 *g* for 20 min at 4°C using a JA 18.1 Beckman rotor. The lipid layer was discarded and the protein free supernatant was filtered through a 0.2-μm Nylon 66 membrane syringe filter (Scharlau, Barcelona, Spain). Samples were frozen at −80°C until use for GSH and GSSG determination. The cell-free extract was aliquoted to avoid repeated freezing and thawing.

Protein-bound GSH (PSSG)

The protein pellets obtained by the above described procedure, were resuspended in 1.0 ml of a pH 12.0 buffer solution (0.1 *M* KH_2PO_4/0.1

M KOH, 1:3, v/v). After addition of a 0.1-ml volume of a fresly prepared 0.03 *M* DTT solution, the mixture was sonicated for 30 s and incubated for 30 min at room temperature. Finally, 0.5 ml of acetonitrile and 0.1 ml of 3.7 *M* perchloric acid solution were added to the mixture. The mixture was kept on ice for 15 min. After centrifugation, the protein-free supernatant was filtered and frozen as described above. Before being analyzed for protein-released GSH, the samples were diluted (1:5, v/v) in mobile phase buffer and 20 μl were injected onto the column for chromatography.

Standard solutions

A standard stock solution containing 10 mg/ml of each GSH and GSSG, was prepared in mobile phase buffer and stored at −80°C. Working standards, within the required concentration range of 0.25 to 10 μg/ml, were obtained by diluting the stock solution with the same buffer, and have been used for the daily assessment of the detector response.

3. Results and discussion

The use of sensitive analytical methods is necessary for the quantitation of total glutathione in small or diluted samples of animal tissues. HPLC coupled to electrochemical detection allows accurate analysis of both GSH and GSSG, and offers additional advantages such as detection of glutathione metabolites, absence of interferences from other biological thiols, and high selectivity and sensitivity. The concentration of glutathione in fish liver changes widely depending on different factors such as nutritional and environmental aspects. In general, the GSSG concentration is much lower than the GSH concentration in animal tissues [19,20]

Accurate determination of glutathione in biological samples depends on adequate sample treatment. Autoxidation of GSH during sample handling can give misleadingly high GSSG levels. Extraction of these compounds from animal tissues is usually carried out in acidic media to precipitate proteins and inhibit oxidation of GSH by endogenous enzymes [9]. However, most of the acids used can promote a fast GSH autoxidation [21]. Nevertheless, perchloric acid has been found to adequately precipitate proteins with minimal GSH autoxidation [21,22]. Under the experimental conditions used here, using an acidic buffer for extraction, no decrease in GSH concentration after storage for 24 h at 4°C has been observed. The recovery of the extraction procedure was calculated by adding standard amounts of GSH and GSSG (up to 100 ng of each) to liver samples prior to extraction. The values found were 89 ± 9% and 83 ± 3% for GSH and GSSG ($n = 6$), respectively.

Optimization of the detector response was carried using the current–voltage curve obtained by plotting the relative peak area *vs.* applied potential. For this purpose, the same amounts of standard (50 ng of each) were injected onto the column, while the potential setting was changed for each run until maximum detector response was achieved. Fig. 1 shows the hydrodynamic voltammograms obtained and a delay of *ca.* 0.2 V in the detector response was observed between GSH and GSSG. The potential setting of detector 1, +0.45 V, was intended as a screen against electroactive species present in the sample; at this potential no signal was observed for both GSH and GSSG. The potential of detector 2 was set at +0.8 V for the following reasons: (i) The detector signal observed for both ana-

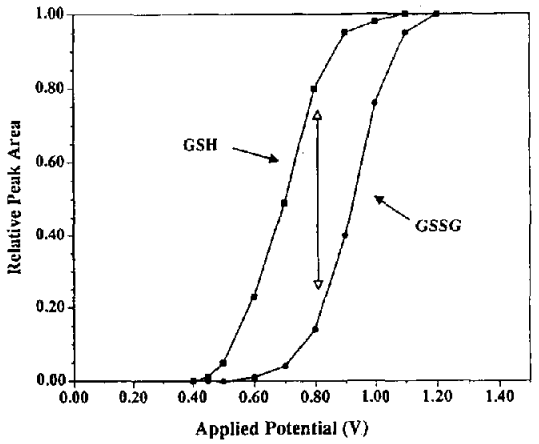

Fig. 1. Hydrodynamic voltamograms for GSH and GSSG.

lytes was sufficient to determine the glutathione levels found in fish liver samples. (ii) Although at potentials higher than +0.8 V higher responses would be obtained, the high background current produced would decrease the electrode life, and a number of other peaks would be observed in the chromatogram. The guard cell, placed between the pumps and the injector, serves to remove interfering electroactive trace impurities in the mobile phase, and was needed to achieve a stable baseline. The detector response was linear in the range of 5 to 200 ng of GSH and GSSG injected onto the column. Linear regression analysis (least-squares) of the standard data gave correlation coefficients of 0.993 and 0.998 for GSH and GSSG, respectively. The detection limits found at a signal-to-noise ratio of 3:1 were 16.2 and 8.12 pmol for the reduced and oxidized forms, respectively, which is at least 100-fold lower than the glutathione levels found in all the samples tested.

A typical chromatogram showing the elution profile of both glutathione forms from fish liver extracts is shown in Fig. 2A. The retention times found were 3.7 min for GSH and 11.5 min for GSSG. Additional peaks in the chromatogram, including other biological thiols and related compounds, such as cysteine, cystine, coenzyme A, *etc.*, were also detected at the potential settings used in our system. Fig. 2B shows the elution profile of a crude cell-free extract spiked with 130 and 65 pmol of GSH and GSSG. As expected, an increase of the peak heights was observed for these compounds, while the other peaks remained constant.

Several experiments have been performed to confirm the identity of the peaks with those of authentic samples of GSH and GSSG. The component eluting at 3.7 min disappeared completely after treatment with NEM [21] and thus could unequivocally be identified as GSH. Chromatograms corresponding to samples before (I), and after (II) NEM treatment are shown in Fig. 3A. As can be seen, a decrease of the GSH peak and appearance of a new peak eluting at 7.8 min corresponding to the NEM–GSH adduct was observed. On the other hand, the peak eluting at 11.5 min was identified as GSSG after incubation of the crude extract with NADPH and glutathione reductase. The chromatogram in Fig. 3B.I, shows a total disappearance of the GSSG peak and a concomitant increase of the GSH peak. The broad peak eluting after GSH, corre-

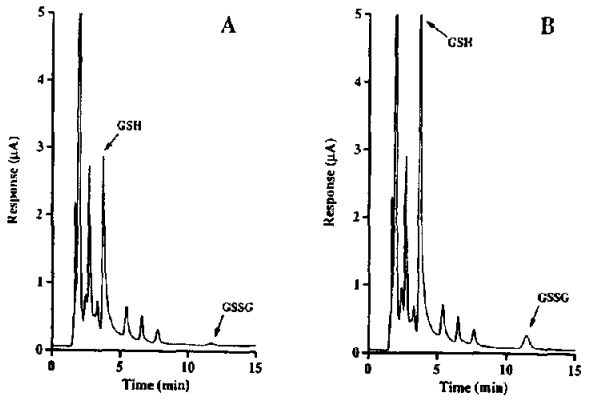

Fig. 2. HPLC-ED of fish-liver cell-free extracts. (A) Sample: 20 μl of crude extract diluted 1:20 in mobile phase. (B) Same as above spiked with 40 ng of each GSH and GSSG. Column: Supelcosil LC-18 (250 × 4.6 mm I.D., 5 mm d.p. and 100 Å pore size). Pre-column: Sulpelguard (20 × 4.6 mm I.D., pelicular reversed-phase cartridge). Mobile phase: 20 m*M* sodium phosphate, pH 2.7. Flow-rate: 1.5 ml/min. Backpressure: 16.5 MPa. Temperature was ambient. Potentials in the Coulochem II multidetector: guard cell, +0.900 V; detector 1, + 0.450 V; detector 2, + 0.800V. Current range: 5 μA full-scale. Retention times: 3.7 and 11.5 min for GSH and GSSG, respectively.

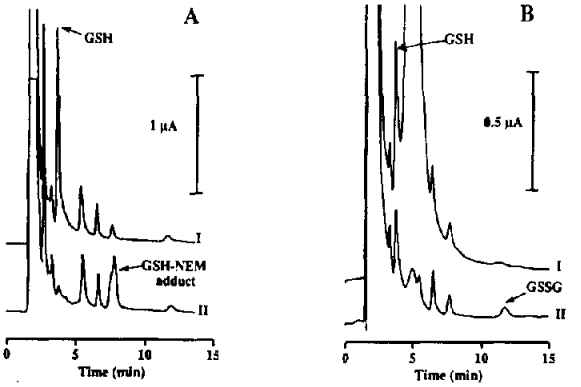

Fig. 3. Identification of GSH and GSSG peaks in crude extract samples. (A) I. Elution profile of a control; II. after treatment with NEM. (B) I. Elution profile after reduction with glutathione reductase and NADPH; II. control sample. Chromatographic conditions were as described in Fig. 1 and sample preparation as described in Experimental.

sponds to the excess of NADPH present in the sample. The chromatogram corresponding to a control sample is shown in Fig. 3B.II. We have also assessed the mass recovery of the chromatographic process. The data were calculated on the basis of the results obtained from spiked, non-spiked and pure standard samples. The assays were performed in triplicate and the respective mean values were used for the recovery calculations. The recoveries found were 99.8% for GSH and 100% for GSSG. Retention times and peak areas obtained were highly reproducible.

In the past, detection of protein-bound GSH was usually carried out by sodium borohydride reduction of mixed disulfides and the released GSH was analyzed by the classical glutathione reductase method [23,24]. When this procedure was applied to our small fish liver samples, a wide variation among the triplicate determinations was observed in all cases. This poor reproducibility could be attributed to different artifacts, such as loss of sample due to foaming and pH changes occurring in the course of the reaction. An alternative method to release the protein-bound GSH is the use of DTT as reducing agent [25]. It should be noted that, when the amount of tissue sample available is not a limiting factor, both methods provide comparable results [26]. Nevertheless, small samples yielded better results using DTT as reducing agent, and therefore this method was selected to analyze the GSH bound to proteins. As a dithiol, DTT was also detected under the experimental conditions used; however, it did not interfere with the measurement of GSH, since retention times of 13.9 and 20.1 min were found for the reduced and oxidized DTT forms, respectively. A typical chromatogram corresponding to the detection of protein-bound GSH is shown in Fig. 4. It should be noted that the DTT concentration is critical, and thus had to be optimized to ensure the total reduction of PSSG and to avoid the inactivation of the porous graphite electrodes in the detector. Part of the DTT added in excess (peak at 13.4 min) was oxidized yielding a peak at 20.1 min (see Fig. 4). The baseline returned to the initial value in less than 30 min after injection, and removal of remaining DTT by ethyl acetate

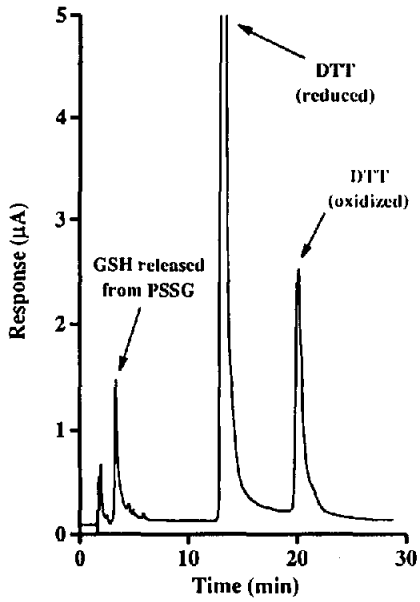

Fig. 4. Elution profile of GSH released from protein disulfides by DTT. The sample injected was 20 μl of reaction mixture (see text for details). Peaks: 3.51 min for GSH released from proteins, 13.9 and 20.1 min for DTT reduced and oxidized forms, respectively. Chromatographic conditions as in Fig. 2.

extraction was not needed [27]. When DTT concentrations higher than 2.8 m*M* were used, the resulting detector overload yielded a high background current level during 2–3 h, which returned slowly to its initial value and thus increased the time of analysis.

The glutathione redox status is a sensitive index which allows the evaluation of the intracellular effects of oxidative stress [28,29]. It is well established that transition metals, such as copper, undergo redox cycling which result in the formation of reactive oxygen species [30]. We have recently shown that fish living in environments polluted by metals and organic xenobiotics undergo oxidative stress [31]. Until recently the determination of the glutathione redox status was a time-consuming process requiring the use of the spectrophotometric assay developed by Tietze [24], which made use of the cyclic reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) in the presence of NADPH and glutathione reductase. A similar approach to that

presented in the present study has been reported by Sofic *et al.* [18], although their coulometric method only detected GSH due to the lower potentials applied, while GSSG was measured after reduction by NADPH and glutathione reductase. The HPLC method described here provides a significant advantage, allowing the simultaneous detection of both glutathione redox forms. Consequently, we decided to apply it to study the intracellular redox status of fish exposed under controlled conditions to Copper(II) ions.

Table 1 shows the levels of GSH, GSSG and PSSG determined in liver samples from *Sparus aurata*, two and seven days after intraperitoneal injection with different Cu(II) doses. A clear case of oxidative stress was observed after 48 h in the fish injected with 1.0 mg/kg of Cu(II). A huge GSSG increase was observed in these animals which displayed a GSSG/GSH ratio 7-fold higher than the corresponding control. In contrast, no such clear-cut result was observed in animals injected with the lower Cu(II) dose, with the exception of some increase in the PSSG level. The results obtained after seven days were somewhat surprising. Thus, the glutathione redox status of fish injected with 0.2 mg/kg Cu(II) was even less oxidized than the corresponding control, and an increase in the oxidized glutathione forms was observed only in the animals injected with the high Cu(II) dose. However, the GSSG/GSH and the (GSSG + PSSG)/GSH ratios were not very different from those of controls, probably as a result of a concomitant increase in GSH content. The results obtained seven days after Cu(II) injection suggest that fish exposed to redox-active metals develop a mechanism to compensate for extreme oxidative stress. Increases in several detoxifying and antioxidative enzymatic activities have been recently described in fish living in a metal polluted environment [31].

4. Conclusion

The aim of the present work was to develop a sensitive and reproducible chromatographic method using electrochemical detection for the rapid and accurate analysis of GSH, GSSG and protein-bound GSH in animal tissues. The wide linear dynamic range in detector responses allows simultaneous determination of GSH and GSSG, in less than 15 min, and of PSSG in less than 30 min. Elution was carried out isocratically and the addition of ion-pairing agents to the mobile phase was not necessary. A real fish-liver sample could be fully processed in our laboratory in 2 h, including sample handling and chromatography. By using an autosampler, 10 samples/day (determination in triplicate) can be easily analyzed.

Table 1
Effect of Cu(II) treatment on the redox status of hepatic glutathione from gilthead seabream (*Sparus aurata*)

Sample	GSH[a]	GSSG[a]	PSSG[b]	GSSG/GSH[c]	GSSG + PSSG/GSH[c]
Two days					
Control	522 ± 11	48 ± 4	90 ± 3	0.18	0.36
Copper (0.2 mg/kg)	574 ± 50	55 ± 2	140 ± 1	0.19	0.44
Copper (1.0 mg/kg)	360 ± 7	227 ± 17	98 ± 3	1.26	1.53
Seven days					
Control	719 ± 35	76 ± 12	96 ± 1	0.21	0.34
Copper (0.2 mg/kg)	688 ± 69	63 ± 13	90 ± 1	0.18	0.31
Copper (1.0 mg/kg)	821 ± 15	196 ± 18	103 ± 1	0.48	0.60

[a]Expressed as nmol/g of liver (mean ± S.D.) of three different determinations.
[b]Expressed as nmol of GSH/g of liver.
[c]Expressed as the ratio of the nanomol of GSH in its oxidized (GSSG and/or PSSG) and reduced forms.

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