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Capillary electrophoresis of glutathione to monitor oxidative stress and response to antioxidant treatments in an animal model

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Abstract

Glutathione plays a central role in metabolism and antioxidant defence. Several factors can influence the analytical efficiency and rapidity of the quantitative determination of glutathione. Procedures in sample pre-treatment have been compared in order to minimize analytical errors. Capillary electrophoresis has been chosen as a more adequate technique for obtaining a rapid and simple method for glutathione and glutathione disulfide determination in the blood and liver of the rat. The methods, once optimised, have been validated and applied for monitoring the oxidative stress in an animal model, such as the rat made diabetic by streptozotocin injection, when the animals are treated with antioxidants and compared with the corresponding controls.
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1. Introduction

Glutathione is one of the most significant components of the collective antioxidant defenses. The glutathione status of a cell (that is, the excess of reduced over oxidized glutathione) is probably one of the most accurate single indicators of the health of the cell. This crucial role of glutathione in antioxidant defense, nutrient metabolism, and regulation of essential pathways for whole body homeostasis presents a challenge, since in order to obtain significant results about its content, continuous research and a large number of samples have to be measured. Glutathione is an ubiquitous molecule that is produced in all organs, especially in the liver [1]. Nevertheless, since blood glutathione concentrations may reflect glutathione status in other less accessible tissues, measurement of both reduced (GSH) and oxidized glutathione (GSSG) in blood has been considered essential as an index of the whole subject oxidative status and as a useful indicator of disease risk in humans.[2,3].

Due to its interest as biomarker, the literature on glutathione is very extensive in relation to both metabolic [1,4] and analytical aspects. Thus, determination is commonly achieved with HPLC by using spectrophotometric [2,5–7], spectrofluorimetric [8–10], and electrochemical [11–13] detectors and more recently capillary electrophoresis (CE). Table 1 summarizes the methods found in the literature for glutathione analysis by CE in liver and blood.

However, a difficult problem in determining glutathione levels is the sample pre-treatment. Sample collection, sample preparation, protein precipitation, and any subsequent sample treatment must be carefully considered, because the oxidation of glutathione to glutathione disulfide, including protein mixed disulfides, must be minimized; otherwise erroneously low values of glutathione and high values of glutathione disulfide and mixed disulfides will be obtained. Conflicting literature values for erythrocyte, plasma, and whole blood thiol and disulfide (cysteine or GSH and cystine or GSSG) values have resulted from the use of different analytical meth-

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Table 1
Glutathione analysis by CE

Sample	Sample treatment		Detectors	Separation conditions	Concentration		Refs.
Blood (humans)	Blood was collected with heparin. MPA was added and the sample was centrifuged		CZE UV–vis 200 nm	Buffer boric acid, bis-tris (pH 8.4) 20 kV. Capillary 75 μm × 57 cm. Normal polarity	($n = 47$) GSH: 486 ± 85 μmol/L. ($n = 47$) GSSG: 553 ± 90 μmol/L. ($n = 47$) GSH/GSSG: 8.1 ± 2.7		[41]
Erythrocytes in blood (rabbits)	Blood was collected with heparin and centrifuged. Plasma and buffy coat were removed and the red cells were washed twice with NaCl isotonic solution. The red blood cells were lysed with an equal volume of cold water and NEM. The stroma were removed by centrifugation. The supernatant was filtered using a Microcon-10 membrane		HPCE UV–vis 200 nm	Buffer sodium phosphate (pH 2.5) 15 kV. Capillary 50 μm × 20 cm. Normal polarity	–		[21]
Tissues: heart and liver, mitochondria (rats)	Sample was rinsed in ice-cold saline solution. The tissues were homogenized in PCA and KOH. The acid-soluble phases were ultrafiltered by centrifugation		CZE UV–vis 200 nm	Buffer boric acid, bis-tris (pH 8.2) Voltage of 30 kV. Capillary 75 μm × 67 cm. Normal polarity	GSH: 8.1 ± 2.6 μmol/mg. GSSG: 0.45 ± 0.15 μmol/mg		[42]
Blood (humans)	Lysis and protein precipitation in EDTA and SSA. The sample was vortex mixed and centrifuged		CZE ED carbon fibre and Au-Hg	Buffer (NaH$_2$PO$_4$-Na$_2$HPO$_4$ (pH 5.8) Voltage of 20 kV. Capillary 25 μm × 57 cm. Normal polarity	GSH: 3.08 × 10^{-4} μmol/L		[43]
Erythrocytes and miocardial tissue (dog and humans)	The blood was vortex-mixed with saponin. Sodium chloride and acetonitrile were added. The tubes were vortex-mixed again and centrifuged		CZE UV–vis 214 nm	Buffer boric acid and Tris (pH 8.0) Voltage of 10 kV. Capillary 50 μm, 30 cm. Normal polarity	Dogs ($n = 6$) GSH: 0.94 μmol/L. GSSG: 0.23 μmol/L. Humans ($n = 6$) GSH: 0.87 μmol/L. GSSG: 0.06 μmol/L		[44]
Erythrocytes in blood (humans)	Blood was collected with EDTA. Blood samples were centrifuged and then washed three times with NaCl. Samples were stored at −80 °C		CZE UV–vis 200 nm	Buffer boric acid (pH 7.8) Voltage of 27 kV. Capillary 75 μm, 37 cm. Normal polarity	(A) GSH (4.256–10.659 μmol/g haemoglobin) GSSG: (0.302–0.507 659 μmol/g haemoglobin)	(B) GSH (5.067–11.199 μmol/g haemoglobin)	[20]
	(A) The solution was deproteinized with, TCA and EDTA	(B) The solution in water was filtered through a Microcon-10 membrane					
Serum and plasma (humans)	Blood was collected with EDTA, or heparin, or citrate as anticoagulant. Samples were centrifuged and stored at −20 °C. Serum and plasma were treated with thiol reducing agent (TCEP) and internal standard (N-acetylcysteine). The solution was deproteinized with, SSA, PCA, TCA, acetonitrile or acetone, vortex mixed and centrifuged. 6-iodoacetamidofluorescein was added for derivatization		CZE LIF Argon ion	Buffer boric acid and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 10) Voltage of 30 kV. E_{X488nm} E_{m515nm} Capillary 50 μm, 85 cm × 50 cm. Normal polarity	–		[29]
Serum and plasma (humans)	Blood was collected with EDTA. Samples were treated with thiol-reducing agent (TCEP) and internal standard (N-acetylcysteine). The solution was deproteinized with acetonitrile, vortex mixed and centrifuged. 6-iodoacetamidofluorescein was added for derivatization		CZE LIF Argon ion 488 nm laser	Buffer boric acid and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 10) Voltage of 30 kV. E_{X488nm} E_{m515nm} Capillary 50 μm, 85 cm × 50 cm. Normal polarity	GSH in serum: 6.51 ± 0.50 μmol/L. GSH in plasma: 7.01 ± 0.51 μmol/L		[23]

ods [3,14]. Main differences come from the deproteinization step [12,15–24]. Moreover, the stability of GSH and its possible oxidation to GSSG during the period between collection and analytical determination have been questioned [3].

After considering different aspects, capillary electrophoresis has been chosen as a more adequate technique for obtaining a rapid and simple method for GSH and GSSG determination in the blood and liver of the rat. The present work will focus on putting together different considerations made by different authors on sample collection, sample treatment and the method of measurement to optimize and validate an analytical method for glutathione analysis in blood and liver. The methods are then applied for monitoring the oxidative stress in an animal model, such as the rat made diabetic by streptozotocin injection [25,26], when the animals are treated with antioxidants and compared with the corresponding controls. The final purpose is to have analytical tools to evaluate the variation of metabolic parameters during in vivo studies that can correlate with the nutraceutical activity of extracts from different natural sources.

2. Materials and methods

2.1. Instrumentation

The separation was performed on a capillary electrophoresis P/ACE 5010 (Beckman, Madrid, Spain) with UV detection at 200 ± 10 nm. It was equipped with an uncoated capillary (Beckman, Madrid, Spain) with 47 cm total length (40 cm effective length) and 50 μm i.d. and was operated at 27 kV. Temperature was maintained at 25 °C. The capillary was conditioned by rinsing in the high-pressure mode (20 psi), in the following order: 0.1 M NaOH (1 min), purified water (1 min) and running buffer (1 min). Buffer vials (4 mL) used for separation were replaced after every four injections.

2.1.1. Liver
The background electrolyte (BGE) for GSH and GSSG analysis in liver was prepared with 0.200 M H_3BO_3 made up pH 8.0 with NaOH. The current generated in such conditions was 27 μA. The injection was by pressure (0.5 psi) for 5 s.

2.1.2. Blood
The BGE for GSH and GSSG analysis in blood was prepared with 0.350 M H_3BO_3 made up pH 8.0 with NaOH. The current generated in such conditions was 69 μA. The injection was by pressure (0.5 psi) for 10 s.

2.2. Chemicals

Alpha-tocopherol (*all-rac*) i.e. Vitamin E, ascorbic acid (vitamin C), streptozotocin (STZ), Triolein, Tween 80, *N*-ethilmaleimide (NEM) and standards were obtained from Sigma (St. Louis, MO, USA). Boric acid 85% and acetroni-

trile (HPLC grade) were from Merck (Darmstadt, Germany). Sodium hydroxide and EDTA-Na_2 from Panreac (Madrid, Spain). All the buffer solutions were prepared with purified water by a Milli-Qplus185 (Millipore, Billerica, MA, USA).

2.3. Animals and samples

Liver and whole blood for method development and validation were obtained from Sprague–Dawley rats bred in San Pablo-CEU University animal quarters. STZ (50 mg/kg) was administered (i.p.) to promote diabetes in the diabetic groups of animals. This day was considered Day 0 of diabetes. Three days after administration of the drug, blood glucose was estimated in a droplet obtained from the saphenous vein with an Accu-Check® system (Roche, Basel, Switzerland). Rats with glucose levels above 250 mg/dL were assigned to experimental groups.

The day of sacrifice, animals were anaesthetised with ketamine/azepromacine (75–100 mg/kg and 2.5 mg/kg, respectively) and blood was obtained by cardiac puncture in EDTA. Blood (100 μL) was rapidly mixed with either cold water (250 μL) plus 134.3 mM EDTA (50 μL), or with the same volume of cold water (50 μL) plus 134.3 mM EDTA (50 μL) and 100 mM NEM (200 μL), depending on the experiment. The hemolysate was immediately ultrafiltered to remove proteins using a micropartition device with molecular weight 30 kDa Microcon-30 (Milipore, Billerica, MA, EEUU) by centrifugation at 36220 *g*, 10 min in a Mikro 22R refrigerated centrifuge (Hettich, Tuttlingen, Germany). The ultrafiltrate was measured by CE immediately.

Livers were excised, placed in liquid nitrogen and kept at −80 °C. Tissue samples were ground in liquid nitrogen and around 100 mg were homogenized in a Potter with 4 ml of a mixture containing AcN/H_2O (62.5:37.5, v/v). 800 μL of the homogenate were transferred to eppendorf tubes and centrifuged at 36220 g for 10 min. The supernatant was immediately measured by CE.

The scheme for sample treatment of both blood and liver is summarised in Fig. 1.

2.4. Experimental design

For Experiment 1, nine diabetic rats were sacrificed after 7 days of non-controlled diabetes, and results were compared with those of nine control rats with no administration.

For Experiments 2 and 3 an antioxidant mixture was developed with 100 mg ascorbic acid as hydrophilic antioxidant, 10 mg alpha-tocopherol as lipophilic antioxidant, dispersed in the vehicle, made up by 100 mg triolein and 10 mg Tween 80 in 0.5 mL saline.

In Experiment 2, one single dose of mixture or vehicle was respectively administered by intragastric probe to the rats of the corresponding group 24 h before sacrifice, which took place 7 days after STZ administration. Therefore, four groups of animals were constituted: DX, diabetic rats with antioxidant; DV, diabetic rats with vehicle; CX, age-matched

RATS

LIVER

100 mg liver+ 2 ml(37.5% H2O + 62.5% AcN)

BLOOD

1: 100 μL blood+ 50 μL EDTA 134.3 mM+250 μL water

or

2: 100 μL blood+ 50 μL EDTA 134.3 mM +50μLwater+200 μL NEM 100mM

Homogenize

2 ml(37.5% H2O + 62.5 % AcN)
Vortex(5s)

Centrifugation
4ºC, 10 min, 36220 g

Analyze
supernatant

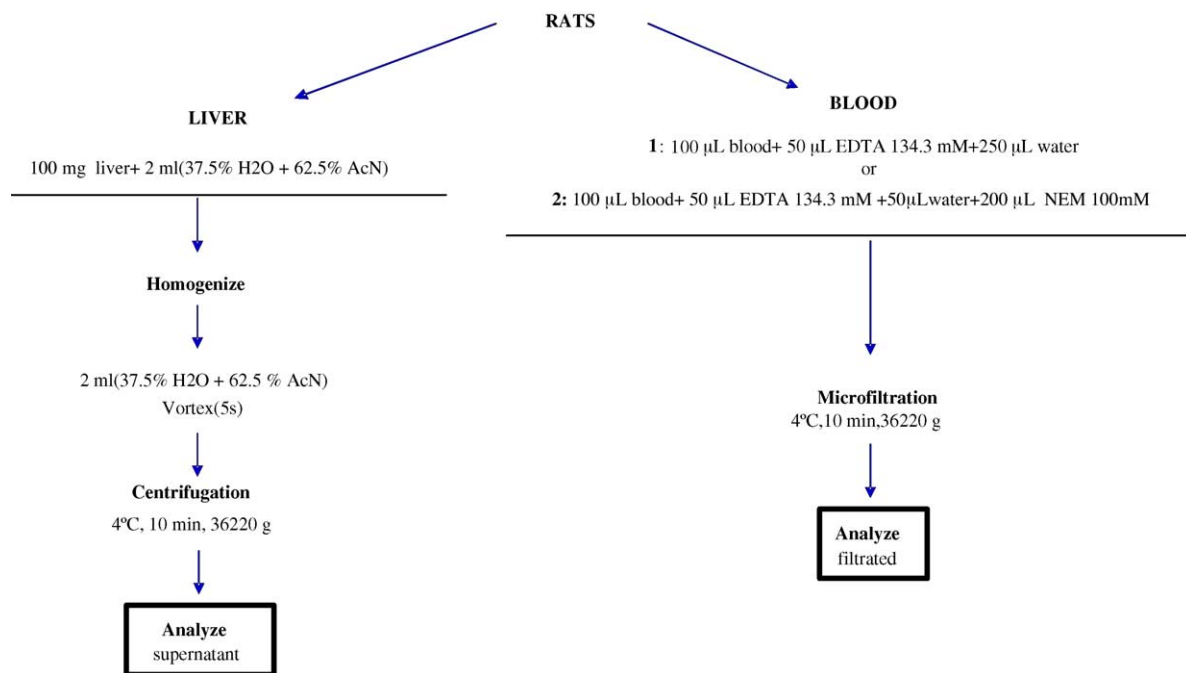**Microfiltration**
4ºC,10 min,36220 g

Analyze
filtrated

Fig. 1. Sample treatment in liver and whole blood for GSH and GSSG analysis.

healthy control rats with antioxidant; CV, control rats with vehicle.

In Experiment 3, rats were sacrificed 1 day later than in Experiment 2 (Day 8), and they received three doses of antioxidants (0, 8 and 24 h), the last dose being 24 h before sacrifice. The groups were named following the same scheme as in Experiment 2.

The experimental protocol for the study has the approval of the animal ethics committee of our institution and it is in agreement with Amsterdam treaty and Spanish legislation (RD223/1988).

2.5. Standards

The standards used for quantification in blood contained 230 μM GSH and 5 μM GSSG in purified water containing 16.8 mM EDTA. When NEM was employed as derivatizating agent, the standard also contained 50 mM NEM.

For liver, standards contained 240 μM GSH and 2 μM GSSG in the same mixture AcN/H$_2$O (62.5:37.5, v/v) as the samples. They were prepared daily and kept cold in an ice bath.

3. Results and discussion

3.1. CE separation of GSH and GSSG: method development

Electrophoretic conditions were optimised starting from those employed by Carru et al. [20,27] for erythrocytes. Capillary length, buffer concentration, potential and pH were

tested. Final conditions are described in the experimental section and the corresponding electropherograms can be seen in Fig. 2 for blood, Fig. 3 for blood with or without NEM and Fig. 4 for liver (vide infra).

3.2. Effect of sample treatment on GSH/GSSG ratio determined by CE

There is little consistency in the literature regarding the procedures for sample preparation employed for the measurement of glutathione in biological tissues [28]. Several authors check carefully the incidence of one particular step in the analytical process, whereas the error was introduced in the previous one. For example, precision obtained after acidic treatment with trichloroacetic acid (TCA) has been exhaustively tested [24], and, indeed, final samples are very stable, but GSH content with that treatment is much lower than with other treatments, as is shown below. Therefore, we decided to evaluate the factors affecting the method from sample collection to the measurement.

Causse and coworkers [29] demonstrated that for thiol quantification in plasma, EDTA gave better results than heparin or citrate as anticoagulant and blood samples were collected in EDTA. As for sample preparation, many different procedures can be found in literature. Some authors affirm the higher stability of GSH in samples precipitated with TCA [5], other authors maintain that metaphosphoric acid (MPA) is the best option [12], sulfosalicilic acid (SSA) and other acids are also employed [10,21]. Meanwhile, the real existence of GSSG in the samples is open to doubt [3].

Therefore, 0.1 M TCA, acetonitrile (AcN), 0.1 M perchloric acid (PCA), 0.1 M HCl, 3 mM desferoxamine (DF),
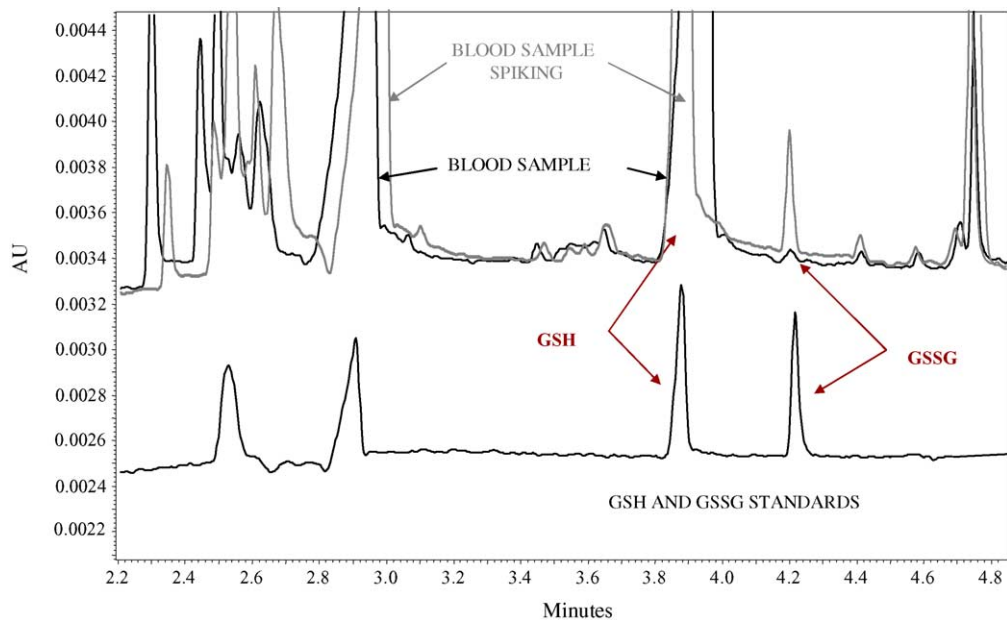
Fig. 2. Electropherograms for GSH and GSSG standards (lower); rat blood without NEM (medium); rat blood with standards added (upper). Conditions: UV detection at 200 nm, voltage 27 kV, capillary untreated 47 cm and 50 μm i.d., 350 mM borate buffer at pH 8.0.

16.8 mM EDTA and only cold water were tested to produce cell lysis followed by microfiltration to eliminate proteins (see Table 2). Also, 0.4 M TCA and AcN were assayed without microfiltration (see Table 2). Blood was mixed with the corresponding reagent 1/3 (v/v). Results of this study are given in Table 2, where a higher ratio GSH/GSSG was the parameter employed for optimization. As previously described [5], 0.4 TCA provided high reproducibility and the highest values when comparing acidic precipitation, that is

the reason why a large number of samples was assayed. Nevertheless, the GSH/GSSG levels were one order of magnitude lower with protein precipitation than with only cold water and values increased when water contained EDTA. This effect could be related not only to the ability of EDTA for metal complexation, also present in desferoxamine, but also to the increase in the osmotic fragility of erythrocytes, the extent of red blood cell haemolysis produced by osmotic stress, previously described [30]. Therefore, cold water with EDTA, for
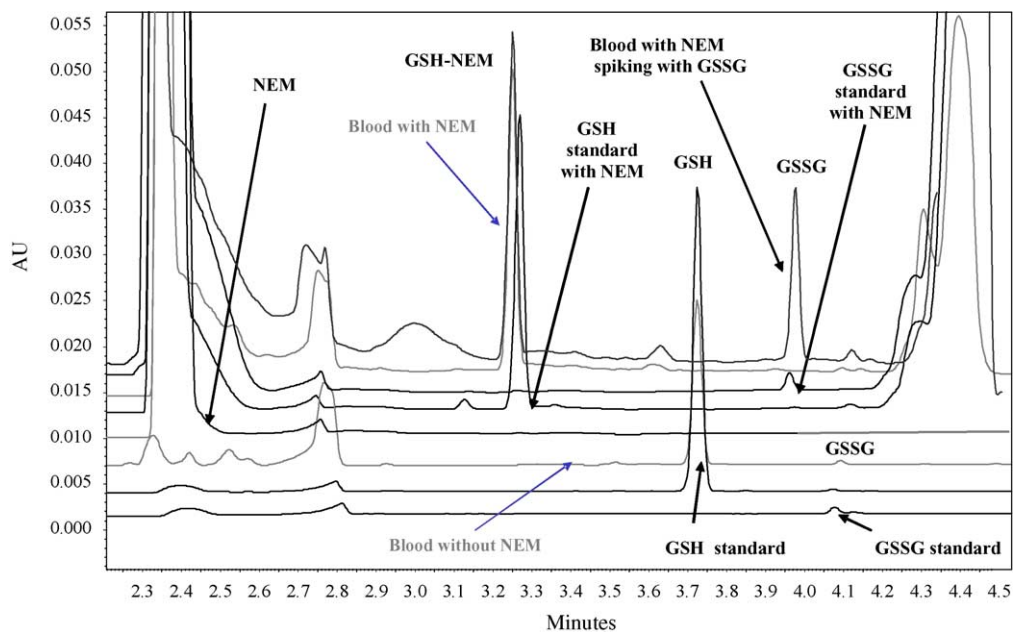

Fig. 3. From lower to upper level: electropherogram of GSSG standard; GSH standard; whole blood treated with water; NEM standard, GSH standard with NEM; GSSG standard with NEM; blood with NEM and blood with NEM spiked with GSSG. For condition see the text.
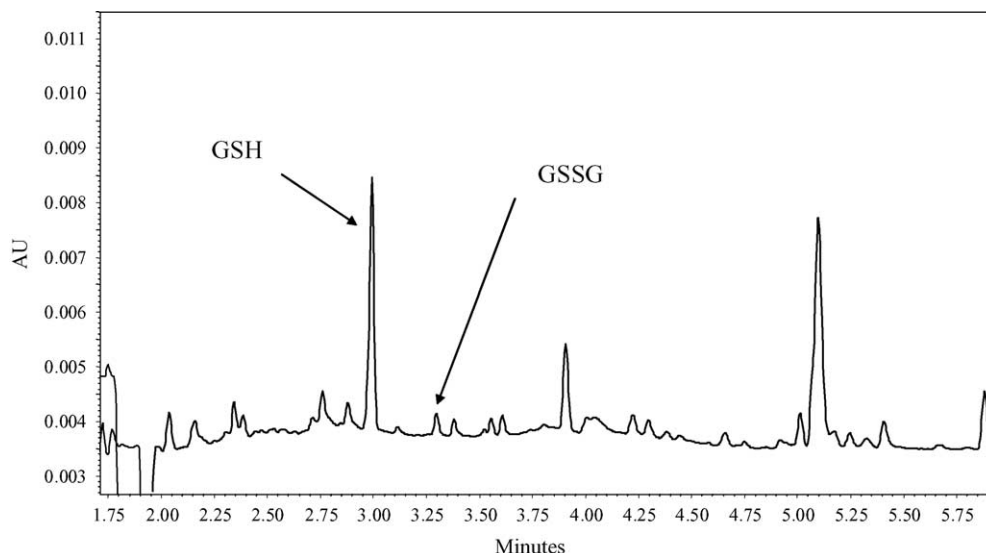
Fig. 4. Electropherograms of rat liver at 200 mM borate buffer at pH 8.0. For other conditions see the text.

haemolysis, and ultrafiltration was the method selected for sample pre-treatment. There were still two aspects to confirm: the selectivity of the method for GSH, that is to demonstrate that the peak corresponds only to GSH, and that the GSH oxidation is really prevented with the working conditions. With these purposes NEM was employed [31,32] because it gives a rapid and quantitative reaction at room temperature with SH groups. Results can be observed in Fig. 2. When NEM was added to blood samples the GSH completely disappeared, therefore, selectivity was proved. Moreover, no differences were found in GSSG concentration when NEM was present or not.

Regarding liver treatment, samples were homogenised in pure water or water containing different proportions of methanol or acetonitrile. Our results confirmed those previously obtained by Shihabi et al. [33], the supernatant was more clear and the electrophoretic profile better defined with 62.5% AcN in the mixture. Moreover, the presence of EDTA, ascorbic acid or a mixture of EDTA and ascorbic acid in the

aqueous part was tested. Results for 6 replicates can be seen in Table 3. The presence of ascorbic acid decreased GSH concentration, and that is justified because GSH protects ascorbic acid from oxidation [34,35]. There was no difference between water alone or with EDTA, therefore liver was treated with pure water and AcN as previously described by other authors [5,8,28,36,37].

3.3. Determination of GSH/GSSG in real samples: method validation

Table 4 includes the results obtained for the main validation parameters for both GSH and GSSG in blood with and without NEM and Table 5 for liver.

Linearity has been determined by a series of two replicates of five levels of standards whose concentrations span up around 50–200% of the expected concentration range. As can be seen in Table 3, the linear regression equation applied to the results gave an intercept not significantly different from zero. The slopes are different from zero in all cases and correlation coefficients are over 0.99 in all cases. Sample linearity and recovery were estimated comparing the values obtained in the linearity test for spiked samples with the corresponding standards linearity, taking into account the initial sample concentrations, which had been previously quantified. It is

Table 2

GSH/GSSG ratio obtained in blood samples with different treatments using precipitation and microfiltration (mean ± S.E.)

Reagents	GSH/GSSG
Microfiltration	
AcN ($n = 3$)	3.43 ± 0.4
TCA, 0.1 M ($n = 2$)	18 ± 5
PCA, 0.1 M ($n = 2$)	1.1 ± 0.3
HCl, 0.1 M ($n = 2$)	0.4800 ± 0.0008
Desferoxamine, 3 mM ($n = 4$)	7.00 ± 0.32
H$_2$O–EDTA, 16.79 mM ($n = 10$)	171.0 ± 22.5
H$_2$O ($n = 4$)	70 ± 23
Precipitation	
TCA, 0.4 M ($n = 13$)	4.0 ± 0.6
AcN ($n = 2$)	8.2 ± 0.4

Table 3

GSH/GSSG ratio obtained in liver samples with different treatments (mean ± S.E.)

Reagents	GSH/GSSG
H$_2$O ($n = 6$)	27.2 ± 3.2
Ascorbic acid, 25 mM ($n = 6$)	21.36 ± 0.96
EDTA, 16.8 mM ($n = 6$)	25.8 ± 2.0
Ascorbic acid, 25 mM + EDTA 14.9 mM ($n = 6$)	19.9 ± 0.7

Table 4
Main validation parameters for the analytical method for the determination and quantification of GSH and GSSG in rat liver

Liver			GSH 120–240	GSSG 1–10
	Range (μmol/L)			
Linearity	Standards	Intercept ± C.I.	43.0 ± 760.9	−4.1 ± 22.6
		Slope ± C.I.	50.2 ± 2.7	98.32 ± 4.1
		r	0.998	0.999
	Samples	Intercept ± C.I.	−37.1 ± 1487.1	12.4 ± 72.8
		Slope ± C.I.	48.6 ± 4.7	98.2 ± 11.6
		r	0.998	0.99991
Accuracy	Standards	Recovery (%)	99.7	101.1
		R.S.D. (%)	3.3	6.2
	Samples	Recovery (%)	95.3	104.4
		R.S.D. (%)	4.5	4.5
Instrumental precision	Standards	n	9	9
		Mean RF (UA/μmol/L)	54.4	102.1
		R.S.D. (%)	3.4	5.7
Standards method precision	Intra-assay	n	6	6
		Mean RF (UA/μmol/L)	51.0	104.1
		R.S.D. (%)	3.0	9.0
	Intermediate	n	6	6
		Mean RF (UA/μmol/L)	51.9	109.7
		R.S.D. (%)	2.8	10.9
Samples method precision	Intra-assay	n	10	10
		Mean (μmol/g)	7.0	0.14
		R.S.D. (%)	5.2	8.9
	Intermediate	n	20	20
		Mean (μmol/g)	7.1	0.14
		R.S.D. (%)	6.0	8.7

Table 5
Main validation parameters for the analytical method for the determination and quantification of GSH and GSSG in rat blood

	Range (μmol/L)		Whole blood without NEM		Whole blood with NEM	
			GSH 120–361	GSSG 2.6–7.8	GSH 115–460	GSSG 2.6–9.9
Linearity	Standards	Intercept ± C.I.	−472.5 ± 1893.8	−85.9 ± 178.2	480.1 ± 999.5	−87.7 ± 101.2
		Slope ± C.I.	132.8 ± 7.4	255.3 ± 38.3	209.9 ± 4.5	439.3 ± 20.4
		r	0.998	0.995	0.9995	0.998
	Samples	Intercept ± C.I.	932.2 ± 1684.3	−74.8 ± 74.5	1983.6 ± 2332.1	−16.58 ± 202.1
		Slope ± C.I.	121.1 ± 6.5	263.8 ± 17.9	192.3 ± 7.9	431.9 ± 36.3
		r	0.998	0.998	0.998	0.998
Accuracy	Standards	Recovery (%)	99.9	100.1	99.3	102.2
		R.S.D. (%)	2.6	3.0	1.04	3.5
	Samples	Recovery (%)	95.2	83.6	93.7	102.0
		R.S.D. (%)	2.5	3.7	1.4	5.5
Instrumental precision		n	10	6	10	6
		Mean RF (UA/μmol/L)	131.4	263.0	215.6	519.1
		R.S.D. (%)	2.6	2.5	1.7	7.8
Standards method precision.	Intra-assay	n	6	6	6	6
		Mean RF (UA/μmol/L)	135.4	266.3	211.0	495.1
		R.S.D. (%)	1.2	5.6	0.7	7.3
Samples method precision	Intra-assay	n	10	10	6	6
		Mean (μmol/L)	903.8	8.5	1017.8	18.5
		R.S.D. (%)	1.4	6.4	4.4	3.4

Table 6
GSH and GSSG concentrations reference values in rat blood

Experimental values	Reference values	Ref.
	GSH:1378.0 μmol/L;	[2]
GSH: 903–1017.8 μmol/L;	GSSG:3.21 μmol/L	
GSSG: 7.5–18.5 μmol/L	GSH: 744.0 μmol/L;	[45]
	GSSG:132.0 μmol/L	
	GSH: 991 ± 184 μmol/L	[46]
	GSSG: 19.3 μmol/L	[5]

curious to point out that a 95.3% GSH recovery in liver samples is compensated with a 104.4% GSSG recovery, which indicates a minor oxidation in GSH during sample treatment (Table 6).

In blood samples, the lower recovery was obtained for GSSG when only water was added (83.6%) and could be related to the rapid reaction of enzymes that destroy GSSG, which is highly toxic for the organism. Nevertheless, GSSG loss is much lower than GSH loss when acidic media is employed to deactivate these enzymes. On the other hand, the possible modification of GSH/GSSG equilibrium when NEM is present due to the decrease in GSH concentration, discussed by some authors [3], does not seem to take place, looking at GSSG recoveries in the presence of NEM.

Therefore, validation parameters and manipulation aspects made the method with NEM more reliable for blood analysis.

Precision provided R.S.D. values, which were under 10% in all cases. This was the acceptance criterion, considering the characteristics of samples and analytes.

Six blood samples were analysed with both methods and results were compared with data in bibliography for the same type of samples. They are summarised in Table 5. There were no significant differences (Student's *t*-test, $p < 0.05$) between results obtained with both methods and all of them were in the range of results in bibliography except for GSSG by Asensi et al. [5]. As blood proved to be the more complicated matrix, some additional assays were developed. Blood samples were stable for no more than 30 min when stored in ice without any treatment, but stability increased to over 3 h when NEM was added after collection. Longer storage times are not recommended because NEM can slowly react with amino groups giving a decrease in the GSH-NEM peak and additional or possible interfering peaks [2,3,5]. Stability was established when responses varied less than 5% for GSH or 10% for GSSG, related to $t = 0$.

Blood has to be analysed fresh, but liver samples can be stored at −80 °C, therefore the stability of stored samples was also checked by comparing the values obtained for GSH and GSSG in a fresh sample and in aliquots of the same liver measured at 7, 14, 28 and 30 days. No differences were found in the results and therefore, liver samples can be stored for at least 1 month at −80 °C for glutathione analysis.

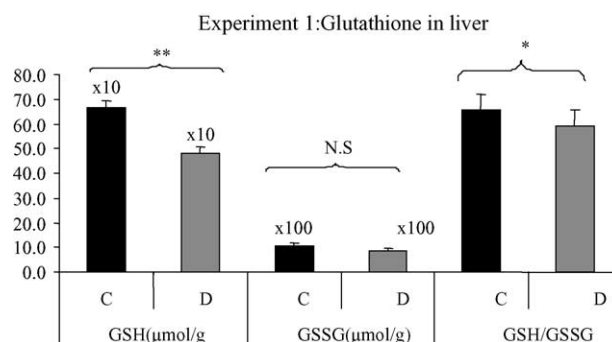LODs, calculated following EURACHEM method [38] were below the expected values for both the analytes in the samples.

Fig. 5. GSH, GSSG, and the ratio GSH/GSSG in control and diabetic rat liver for the Experiment 1. (*$p < 0.05$; **$p < 0.01$). N.S.: non-significant; C: control rats; D: diabetic rats.
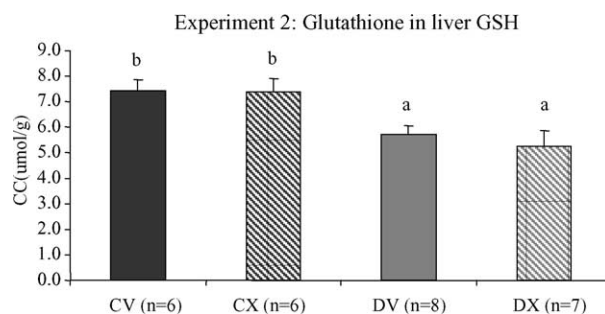

Fig. 6. GSH in control and diabetic rat liver for the Experiment 2. The same letters indicate homogeneicity of the means after multiple range test ($p < 0.05$). Abbreviations: CV, control rats with vehicle; CX, age-matched healthy control rats with antioxidant; DV, diabetic rats with vehicle; DX, diabetic rats with antioxidant.

3.4. GSH/GSSG for animals under antioxidant treatments

Results obtained with the experimental design described above in liver are summarised in Figs. 5–7. Results from control and diabetic animals (Experiment 1; Fig. 5) were statistically compared with a Student's *t*-test and showed a significant higher amount ($p < 0.01$) of GSH in control than in the liver of diabetic animals. Meanwhile, the effect of one and three doses of antioxidants was compared with the
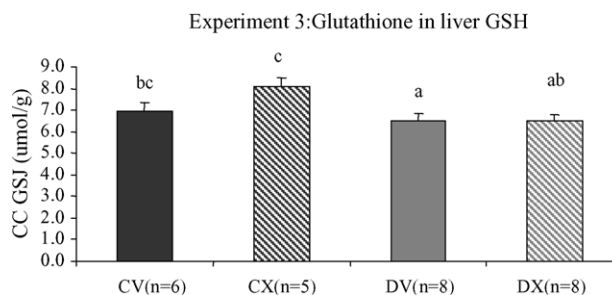

Fig. 7. GSH in control and diabetic rat liver for the Experiment 3. The same letters indicate homogeneicity of the means after multiple range test ($p < 0.05$). Abbreviations: CV, control rats with vehicle; CX, age-matched healthy control rats with antioxidant; DV, diabetic rats with vehicle; DX, diabetic rats with antioxidant.
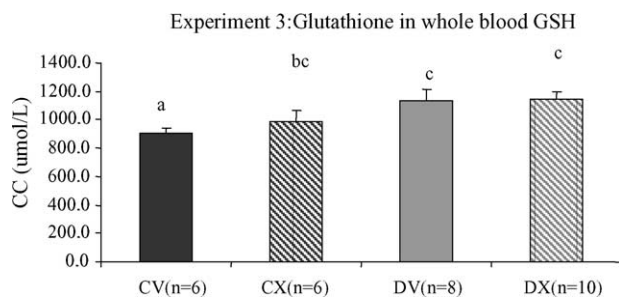
Fig. 8. GSH in control and diabetic rat whole blood for the Experiment 3. The same letters indicate homogeneicity of the means after multiple range test ($p < 0.05$). Abbreviations: CV, control rats with vehicle; CX, age-matched healthy control rats with antioxidant; DV, diabetic rats with vehicle; DX, diabetic rats with antioxidant.

Bonferroni multiple-range test after one-way ANOVA. One dose of antioxidant showed no change in GSH of control versus diabetic animals (Experiment 2; Fig. 6), however, when three doses were administered GSH in the liver of diabetic animals was not different to the values in controls (Experiment 3; Fig. 7).

Gluthathione in blood was measured only in the experiment with three doses, because samples cannot be stored and the method was not validated for previous experiments. It is noteworthy in Fig. 8 that GSH was significantly increased in diabetic animals versus their controls. That was previously observed by Kondo et al. [39] and could be justified by an increased endogenous synthesis [40].

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