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Simultaneous high-performance capillary electrophoretic determination of reduced and oxidized glutathione in red blood cells in the femtomole range

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Abstract

This paper describes a high-performance capillary electrophoretic (HPCE) method which allows a quick, simultaneous and quantitative determination of reduced (GSH) and oxidized (GSSG) glutathione in mammalian red blood cells using a Supelco-bonded hydrophilic phase capillary CElect-P150. The extraction procedure of GSH and GSSG from erythrocytes using Microcon-10 membranes is very simple and allows a correct evaluation of these compounds present in the red blood cells. Furthermore, the HPCE method does not require removal of the excess N-ethylmaleimide used to block the glutathione in its reduced state, making the simultaneous evaluation of GSH and GSSG possible in a very short time (ca. 4 min), with a sensitivity at femtomole level.

1. Introduction

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH), an essential tripeptide present in virtually all animal cells, is a component of a pathway that uses reduced nicotinamide–adenine dinucleotide phosphate (NADPH) to maintain the cellular redox state. It has an essential role in maintaining both proteins and other compounds such as ascorbate, α-tocopherol, etc. in their reduced states. Furthermore, it is involved in the reduction of ribonucleotides to deoxyribonucleotides (precursors of DNA) and is implicated in protecting the cell against oxidative damage, free radical damage and other types of toxicity [1]. Glutathione can be present in the cells in its oxidized (GSSG) and reduced states (GSH). Usually, most intracellular glutathione is in its reduced state, which in most tissues represents more than 99% of the total glutathione (GSH + GSSG) [2]. Commonly, GSH is present in the cell in 1–2 m*M* concentrations, while the concentration of GSSG is at the μ*M* level. However, the levels of oxidized and reduced glutathione can change significantly upon oxidative stress, and their evaluation provides useful information about the redox and detoxification status of cells and tissues [3–6]. Several procedures for the determination of GSH and GSSG from different biological sources have been reported in the literature: chemical [7] and enzymatic [8–10] methods, high-performance liq-

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uid chromatography [2,11–19], flow cytometry [20–22] and, more recently, capillary electrophoresis (CE) [23,24]. GSH and GSSG can be detected using spectrophotometric [25,26], spectrofluorimetric [27–33] and electrochemical detectors [11,15,17,34–39] with different levels of sensitivity ranging from nmol [14,16] to pmol [12,13]. Correct measurement of glutathione disulfide is difficult both because of its low concentration and the ease with which GSH is oxidized to GSSG in biological extracts. One of the most common methods utilized for GSSG determination in red blood cells involves an enzymatic reaction using NADPH-dependent glutathione reductase which ensures high specificity and good sensitivity [40]. However, in order to prevent undesirable oxidation of GSH in the course of sample treatment, N-ethylmaleimide (NEM) must be added to block free thiol groups [10,40–42]. This reagent interferes with the subsequent enzymatic determination of GSSG by inhibiting the glutathione reductase. Furthermore, this method requires several extractions with diethyl ether to remove the excess NEM, followed by flushing with nitrogen to eliminate this diethyl ether. These numerous steps make this method laborious and time-consuming and, moreover, the additional manipulations required may lead to an inaccurate evaluation of glutathione. In this paper, we describe a procedure which allows a quick CE analysis of the GSH-NEM derivative and GSSG in the red blood cells in conjunction with a simple extraction procedure using a Microcon-10 membrane. This filtration procedure does not require the use of protein-precipitating agents (such as perchloric acid, metaphosphoric acid, trichloroacetic acid, etc.) commonly used to minimize the oxidative change of GSH to GSSG. This is an important point because Reed et al. [14] have shown that the use of these protein-precipitating agents is unsuitable for the correct determination of GSH and GSSG in whole blood samples. Furthermore, the CE method described here, using a Supelco-hydrophilic bonded phase capillary CElect-P150, allows the analysis of GSH and GSSG in red blood cells with a sensitivity at fmol level.

2. Experimental

2.1. Chemicals

GSH, GSSG and NEM were obtained from Sigma (St. Louis, MO, USA). Analytical reagent-grade potassium dihydrogenphosphate was purchased from Merck (Darmstadt, Germany). Microcon-10 microconcentrators were purchased from Amicon (Beverly, MA, USA). Filters (0.22 μm) were obtained from Millipore (Bedford, MA, USA). The water used for the experiments was doubly distilled. All buffers were filtered through a Millipore filter (0.22 μm) before HPCE analysis.

2.2. HPCE apparatus

CE analysis was performed using an automated P/ACE 2100 system (Beckman Instruments, CA, USA) interfaced with an IBM 55sx computer using System Gold software for control and data collection. The P/ACE 2100 system, equipped with UV detector, automatic injector and autosampler, was fitted with a 30 kV high-voltage power supply with a current limit of 250 μA.

2.3. Capillary

The capillary used throughout this work was a 27 cm Supelco bonded hydrophilic phase CElect-P150 (50 μm I.D. $\times$ 363 μm O.D.), with the detector cell at 20 cm (Supelco, Bellefonte, PA, USA). The capillary was assembled in a Beckman cartridge.

2.4. Preparation of samples

Whole blood was collected using heparin as anticoagulant. Rabbit red blood cells with a percentage of reticulocytes ranging from 40 to 60% were obtained from rabbits made anaemic by phenylhydrazine administrations [43]. The blood samples were centrifuged at 5000 g (3000 rpm) for 10 min, plasma and buffy coat were removed and the red cells were washed twice

with 0.9% (w/v) NaCl isotonic solution. In some experiments, the packed cells were resuspended 1:10 in the presence or absence of 0.1 m*M* Fe^{2+} and 10 m*M* ascorbate and then incubated for 90 min at 37°C, in a shaking water bath. In all experiments the red blood cells were lysed adding an equal volume of cold water in presence of (50 m*M*) NEM and leaving the solution in ice for 20 min. The stroma were removed by centrifugation at 14 000 rpm for 10 min. The supernatant was filtered using a Microcon-10 membrane (cut-off M_r 10 000) at 10 000 rpm for 5 min and the filtered solution was directly analyzed by HPCE.

2.5. CE analysis

The analysis was performed applying the sample under nitrogen pressure for 5 s using a sodium phosphate (35 m*M*, pH 2.1) buffer as electrolyte. The separating conditions of 15 kV were achieved in 1 min and held at a constant voltage for 9 min. The experiments were carried out at 25°C and detection was performed by UV absorption at 200 nm. After the analysis, preconditioning of the capillary using acid or basic treatments is not necessary, and new analyses can be performed by equilibrating the capillary using the electrolyte buffer for about 2 min.

2.6. Spectrophotometric determination of GSH and GSSG

The colorimetric method of GSH determination and the enzymatic assay for the GSSG evaluation were performed as described by Beutler [44].

3. Results and discussion

3.1. Separation of GSH and GSSG by HPCE

Fig. 1A shows the simultaneous separation of a 500 μM standard mixture of GSH and GSSG by CE using a Supelco CElect-P150 hydrophilic bonded phase capillary. As shown in the electropherogram, the analysis of the oxidized and reduced glutathione can be performed in a short time (less than 4 min) making this method significantly quicker than other high-performance liquid chromatographic, enzymatic or colorimetric procedures. The use of NEM to block the GSH in its reduced state has been suggested, however, in order to obtain a correct

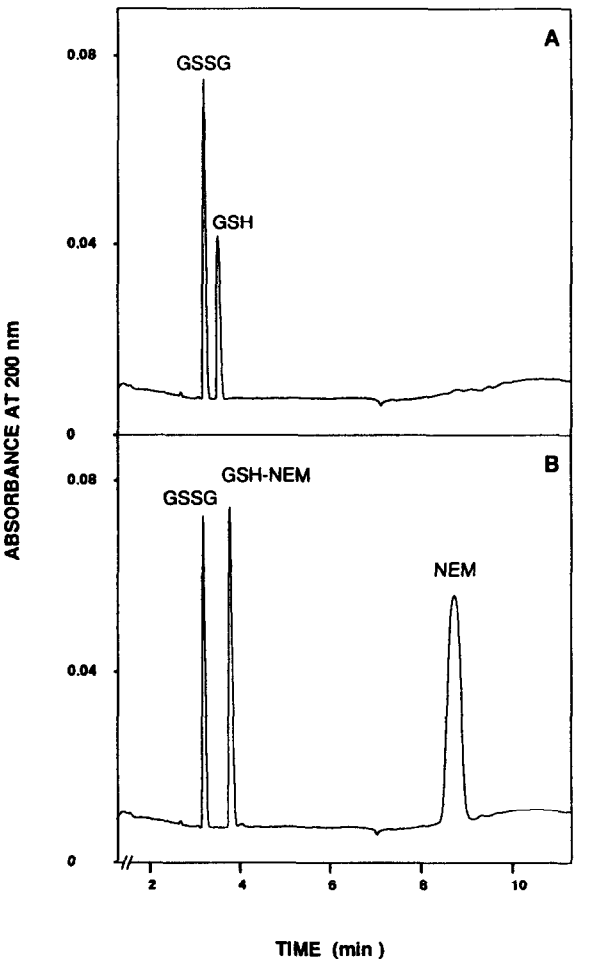

Fig. 1. Separation of standard mixture of GSH and GSSG by CE using the Supelco hydrophilic bonded phase capillary CElect-P150. (A) Separation of standard mixture of GSH and GSSG. (B) Separation of GSH and GSSG in presence of an excess of NEM. The analysis was performed applying the sample under nitrogen pressure for 5 s and using sodium phosphate (35 m*M*, pH 2.1) buffer as electrolyte. The experiment was carried out at 25°C and the detection was performed by UV absorption at 200 nm.

evaluation of GSH and GSSG in biological fluids, cells and tissues [10,40–42]. Fig. 1B shows the analysis of the GSH-NEM derivative and GSSG by CE under the same experimental conditions described in Fig. 1A. As shown in the electropherogram, the use of NEM does not interfere with the separation of these two compounds, allowing an even better separation. In fact, NEM reduces the electrophoretic mobility of the GSH-NEM derivative, increasing the separation time between GSSG and GSH-NEM by about 20 s. Under these conditions it is possible to correctly evaluate the two compounds even when one is present in great excess as compared to the other, as usually occurs in biological samples [2]. Furthermore, the GSH-NEM derivative detected at 200 nm shows a higher extinction coefficient, compared to the GSH absorption, showing the increased sensitivity of factor 2 (Fig. 1B). As reported in Fig. 1B, the excess of NEM does not interfere with the CE analyses of the GSH-NEM derivative and GSSG, since it is eluted from the capillary with a migration time of about 9 min. However, it is not necessary to wait for the end of the analysis (ca. 9 min) before injecting another sample, since it is possible to remove the excess NEM from the capillary by performing the equilibrating procedure with the phosphate electrolyte buffer. Under these conditions, analyses of the GSH-NEM derivative and GSSG can be performed every 5–6 min. This, together with the possibility of automating the analysis, makes this procedure of interest for routine analyses as in the case of clinical applications or in other biochemical studies.

3.2. GSH and GSSG calibration curves

Figs. 2 and 3 show the calibration curves for GSH and GSSG obtained by injecting a standard solution of reduced and oxidized glutathione ranging from 1 to 2000 μM. As shown in the figures, there is a very good correlation between the amount of GSH and GSSG injected and that detected by CE, even at low concentrations (1–100 μM). Furthermore, the reproducibility has been tested from analysis to analysis when a 50

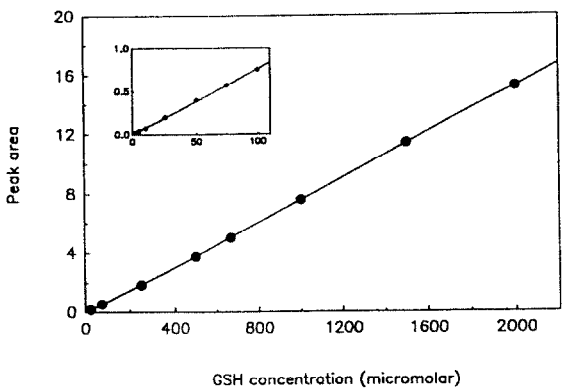

Fig. 2. Calibration curve of GSH-NEM. The experiment was performed injecting standard solutions of GSH-NEM ranging from 2 to 2000 μM. The inset shows the linearity of calibration curve at low standard concentrations (1–100 μM). Analysis conditions as in Fig. 1 ($R > 0.999$).

μM solution of GSH and GSSG is repeatedly injected. The relative standard deviations (R.S.D.s) determined on the basis of six different injections, 0.226% for GSH and 0.229% for GSSG, show that there is a very good correlation between the values of the standard solution of reduced and oxidized glutathione and that experimentally determined by HPCE. The same experiment was performed using a real sample obtained from red blood cells exposed to an oxidant system (Table 1). Also in this case the sample was injected six consecutive times and

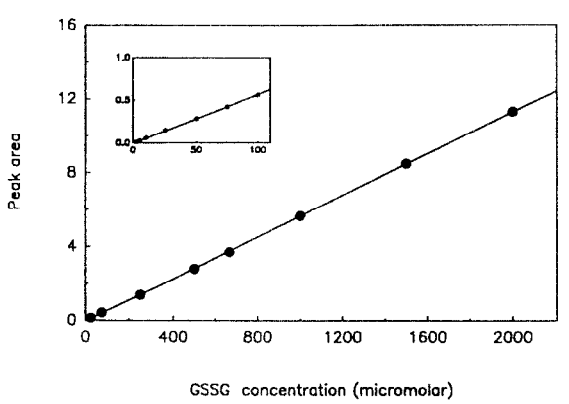

Fig. 3. Calibration curve of GSSG. The experiment was performed injecting standard solutions of GSSG ranging from 2 to 2000 μM. The inset shows the linearity of calibration curve at low standard concentrations (1–100 μM). Analysis conditions as in Fig. 1 ($R > 0.999$).

Table 1
Reproducibility of GSH and GSSG analysis in red blood cells by HPCE

Injection No.	GSH		GSSG	
	Migration time (min)	Concentration[a]	Migration time (min)	Concentration[a]
1	4.487	206.23	3.600	534.03
2	4.407	202.07	3.541	536.34
3	4.512	202.91	3.615	542.37
4	4.510	205.25	3.615	544.24
5	4.444	198.31	3.572	528.28
6	4.458	201.62	3.578	532.07
R.S.D. (%)	0.92	1.39	0.80	1.14

The sample used in the experiment was obtained from red blood cell suspension incubated for 30 min in the presence of an oxygen radical-generating system represented by Fe^{2+}/ascorbate. After the incubation the red blood cell suspension was processed as described in the Experimental section and the sample obtained was injected six consecutive times. The migration times and the concentrations of GSH and GSSG determined by HPCE analysis are reported.
[a] The data reported are expressed as nmol/ml hemolysate (1:1).

the data reported in Table 1 show the high reproducibility of the migration times of GSH and GSSG together with an experimental reproducible determination of their amounts. The detection limit, calculated from electropherograms obtained using the experimental conditions described above, indicates that it is possible to detect the GSH and GSSG present in samples at a concentration of 0.5–1 μM, corresponding to about 10–20 fmol. These results prove that the CE method developed is reliable and can be used for quantitative determinations of GSH and GSSG in biological samples.

3.3. Recovery of exogenous GSSG

The extraction procedure for the determination of glutathione in red blood cells was performed using a Microcon-10 membrane to filter the erythrocyte lysate.The reliability of this extraction procedure was checked by adding an exogenous GSSG solution to the hemolysate. The red blood cell lysate was diluted with an equal volume of 2 mM GSSG standard solution and the sample was then filtered for 5 min at 10 000 rpm using the Microcon-10 membrane. The filtered solution was then directly analyzed by CE. The complete recovery of the exogenous GSSG (1.007 μmol/ml of hemolysate 1:1), demonstrates the validity of the extraction procedure used. Furthermore, in order to assure the best analytical conditions, we performed various experiments either using low pressure of nitrogen or injecting the sample electrokinetically. The results obtained show that, for an accurate analysis of GSSG and GSH by CE using a CElect-P150 capillary (27 cm × 50 μm I.D.), it was necessary to inject the sample under pressure for 5 s. Injection times lower than 5 s can significantly influence the reproducibility of the amount of sample injected, while higher injection times can cause overloading.

3.4. Determination of GSH and GSSG in red blood cells

The procedures reported in the literature for evaluating the reduced and oxidized glutathione in red blood cells show some drawbacks, due to the extraction and analysis methods used [2,10,45]. In fact, the extraction must be performed using excess NEM to block the GSH in its reduced state, with a subsequent removal of the excess NEM from the sample requiring additional tedious and time-consuming steps. The CE method described in this paper together

with an appropriate extraction procedure, which does not require the use of protein precipitating agents, was shown to be suitable for the simultaneous analysis of GSH and GSSG in mammalian erythrocytes. We tested this procedure evaluating the levels of GSSG and GSH in human erythrocytes from adults and umbilical cord blood, rabbit reticulocytes and in the red blood cells exposed "in vitro" to an oxygen radical generating system. Fig. 4 shows the electropherograms of glutathione content in rabbit reticulocytes and human fetal erythrocytes. Fig. 5 shows how the levels of GSSG and GSH in human erythrocytes exposed to 0.1 mM Fe^{2+} and 10 mM ascorbate

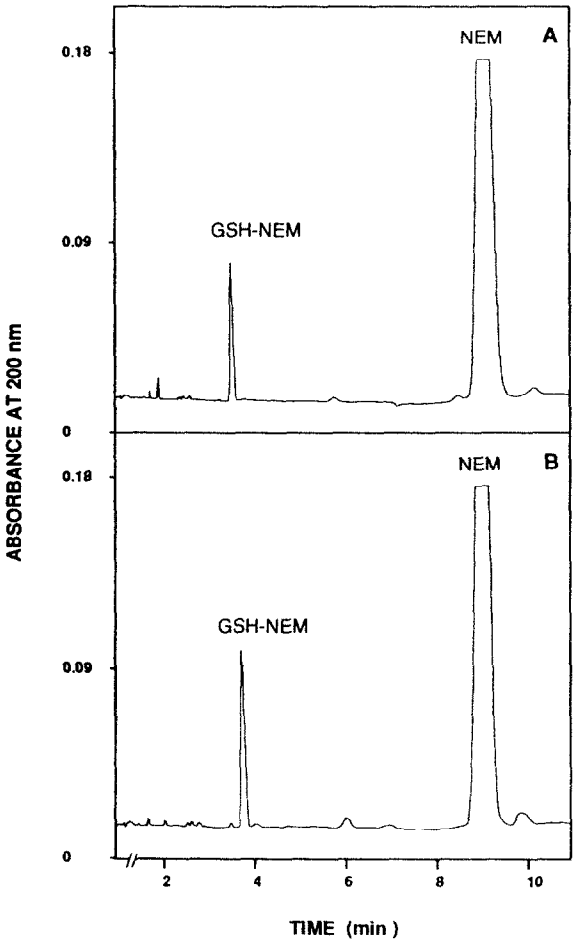

Fig. 4. CE analysis of GSH and GSSG levels in the human red blood cells from umbilical cord blood (A) and in rabbit reticulocytes (B). The red cells were lysed in presence of excess of NEM and deproteinized as described in the Experimental section. The levels of GSH were 1.17 and 1.68 μmol/ml of hemolysate (1:1) in umbilical cord blood and in rabbit reticulocytes, respectively. The GSSG concentrations were not detectable, being lower than 1 μM. Analysis conditions as in Fig. 1.

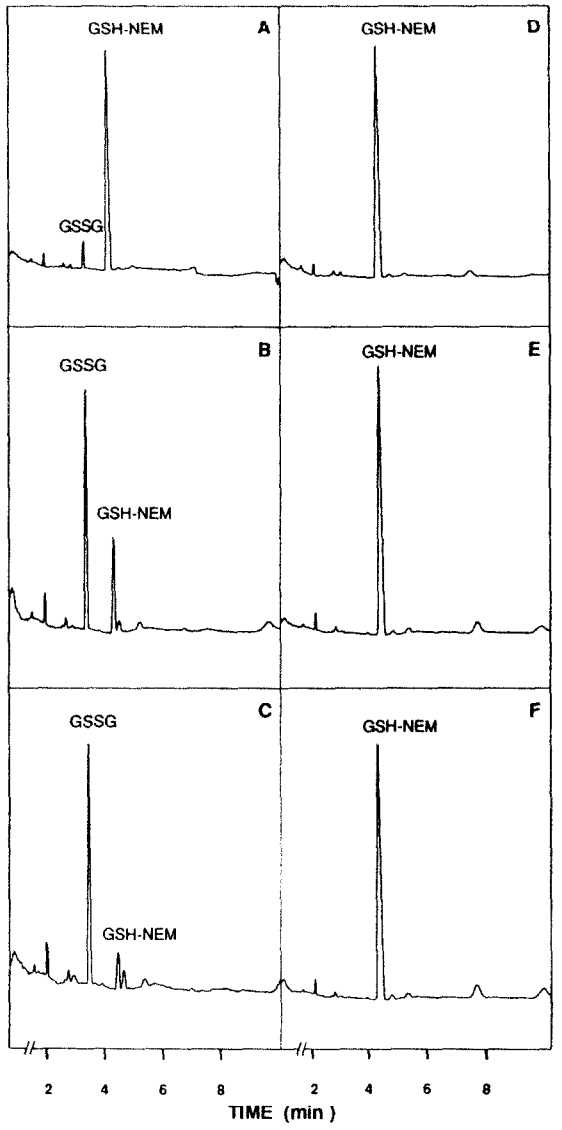

Fig. 5. GSH and GSSG levels in human red blood cells exposed to Fe^{2+}/ascorbate oxygen radicals generating system. (A) Red blood cells at starting point of exposure to the Fe^{2+}/ascorbate system; (B) red blood cells after 30 min of exposure; (C) red blood cells after 60 min of exposure; (D), (E) and (F) red blood cell controls incubated 0, 30 and 60 min, respectively, at 37°C. Analysis conditions as in Fig. 1.

changed. This oxidant radical generating system is able to produce free radicals which can provoke an intracellular metabolic impairment modifying the redox state of cells significantly [46]. As shown in Fig. 5A, B and C the level of GSH in the erythrocytes exposed to this oxidant system undergoes a dramatic drop, with a simultaneous increase in GSSG concentration from 0.020 μM to 0.40 mM. A fraction of GSSG thus formed in the erythrocytes incubated with iron/ascorbate flows-out of the cells, and can be detected in the external medium. Fig. 5D, E and F shows the electropherograms of GSH and GSSG in the human red blood cells not exposed to iron and ascorbate. As shown in the figure, incubation of intact red blood cells up to 60 min at 37°C, does not provoke any significant change of the GSH levels.

Furthermore, we have also compared the GSH and GSSG levels determined by spectrophotometric [44] and CE analyses in human red blood cells from normal adults and in the erythrocytes exposed to the iron/ascorbate system (Table 2). As shown in the table, there is a good relationship between the GSH values obtained spectrophotometrically and those determined by HPCE analysis. However, using the procedure described in this paper, the level of GSH in the human erythrocytes is about 10–12% higher compared to the values obtained using the method proposed by Beutler [44], while the levels of GSSG are not detectable. In fact, our data show that the concentration of GSSG in human red blood cells from whole blood samples collected and immediately used for the analysis is less than 1 μM. This result is in agreement with that obtained also by Eyer and co-workers [47,48]. Furthermore, the exposure of intact red blood cells "in vitro" to an oxidant system changes the intracellular GSH/GSSG ratio with a significant increase of oxidized glutathione that can be correctly evaluated (Table 2). The results reported in the table show that, at different incubation times, the GSH and GSSG levels reflect a correct evaluation of the total amount of glutathione equivalents in the red blood cells. In addition, it should be pointed out that using this procedure the amount of red blood cells required for a correct evaluation of GSH and GSSG is 100–200 μl, significantly lower than that used by other methods [44]. For example the evaluation of GSSG, according to Beutler [44] requires the use of 2.5 ml of packed erythrocytes.

In conclusion, the extraction of GSH and GSSG from red blood cells using Microcon-10 membrane, together with their simultaneous

Table 2
Levels of GSH and GSSG in human red blood cells using different procedures of extraction and analysis

Sample	Spectrophotometry[a]		HPCE	
	GSH	GSSG	GSH	GSSG
Control	5.14 ± 0.58	0.021 ± 0.0014	5.89 ± 0.36	N.D.
t_0	5.22	0.08	5.70	0.11
t_{30}	1.45	1.85	1.39	2.20
t_{60}	0.27	1.81	0.29	2.16
S_{60}	N.D.	0.013	N.D.	0.015

The data referred to the "control" were obtained collecting the blood samples from 10 healthy adult subjects and immediately used for the evaluation of GSH and GSSG levels after removal of plasma and buffy coat. A sample of intact red blood cells was also incubated up to 60 min, at 37°C, in the presence of Fe^{2+}/ascorbate system. At various incubation times (t_0,t_{30} and t_{60}) an aliquot of red blood cells was collected and analyzed for the GSH and GSSG levels. Under these experimental conditions, a certain amount of the intracellular GSSG flows-out from the cells and can be detected in the external medium (S_{60} corresponds to the supernatant after the incubation of intact erythrocytes for 60 min). All data are expressed as μmol/g hemoglobin except S_{60} that is expressed as μmol/ml external medium. N.D. = Not determined.

[a] The methods used were from Beutler [44].

evaluation by CE represent a reliable and simple method to evaluate the levels of these compounds in mammalian erythrocytes even when low amounts of blood are available.

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