DETERMINATION OF GLUTATHIONE AND GLUTATHIONE DISULFIDE IN HUMAN WHOLE BLOOD USING HPLC WITH COULOMETRIC DETECTION: A COMPARISON WITH FLUORESCENCE DETECTION

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We describe a relatively simple method for the determination of glutathione (GSH) and glutathione disulfide (GSSG) in human whole blood. We have used an HPLC with coulometric electrochemical detection for the simultaneous measurement of GSH and GSSG. Diluted and filtered trichloroacetic acid extracts were injected directly into the HPLC system and were eluted isocratically on a Polaris 5u C18-A, 250 × 4.6 mm analytical column. Glutathione in samples extracted with trichloroacetic acid and diluted with 1.0 mM hydrochloric acid was stable at 4 °C for at least 8 h. The analytical performance of this method is satisfactory: the intra-assay and inter-assay coefficients of variation were below 10%. Quantitative recoveries from spiked whole blood samples were at intervals 91.6–97.6% for GSH and 85.0–104.4% for GSSG. The linear range is 5.0–2000.0 μmol/l, with a detection limit of 2.1 μmol/l (signal-to-noise ratio = 3) for GSH and 2.0–250.0 μmol/l, with a detection limit of 0.9 μmol/l for GSSG.

Keywords: Glutathione; Glutathione disulfide; Glutathione stability; Liquid chromatography; Coulometric electrochemical and fluorescence detection.

The tripeptide, glutathione (GSH; γ-glutamyl-cysteinyl-glycine) is the most abundant low molecular weight antioxidant synthesized in cells. It plays important roles in biological systems, including the synthesis of proteins and DNA, metabolism, enzyme activity, detoxification of potentially harmful endogenous compounds and xenobiotics, the maintenance of redox potential, amino acid transport, etc.[1–4]. GSH acts as a recyclable antioxidant through the formation of glutathione disulfide (GSSG) and subsequent en-

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zymatic reduction through the action of glutathione reductase (Fig. 1). When mammalian cells are exposed to increased oxidative stress, the ratio of GSH/GSSG is decreased. This ratio is used to evaluate an oxidative stress in humans[5–9].

During the past thirty years, there has been an increasing interest in the simultaneous determination of GSH and GSSG. Several methods are available for the determination of GSH and GSSG in biological samples, but few are suitable for direct analysis in routine use. Up to date, a range of analytical methods is available, such as enzymatic[10,11], chromatographic, electrophoretic[12], electroanalytical[13–15], chemiluminescence[16,17], spectrofluorimetric[18,19] or ^{1}H NMR [20,21]. Commonly used procedure for measuring GSH and GSSG is HPLC with various detection techniques. HPLC with ultraviolet and fluorescence detection requires derivatization[22–25]. The derivatization is time-consuming and it is more difficult to avoid oxidation of

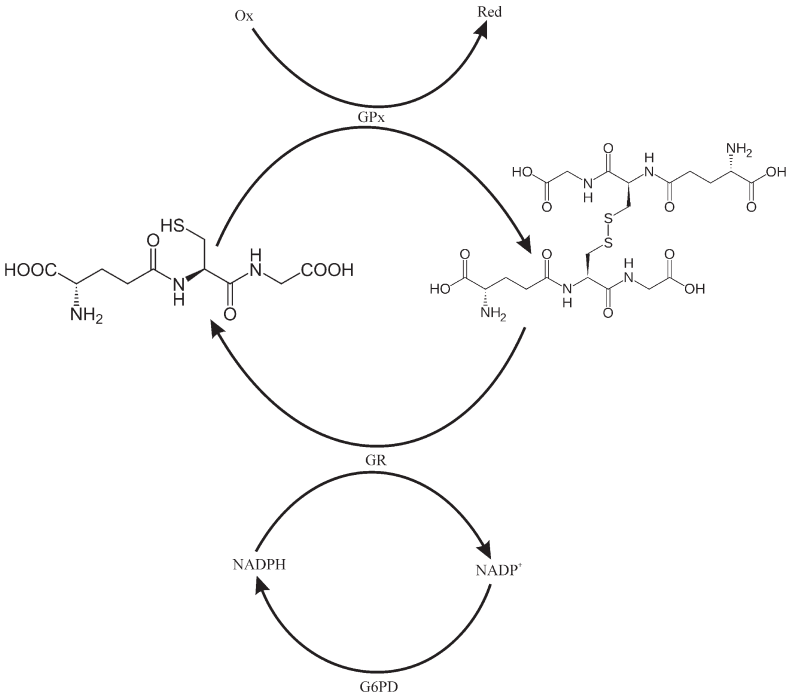

FIG. 1
Glutathione redox cycle. GPx, glutathione peroxidase; GR, glutathione reductase; $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; G6PD, glucose-6-phosphate dehydrogenase

GSH. Therefore, a simple, selective, sensitive and rapid method for the analysis of GSH and GSSG in biological samples is still desired. HPLC with electrochemical detection (HPLC-EC), using either amperometric or coulometric electrodes, can measure GSH and GSSG directly[26–30]. Moreover, these techniques avoid typical problems associated with derivatization procedures. Numerous methods have been developed for the determination of thiols using HPLC-EC, while only few of them enable simultaneous measurement of GSH and GSSG [26,28,29]. HPLC-MS assays were developed for the determination of GSH and GSSG too[31–34].

Oxidation of GSH during sample preparation represents a major problem. Recent data suggested that GSSG could be only a sample preparation artifact[35–38].

The aim of this study was to develop a rapid and simple HPLC-EC method for the measurement of both GSH and GSSG in whole blood, to compare it with existing HPLC-FD and to find out, whether GSSG is only a sample preparation artifact.

MATERIAL AND METHODS

Reagents and Chemicals

Reduced glutathione, glutathione disulfide, *N*-ethylmaleimide (NEM), orthophthaldialdehyde (OPA), sodium hydroxide, hydrochloric acid, phosphoric acid, metaphosphoric acid, 5-sulfosalicylic acid, trichloroacetic acid, perchloric acid, sodium hydrogenphosphate, sodium dihydrogenphosphate, sodium chloride and EDTA were obtained from Sigma Chemical Company (St. Louis (MO), USA). HPLC-gradient grade methanol, ethanol and acetonitrile were from Merck (Darmstadt, Germany). All the other chemicals were of analytical grade. GSH and GSSG solutions were prepared daily in 1.0 mM hydrochloric acid and stored at 4 °C until used.

Instrumentation

Chromatographic analysis was performed with a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-10ADvp solvent delivery system, SIL-10ADvp autosampler, CTO-10ASvp column oven, RF-10Axl fluorescence detector and SCL-10Avp system controller. Data were collected digitally with Clarity chromatography software (DataApex, Prague, Czech Republic). In the case of electrochemical detection, a liquid chromatograph consisted of LC-10ADvp solvent delivery system, injection valve Rheodyne 9725i (Rheodyne, L.P.,

Rohnert Park (CA), USA), with 10-µl loop and Coulochem® III electrochemical detector (ESA Laboratories, Inc., Chelmsford (MA), USA).

Subject and Samples

Blood was collected from a group of healthy blood donors (n = 69, 34 women in the age 28–61 years, mean age 46 years and 35 men in the age 27–59 years, mean age 47 years) into tubes with EDTA (Vacuette, No. 454246, Greiner Labortechnik Co., Kremsmünster, Austria). The tube interior of EDTA tubes is spray dried with K_2EDTA (1.8 mg anhydrous K_2EDTA/ml of blood). None of the studied subjects exhibited renal, hepatic, gastrointestinal, pulmonary or oncological diseases. A written informed consent was obtained from all participants before starting the protocol and the Hospital Committee on Human Research (Regional Hospital of Pardubice, Czech Republic) approved the study.

Sample Preparation

Protein precipitation was done immediately after blood taking. To 200 µl of the whole blood, cold 10% metaphosphoric acid (HPLC-FD) or 10% trichloroacetic acid (HPLC-EC) was carefully added (400 µl). This way treated samples were transported to the laboratory (at about 0 °C, within 1 h). After centrifugation (22 000 × g, 4 °C, 10 min) supernatants were transferred into 1.5-ml propylene tubes (50 µl for the determination of GSH and 200 µl for the determination of GSSG using HPLC-FD, and 20 µl for the determination of both GSH and GSSG using HPLC-EC) and immediately stored at –80 °C.

A stock solution of GSH (about 10.0 mmol/l) and GSSG (about 2.0 mmol/l) was prepared in 1 mM hydrochloric acid. The stock solutions were further diluted with 1.0 mM hydrochloric acid containing EDTA (1.8 mg anhydrous K_2EDTA/ml) to give a series of working standards. To 200 µl of the standard, cold 10% metaphosphoric acid (HPLC-FD) or 10% trichloroacetic acid (HPLC-EC) was carefully added (400 µl). This way prepared standards were subjected to the same procedure as described below for whole blood supernatant.

For the recovery experiment, 10 µl of GSH or GSSG standard of different concentrations were added to 190 µl of whole blood. Next steps were the same as for whole blood sample preparation.

Determination of GSH and GSSG Using HPLC-EC

To 20 μl of the whole blood supernatant or standard solution, 980 μl of 1.0 mM hydrochloric acid were added. The mixture was filtered through a 0.20-μm nylon filter (4 mm diameter, Supelco, Bellefonte (PA), USA) and stored at 4 °C.

Determination of GSH and GSSG Using HPLC-FD

Whole blood GSH and GSSG were measured as previously reported[39]. Briefly, for the determination of GSH, to 50 μl of the supernatant of whole blood or standard, 1.0 ml of 0.1% EDTA in 0.1 M sodium hydrogenphosphate, pH 8.0, was added. To 20-μl portion of this mixture, 300 μl of 0.1% EDTA in 0.1 M sodium hydrogenphosphate, and 20 μl of 0.1% OPA in methanol, were added. The mixture was incubated in dark at 25 °C for 15 min. For GSSG determination, a 200-μl portion of supernatant of whole blood or standard was incubated in dark at 25 °C with 200 μl of 40 mM NEM for 25 min. To the mixture, 750 μl of 0.1 M sodium hydroxide were added. To 20-μl portion of this mixture, 300 μl of 0.1 M sodium hydroxide, and 20 μl of 0.1% OPA in methanol, were added. The mixture was incubated in dark at 25 °C for 15 min.

Chromatographic Analysis

HPLC-EC

The reverse phase Polaris 5u C18-A, 5-μm (250 × 4.6 mm i.d.) column was produced by Varian (Varian, Torrance (CA), USA). An isocratic elution with a mobile phase, which consisted of 25 mM sodium dihydrogenphosphate and methanol (94:6, v/v) adjusted to pH 3.0 with 85% phosphoric acid, was performed at 25 °C at a flow rate of 0.5 ml/min. GSH and GSSG were detected following HPLC separation with a Coulochem® III detector equipped with a dual analytical cell (Model 5010) and a guard cell (Model 5020). The guard cell was connected in line before the injector and used to remove oxidizable impurities in the mobile phase. The dual analytical cell contained two flow-through porous graphite, four counter and four reference electrodes in series. Reference electrode was the α-hydrogen|palladium electrode. A carbon filter was placed before the guard cell and between the injector and the analytical column, a PEEK filter between the analytical column and the analytical cell. For optimum detection of GSH and GSSG, the

electrode potentials for the guard cell, E1 and E2, were set at +1000, +750 and +950 mV, respectively. Gain ranges were 10 and 2 μA. The hydrodynamic voltammogram analysis was performed to optimize conditions for the accurate determination of GSH and GSSG. It was carried out by injection of 10 μl mixed solution of GSH and GSSG (10.0 and 2.0 μmol/l, respectively) and measuring the current produced by GSH and GSSG at the electrodes. Before injection of the first sample, the potential at the electrodes (except the guard cell) was increased stepwise from +200 in 100 mV increments to the final working potential, and the HPLC system was equilibrated with the mobile phase at a flow rate of 0.5 ml/min for approximately 3 h. When not running samples overnight, the flow rate of a mobile phase was set at 0.1 ml/min with guard and analytical cells voltages +500, +300 and +450 mV, respectively. After about 50 injections, the electrode potentials were set to –400 mV at each electrode for 1 h with the mobile phase at a flow rate of 0.5 ml/min, followed by 20-min water rinse and by 60-min methanol rinse at a flow rate of 0.5 ml/min (electrodes off). This procedure was used to remove impurities from electrodes to achieve electrode sensitivity and baseline stability.

HPLC-FD

Chromatography of GSH and GSSG after their derivatization with OPA was accomplished using an isocratic elution on a Discovery C_{18}, 150 mm × 4 mm i.d., 5 μm analytical column fitted with a Discovery C_{18}, 20 mm × 4 mm, i.d., 5 μm guard column (Supelco, Bellefonte (PA), USA) at 37 °C. The mobile phase consisted of 15% methanol in 25 mM sodium hydrogenphosphate (v/v), pH 6.0. The flow rate was kept constant at 0.5 ml/min. Optimum response of the fluorescent derivate was observed when the excitation and emission wavelengths were set at 350 and 420 nm, respectively[39].

Additional Analyses

Hemoglobin in the whole blood was measured with the set HEMOGLOBIN (Lachema, Brno, Czech Republic). Briefly, 5.00 ml of working solution (0.8 mM potassium cyanide and 0.5 mM potassium ferricyanide in 1.1 mM *N*-methyl-D-glucamine buffer, pH 8.3) were mixed with 0.02 ml of blood sample or standard solution in a test tube. After incubation (room temperature, 10 min) the absorbance was read at 543 nm against working solution

(spectrophotometer Shimadzu UV-1700 PharmaSpec; Shimadzu, Kyoto, Japan).

Statistical Analysis

The data are presented as mean values ± S.D. Differences between the women and men were analysed with the use of the Student's t-test. Analysis of correlation was carried out using Spearman Rank Order Correlation and comparison of HPLC-EC with HPLC-FD method using linear regression (software QCexpert, Trilobyte, Pardubice, Czech Republic). A $p < 0.05$ value was considered statistically significant.

RESULTS AND DISCUSSION

The Effectiveness of Various Protein Precipitants and GSH Stability

Oxidation of GSH during a sample preparation is major problem at its measurement. Many of the protein precipitants are acids, which not only precipitate proteins but also prevent oxidation of GSH and avoid GSH and GSSG degradation catalyzed by γ-glutamyl transferase. Some authors state that a portion of GSH was oxidized during protein precipitation and an acid alone was not sufficient to prevent GSH oxidation, hence the acidification of samples slows GSH oxidation, it does not prevent it completely[35–38]. They were convinced that only simultaneous use of an alkylating agent and a strong deproteinizing acid quite prevents oxidation of GSH [38,40]. Using NEM as a –SH group blocker is very effective, but blocking GSH with NEM makes GSH electroinactive so that electrochemical detector cannot be used.

We have investigated stability of GSH in whole blood before protein precipitation immediately after blood taking. Blood was stored at 0 °C and aliquots were analysed at 5-min time intervals for 60 min. Levels of GSH were practically stable for at least 60 min (Fig. 2). It is in agreement with results of Steghens et al.[38]. However, levels of GSSG increased at average about 20% (Fig. 2). As the concentration of GSSG is substantially lower than that of GSH, only slight autooxidation of GSH to GSSG may result in significant overestimation of GSSG level.

We have tested number of protein precipitants: metaphosphoric acid (10%), perchloric acid (1.0 mol/l), trichloroacetic acid (10%) and 5-sulfosalicylic acid (10%). Only trichloroacetic acid as a protein precipitant led to satisfactory recoveries and did not interfere with HPLC analysis.

We have tested various solutions for dilution of supernatant, as phosphate buffers with different concentrations and pH, water, 1.0 mM hydrochloric acid and a mobile phase. When using 1.0 mM hydrochloric acid as a diluent solution, we have obtained the best results with respect to the stability of GSH and HPLC assay.

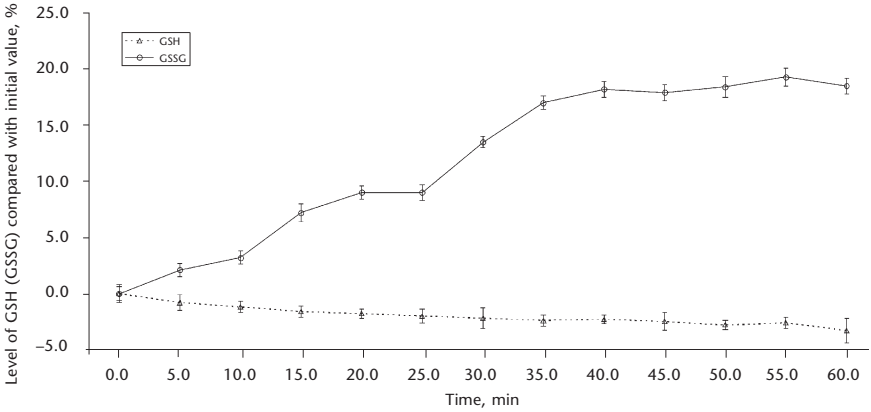

FIG. 2
Stability of whole blood GSH expressed as a relative increase of GSSG during blood storage at 0 °C for 60 min. A decline of GSH concentration is practically insignificant. Mean ± S.E.M (standard error of the mean) of triplicate assays is recorded. Initial levels were 1056.8 ± 17.9 μmol/l for GSH and 23.0 ± 0.4 μmol/l for GSSG

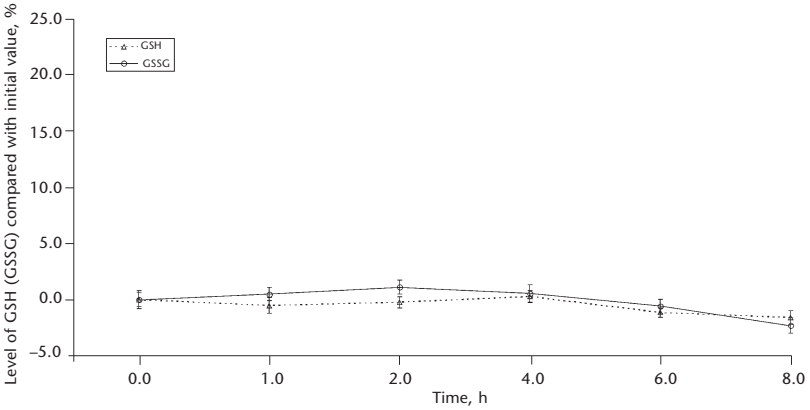

FIG. 3
Stability of GSH and GSSG in blood samples precipitated with trichloroacetic acid and diluted with 1.0 mM hydrochloric acid at 4 °C. Mean ± S.E.M of triplicate assays is recorded. Initial levels were 965.4 ± 14.7 μmol/l for GSH and 27.1 ± 0.8 μmol/l for GSSG

GSH samples extracted with trichloroacetic acid and diluted with 1.0 mM hydrochloric acid were stable at 4 °C for at least 8 h. Levels of GSSG practically did not increase (Fig. 3). These results are in conflict with data of Steghens et al.[38]. They suggest that metaphosphoric acid, as a protein precipitant, alone was not sufficient to prevent GSH oxidation. They found that the mean value for GSH was 1219 μmol/l (protein precipitation with metaphosphoric acid + NEM) against 761 μmol/l (protein precipitation with metaphosphoric acid without NEM). Moreover, GSSG was barely detectable in the presence of NEM, whereas its mean value was 28 μmol/l without NEM. Their results suggest that a portion of the GSH is oxidized during protein precipitation and our results show that GSH is quite stable in trichloroacetic acid (HPLC-EC) and metaphosphoric acid (HPLC-FD) extracts for at least 8 h (after protein precipitation)[39]. Indeed different values can result because the GSH-NEM adduct is unstable and excess of NEM can be caused by GSSG degradation[41–43].

High-Performance Liquid Chromatographic Assay of GSH and GSSG

GSH and GSSG were separated on a reverse-phase column using an isocratic system of methanol and sodium dihydrogenphosphate. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. Standard solutions of GSH and GSSG as well as pooled whole blood were used for study of the mobile phase composition. Several mobile phases (namely buffers containing methanol, ethanol and acetonitrile) were assayed. Optimization of the separation was obtained after studying the effect of the concentration of sodium dihydrogenphosphate (from 5.0 to 100.0 mmol/l) and methanol concentration. The retention behavior was studied in dependence of pH value of the mobile phase in the range 2.5–3.5. The optimal pH 3.0 was chosen for the best separation and detection of GSH and GSSG. Column temperature was changed from 25 to 45 °C. The mobile phase conditions leading to the best separation were: 6% methanol in 25 mM sodium dihydrogenphosphate, pH 3.00 ± 0.02. Optimal temperature interval was from 25 to 30 °C. The criteria were the resolution, electrode sensitivity, baseline stability and the analysis speed. HPLC chromatograms of GSH and GSSG in a standard solution and human whole blood are shown in Figs 4 and 5.

The analysis of the GSH and GSSG voltammograms (Fig. 6) suggest that the thiol group of GSH is oxidized by the electrode at potentials above +300 mV and the amino group oxidation of GSH and GSSG occurs at a potential above +800 mV. The potentials were set near the potentials at which

the GSH and GSSG voltammograms reached the plateau providing maximum sensitivity for GSH and GSSG (+750 and +950 mV, respectively). The guard cell was set at potential +50 mV above that of detector 2 to oxidize the oxidizable impurities in the mobile phase.

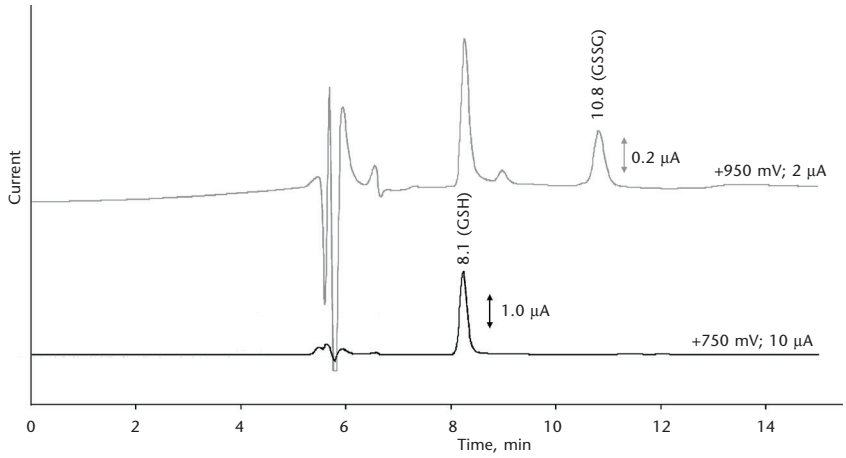

FIG. 4
Chromatogram of standard solution of 1000 μM GSH (67 pmol on column) and 50.0 μM GSSG (3.3 pmol on column). HPLC conditions: mobile phase 6% methanol in 25 mM sodium dihydrogenphosphate, pH 3.0, 0.5 ml/min, column Polaris 5u C18-A, 250 × 4.6 mm, 25 °C

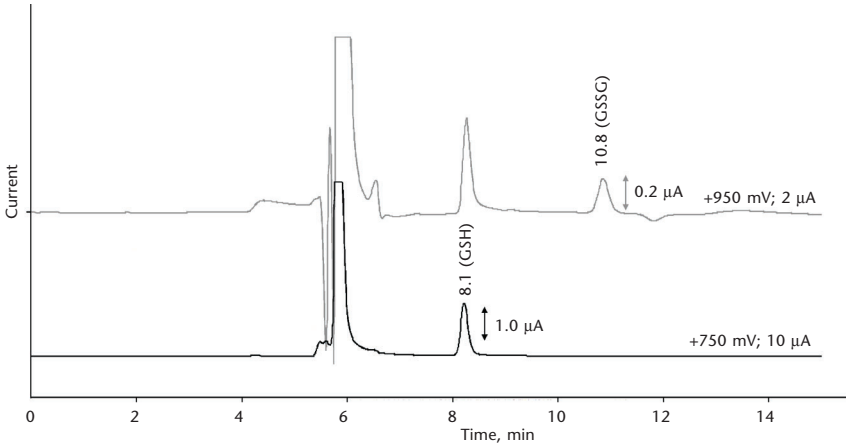

FIG. 5
Chromatogram of human whole blood extract. The blood contained GSH at 689 μmol/l (5.10 μmol/g of hemoglobin) and GSSG at 34.0 μmol/l (0.25 μmol/g of hemoglobin). HPLC conditions: see Fig. 4

Precision of GSH and GSSG analysis for whole blood samples are shown in Table I and II. To determine the within-day precision, the whole blood samples were analysed ten times in the same day under the same conditions. Similarly, results on the between-day precision were obtained on the same whole blood samples, which were analysed in 12 different days. The

TABLE I
Precision of GSH determination in whole blood samples

n	Mean ± S.D., μmol/l	CV, %
A) Precision (within-day)		
10	530.1 ± 10.6	2.0
10	860.9 ± 18.5	2.1
10	1290.7 ± 21.9	1.7
B) Precision (between-day)		
12	911.1 ± 51.1	5.6
12	1409.4 ± 76.6	5.4

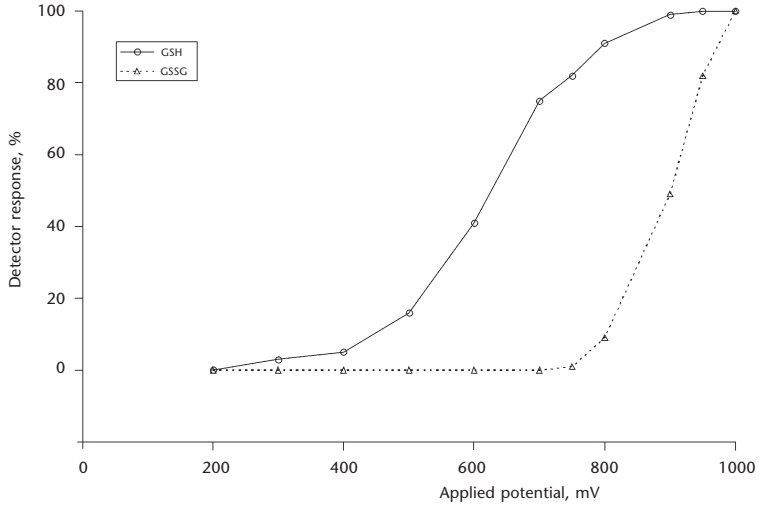

FIG. 6
The hydrodynamic voltammogram generated by repeated injections of GSH and GSSG. The voltammogram was developed by plotting relative peak current produced by injection of 10.0 μM GSH and 2.0 μM GSSG at various oxidation potentials

coefficients of variation were below 10%. The spike recoveries, obtained after the protein precipitation with 10% trichloroacetic acid and dilution with 1.0 mM hydrochloric acid, ranged between 91.6–97.6% for GSH and 85.0–104.4% for GSSG (Fig. 7). The calibration curve (11-point for a determination of analytical parameters and 7-point for a routine analysis) was linear in the whole range tested: 5.0–2000.0 μM GSH and 2.0–250.0 μM GSSG (Fig. 8). The calibration curve parameters obtained as average from ten standard curves are shown in Table III. The lowest concentration that can be quantified with acceptable accuracy and precision was 5.0 μmol/l (333 fmol/inject) for GSH and 2.0 μmol/l (133 fmol/inject) for GSSG. Furthermore, limits of detection for GSH and GSSG, defined as signal-to-noise (*S/N*) ratio of 3:1, were 2.1 μmol/l (140 fmol/inject) and 0.9 μmol/l (60 fmol/inject), respectively.

Comparison with HPLC-FD

The values obtained using HPLC-FD method published previously[39] are comparable to those obtained by present HPLC-EC (Fig. 9). There is less than 10% difference both in the GSH and in GSSG levels measured by HPLC-FD method and HPLC-EC one. Despite of the derivatization is considered as more difficult to avoid GSH oxidation, we obtained practically similar concentrations.

The major advantages of electrochemical detection are simultaneous determination of both GSH and GSSG and rapid sample preparation without time-consuming precolumn derivatization. GSH is easily measured by elec-

TABLE II
Precision of GSSG determination in whole blood samples

n	Mean ± S.D., μmol/l	CV, %
A) Precision (within-day)		
10	11.1 ± 0.4	3.6
10	35.4 ± 1.1	3.1
10	85.6 ± 2.2	2.6
B) Precision (between-day)		
12	14.5 ± 1.2	8.3
12	74.1 ± 5.1	6.9

trochemical detection because the thiol group is oxidized at a relatively low potential. A selection of the adequate potentials and the diluting of a supernatant (fifty times) eliminate other electroactive compounds. On the

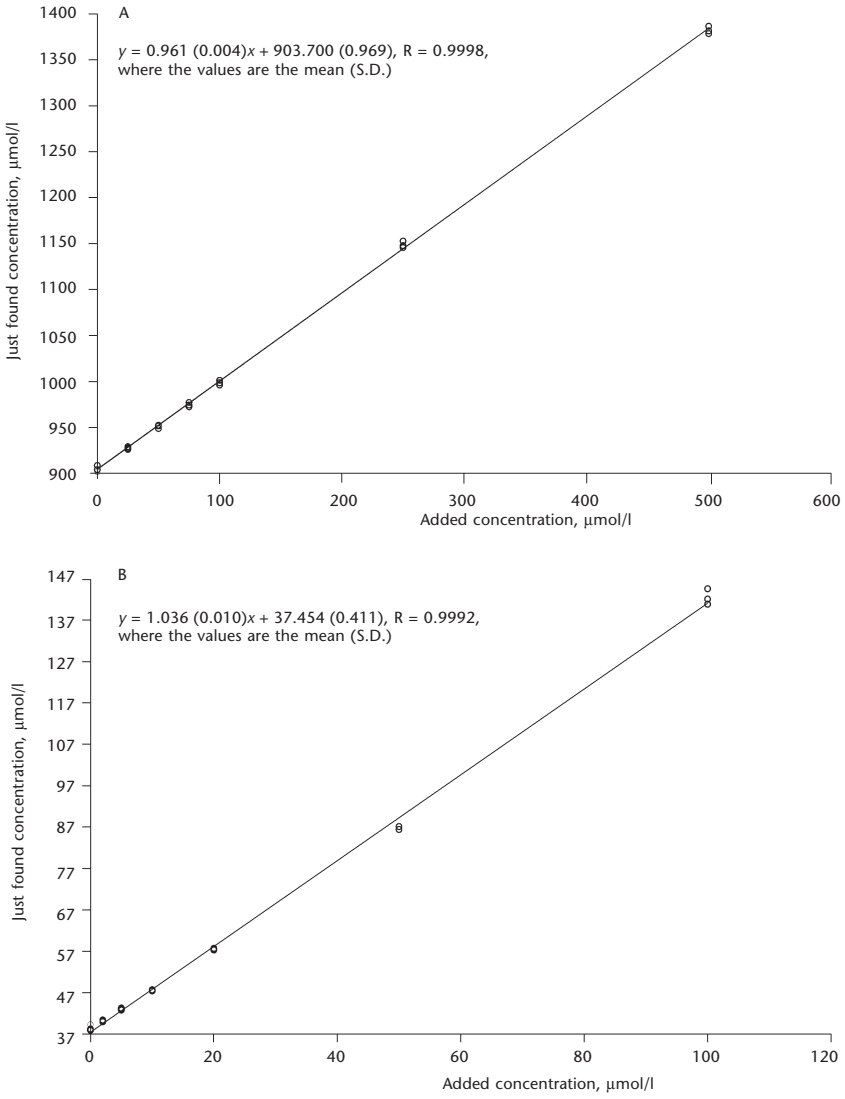

FIG. 7

Recovery experiment: GSH (A) and GSSG (B). Values of triplicate assays are recorded. Slopes correspond to the mean recovery 96.1% (S.D. = 2.0%) for GSH and 95.4% (S.D. = 6.1%) for GSSG

other hand, the determination of GSSG using HPLC with electrochemical detection is difficult because the amino group oxidation of GSSG occurs at high potentials.

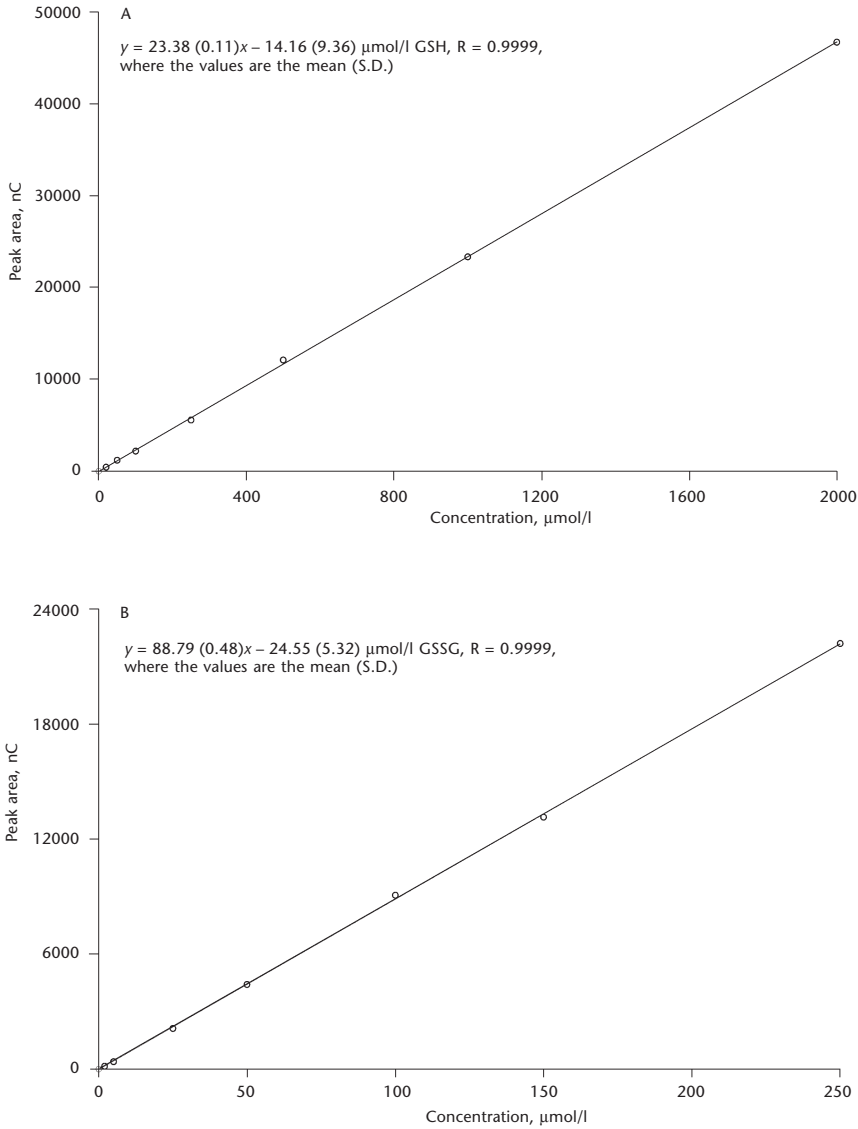

FIG. 8
Typical calibration curve for HPLC quantification of GSH (A) and GSSG (B)

Coulometric electrochemical detection offers several advantages over the commonly used amperometric electrochemical detection. First of all in the amperometric mode about 5% of the analyte is oxidized on the surface of an electrode; with a coulometric detector, close to 100% of the analyte is oxidized in dual flow-through porous graphite electrodes. Therefore, the use of coulometric electrochemical detection provides high sensitivity and specifity required for GSH and GSSG analyses in biological samples.

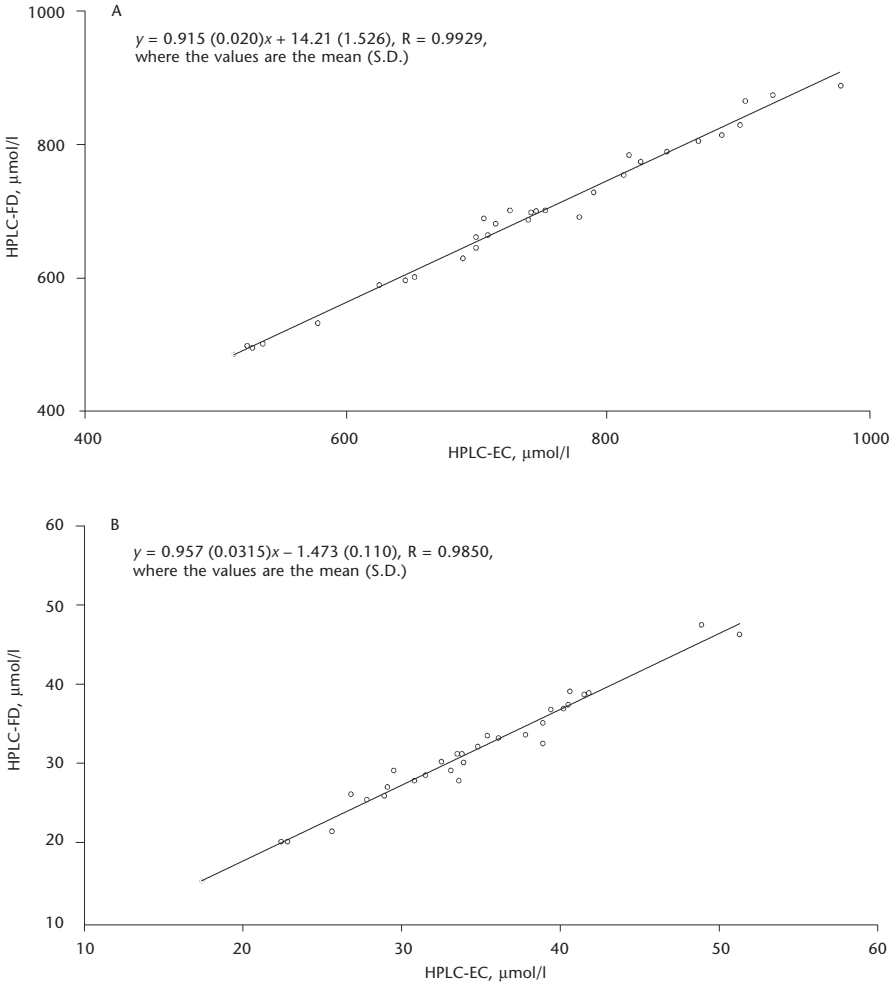

FIG. 9
Plot of the GSH (A) and GSSG (B) concentrations in blood obtained by the HPLC-FD method against those obtained by the HPLC-EC one

The disadvantages of HPLC-EC compared with HPLC-FD are worse robustness and longer time required to achieve baseline stability (more than 3 h against 30 min).

The Determination of GSH and GSSG in Human Whole Blood

Reference ranges for GSH, GSSG and GSH/GSSG ratio established for blood donors are shown in Table IV. The normal values for GSH and GSSG in whole blood differ between laboratories. Especially levels of GSSG and a ratio of GSH/GSSG range at wide interval. Some authors detected practically no levels of GSSG and suggested that GSSG could only be a sample preparation artifact[35–38]. We found no significant differences in GSH and GSSG concentrations between women and men (Table IV). In addition, we ob-

TABLE III
Average parameters of 10 calibration curves for the HPLC-EC method

Standard	Regression equation	Mean slope 95% confidence interval	Intercept (μmol/l)[b] 95% confidence interval	Correlation coefficient
GSH[a]	$y = 23.49x - 21.23$	23.49 (22.99–23.79)	0.8 (0.5–1.3)	0.9999
GSSG[a]	$y = 88.52x - 21.55$	88.52 (87.12–90.01)	0.3 (–0.1–0.7)	0.9998

[a] Eleven-point for determination of analytical parameters and seven-point for routine analysis. [b] The x-intercept (in μmol/l) is the point at which the line crosses the x axis (where the y value equals 0).

TABLE IV
Preliminary reference ranges of GSH, GSSG and GSH/GSSG ratio in the group of blood donors. The data are presented as mean ± S.D. (in μmol/g of hemoglobin). Differences between women and men were analysed with the use of the Student's t-test

Group	GSH	GSSG	GSH/GSSG ratio
All (n = 69)	5.16 ± 1.19	0.19 ± 0.13	23.9 ± 9.8
Women (n = 34)	5.21 ± 1.26	0.19 ± 0.13	25.1 ± 10.5
Men (n = 35)	5.07 ± 1.09	0.21 ± 0.10	21.1 ± 6.2
p	0.187	0.215	0.112

served no significant correlation of GSH and GSSG concentrations, and ratio of GSH/GSSG with age (R = 0.3112, p = 0.147, R = 0.2845, p = 0.191 and R = 0.2949, p = 0.173, respectively).

CONCLUSIONS

We have developed a relatively rapid, simple and very sensitive HPLC method with coulometric electrochemical detection for the simultaneous determination of GSH and GSSG in human whole blood. Sophisticated sample preparation significantly prevents glutathione oxidation and glutathione disulfide degradation. Within 7 months, we have carried out more than 1000 analyses. We estimated that lifetime of the analytical column was at least 1000 injects. Presented method is inexpensive and suitable for clinical trials.

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