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Short communication

# Optimization of the principal parameters for the ultrarapid electrophoretic separation of reduced and oxidized glutathione by capillary electrophoresis

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**Abstract**

Several factors can influence the analytical efficiency and rapidity of the quantitative determination of erythrocyte glutathione by capillary zone electrophoresis (CZE). We optimized the time, efficiency and resolution of the electrophoretic separation of reduced (GSH) and oxidized (GSSG) glutathione by studying the influence of the most important factors affecting the separation, i.e. the pH and ionic strength of the electrolyte solution, the capillary length and temperature. Best results in the shortest time are obtained at 25 °C, using an uncoated 37 cm × 75 μm i.d. capillary and a 300 mmol/l borate buffer pH 7.8. These conditions give a good reproducibility of the corrected peak areas (R.S.D. 1.41 and 1.31%) and of the migration time (R.S.D. 0.22 and 0.26%) for GSH and GSSG, respectively. The high concentration buffer, besides permitting a good resolution of standard GSH and GSSG mix, allows also *N*-nitrosoglutathione detection. By shortening the capillary length to 27 cm, the separation time of GSH and GSSG can be further decreased to less than 60 s. This shortened method, the most rapid described in literature, can detect and quantify GSH in red blood cells despite a loss of sensitivity. To compare the new method here described with the Beutler colorimetric method, the data relative to the GSH content of red blood cells from young normal subjects were analyzed by the Passing and Bablok regression and the Bland–Altman test.


*Keywords:* Glutathione; Nitrosoglutathione

## 1. Introduction

The tripeptide glutathione (γ-L-glutamyl-L-cystenyl-glycine), the most abundant low-molecular mass thiol in eukaryotic cells [1–3] is found in vivo in both reduced (GSH) and oxidized (GSSG) forms. A large number of studies have demonstrated that the GSH/GSSG ratio can be considered as a reliable index of the cellular redox state. Accumulating evidences show that the redox state is actively regulated and controls a variety of cell responses, such as cell proliferation, differentiation, apoptosis,

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death. The cell's fate, in fact, is influenced by the dynamic equilibrium between oxidants, that may alter protein functions by modifying redox-sensitive amino acids, and the "redox buffering" capacity of intracellular thiols, that counteracts intracellular oxidative stress. In this respect, the GSH/GSSG ratio, being an index of the redox state, appears to be a predictive index of cellular physio/pathology. Several authors reported that low intracellular level of GSH is a relevant risk factor for irreversible cellular damage due to oxidative stress [4,5]. Moreover, there is an evidence that GSH depletion is correlated with a variety of diseases including diabetes [6], human immunodeficiency virus (HIV) infection [7], rheumatoid arthritis [3], cystic fibrosis [8] and Parkinson's disease [5]. In their complex, these data point to the clinical relevance of GSH and GSSG assessment. Several procedures for GSH and GSSG measurement have been reported, although, no reference method has been defined yet [9–16]. For the development of a sensitive and reliable method, the optimization of both pre-analytical and analytical procedures must be accomplished [9]. We have recently described a very rapid high-performance capillary electrophoresis method that takes less than 10 min to be completed, starting from packed red blood cells [17]. The use of Microcon-10 membrane filtration minimises the GSH oxidation occurring during the acid precipitation of the sample. The filtered sample can be directly analysed and a 90 s run allows the separation and quantification of GSH and GSSG. To further improve the analytical efficiency and rapidity of this quantitative determination of erythrocyte glutathione by capillary zone electrophoresis (CZE), we have evaluated the influences exerted on the separation by several electrophoretic factors, namely the pH and ionic strength of the electrolyte solution and the capillary length and temperature.

## 2. Experimental

### 2.1. Chemicals and supports

GSH, GSSG, *S*-nitrosoglutathione (GSNO), boric acid, 5,5′-dithiobis(2-nitrobenzoic acid), EDTA, NaCl were obtained from Sigma (St. Louis, MO, USA). GSH, GSSG and buffer solutions were freshly prepared before each analysis.

### 2.2. Red blood samples

Blood samples from 41 healthy young volunteers were collected by venipuncture into EDTA-containing tubes, and immediately processed. Red blood cells were separated from plasma by centrifugation (5000 g for 5 min) followed by three washing with 0.9% (w/v) NaCl. Red blood cells were stored at −80 °C and processed within three days. Three volumes of cold (4 °C) water were added to one volume of packed cells. Sample was then filtered using a Microcon–10 membrane (cut-off $M_r$ 10 000) at 5000 × *g* for 5 min and the filtered solution was directly analysed both by CZE and by colorimetric method after 5,5′-dithiobis(2-nitrobenzoic acid) derivatization as described by Beutler [18].

The GSH and GSSG concentration was expressed as μmol/g haemoglobin, the latter measured by a standard colorimetric method (Drabkin).

### 2.3. Capillary electrophoresis apparatus

The CZE analysis was performed using a P/ACE 5510 system equipped with a UV-Vis detector, automated injector and autosampler, (Beckman Instruments, Palo Alto, CA, USA). The P/ACE 5510 system was fitted with a 30 kV power supply with a current limit of 250 μA. The dimension of the fused-silica capillary was 75 μm i.d. (internal diameter) and the length was manually modified in our laboratory in order to obtain the best condition. The linear vertical dimension of the window in the Beckman cartridge was 800 μm. The analysis was performed applying 16 nl of sample (either standard mixture or red blood cell lysate) under nitrogen pressure for 2 s. The separating conditions (28 kV, 90 μA, normal polarity, with electroendoosmotic flow (EOF), at 25 °C with the detector at 200 nm) were reached in 30 s and held at constant voltage for the separations. Capillary was rinsed between runs with 0.1 mol/l NaOH for 30 s and then with running buffer for 1 min. The injection volume was calculated by Beckman CE expert software, the corrected peak areas, the efficiency and resolution by Beckman P/ACE station version 1.0 software.

## 3. Results and discussion

### 3.1. Optimization of the principal parameters

To elucidate the influence of pH and buffer concentration on the resolution, corrected peak area, migration time and efficiency of the method, we employed a standard mixture of 0.5 mmol/l GSH and GSSG with a capillary of 37 cm × 75 μm i.d. The buffer concentration was varied from 100 to 300 mmol/l borate and the pH from 6.2 to 8.6. The better peak resolutions ($R_s = 2.0$) were obtained at 300 mmol/l buffer concentration and at pH higher than 7.8. The migration time increased by increasing buffer concentration and pH. A similar dependence was observed for the efficiency and the corrected peak areas. In the complex, 300 mmol/l borate and pH 7.8 gave the best electropherogram with a resolution of 1.98 and migration time less than 1.6 min. The influence of capillary temperature was evaluated utilising a 37 cm capillary length, 300 mmol/l borate run buffer at pH 7.8. The temperature was increased of 5 °C between 20 and 45 °C. Although at 25 °C the migration time was longer than at 45 °C (1.6 and 1.1 min, respectively), a better resolution (1.98 and 1.32, respectively), a higher efficiency of GSH and GSSG peaks (29 829 and 32 250 versus 21 250 and 21 890, respectively) and higher corrected peak areas of GSH and GSSG (98 409 and 197 646 versus 96 045 and 191 267, respectively) were obtained. We evaluated also the influence of the temperature on the R.S.D. of the method by performing 15 consecutive injections of the GSH and GSSG standard mixture. Good R.S.D. were obtained at all temperatures tested (<0.4% for migration time, <1.6% for peaks area and <3% for resolution). A capillary temperature of 25 °C was selected since it yielded good resolution and reproducibility with shorter migration time and lower current required (90 μA at 25 °C versus 140 at 45 °C). The influence of the capillary length on the peak migration in 300 mmol/l borate run buffer at pH 7.8 at 25 °C. With a 57 cm capillary the resolution was very high (5.60) and also the efficiency (54 160 and 68 224, respectively) and the corrected peak areas (140 470 and 290 758, respectively) of GSH and GSSG were very good. However, because of the longer running distance a longer migration time (about 5 min) was necessary for the separation of GSH and GSSG. As expected, when a shorter capillary was utilised (27 cm) a very rapid separation time was obtained (<1 min). However, the higher current required (140 μA) caused a loss of efficiency and decreased corrected peak areas for GSH and GSSG. This loss of sensibility represents a limit of the method when samples containing low GSH concentration are analysed but it is uninfluential in the case of GSH rich samples, i.e. red blood cells. The R.S.D., calculated from 15 consecutive injections of the GSH and GSSG standard mixture, are good for all the capillary lengths tested (<1% for migration time, <2.8% for corrected peak areas and <2.7% for resolution). In their complex, the results indicate 37 cm as the best capillary length because of the good resolution, sensibility, reproducibility and migration time obtained. Fig. 1 show a typical electropherogram of a standard mixture (a) and erythrocyte sample (b), obtained in

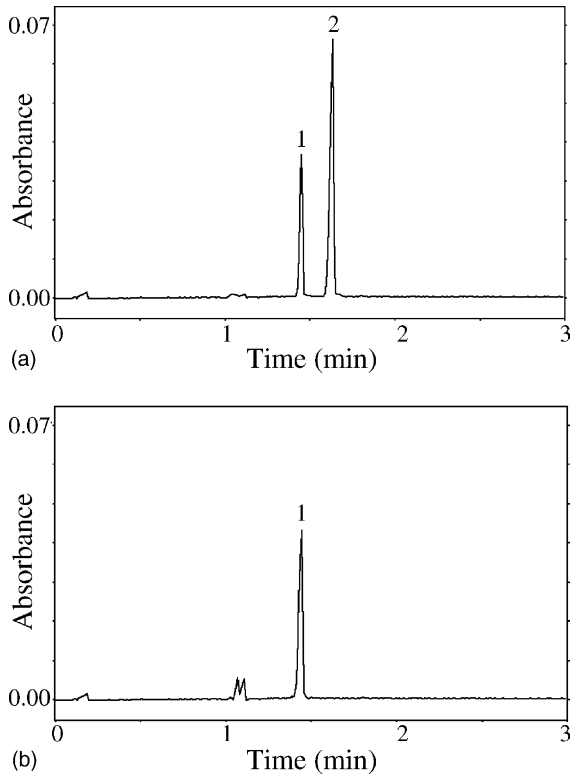


Fig. 1. Separation of GSH (1) and GSSG (2) standard (a) and erythrocyte sample (b) by capillary electrophoresis in the optimized conditions: capillary 37 cm × 75 μm i.d., borate buffer 300 mmol/l, pH 7.8, 28 kV at 25 °C. Migration time is 1.419 ± 0.003 for GSH and 1.557 ± 0.003 for GSSG.

Table 1
Precision of the assay

| | Intra-assay ($n = 10$) | | | Inter-assay ($n = 10$) | | |
|---|---|---|---|---|---|---|
| | Mean (μmol/g Hb) | S.D. (μmol/g Hb) | R.S.D. (%) | Mean (μmol/g Hb) | S.D. (μmol/g Hb) | R.S.D. (%) |
| GSH | 7.14 | 0.22 | 3.1 | 7.39 | 0.33 | 4.5 |
| GSSG[a] | 6.12 | 0.21 | 3.4 | 6.04 | 0.28 | 4.7 |

[a] GSSG standard was added during sample preparation.

the optimized conditions. However, the shorter capillary (27 cm) appears to be the best choice when a very rapid analysis of erythrocyte glutathione is required.

### 3.2. Validation method

The suitability of the optimised method for the analysis of GSH and GSSG was evaluated in a 37 cm × 75 μm i.d., 300 mmol/l borate run buffer, pH 7.8 at 25 °C. Within-run precision (intra-assay) of the method was evaluated by injecting the same red blood cell sample 10 times consecutively, while between-run (inter-assay) precision was determined by injecting the same red blood cell sample on 10 consecutive days. As shown in Table 1, precision tests indicate a good repeatability of method (intra-assay R.S.D. < 3.5%, inter-assay R.S.D. < 4.7%). GSH and GSSG recovery was determined by adding authentic standards to red blood cell extracts. The recovery, evaluated at three different concentrations, was 101.1% and 101.3% for GSH and GSSG, respectively (Table 2). To determine the lower detection limit, serial water dilution of GSH and GSSG were injected and the concentration giving the smallest observable peak was identified: the detection limit for a signal-to-noise ratio of 3 was 75 fmol for GSH and 37.5 fmol for GSSG when we injected 16 nl. The calibration curves for GSH and GSSG obtained by five replicate show a correlation coefficient $R^2 = 0.9993$ (R.S.D. = 4.8%) and $R^2 = 0.999$ (R.S.D. = 3.9%), respectively, ensuring a linear response over a concentration ranging from 0.125 to 2 mM (data not shown). The accuracy of the CZE performance was assessed by comparison with Beutler colorimetric method. Blood samples from 41 healthy volunteers were analysed and erythrocyte GSH concentration was determined. Passing and Bablok regression analysis showed a linear relationship between the two methods, i.e. $y = -0.1589 + 1.0125x$. The 95% confidence interval (CI) of the $y$-intercept and slope were: intercept = −0.1589 (95% CI, −0.6941 to +0.2380); slope = 1.0125 (95% CI, 0.9531 to 1.0876) [19]. Cusum test for linearity showed no significant deviation from linearity ($P > 0.10$). The correlation coefficient among the two procedures demonstrated a good agreement ($R^2 = 0.9715$). An additional analysis by the Bland–Altman test, obtained by plotting the difference in GSH concentration measured by the CZE and the Beutler, showed a proportional bias between the two methods [20]. The mean bias ± 1.96 S.D. was between −0.484 and 0.580 μmol/g Hb.

Table 2
Recovery of the assay measured in a human red blood cells sample as mean of the tree replicate

| | Sample (μmol/g Hb) | Standard added (μmol/g Hb) | Measured (μmol/g Hb) | Recovery **(%)** |
|---|---|---|---|---|
| GSH mean (S.D.) | 7.14 | 1.47 | 8.81 | 102.3 |
| | | 2.94 | 10.25 | 101.7 |
| | | 5.87 | 12.92 | 99.3 |
| | | | | 101.1 (1.6) |
| GSSG mean (S.D.) | 0 | 0.29 | 0.30 | 102.0 |
| | | 1.47 | 1.51 | 102.8 |
| | | 2.94 | 2.91 | 99.2 |
| | | | | 101.3 (1.9) |

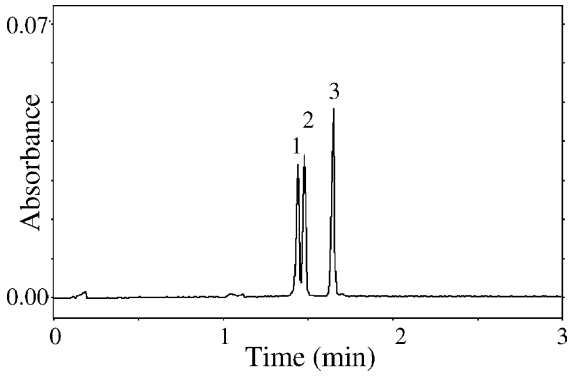


Fig. 2. Separation of GSNO (1), GSH (2) and GSSG (3) by capillary electrophoresis. Conditions: 37 cm × 75 μm i.d., borate buffer 300 mmol/l, pH 7.8, 28 kV at 25 °C. Migration time is 1.384±0.004 for GSNO, 1.419±0.003 for GSH and 1.557±0.003 for GSSG.

### 3.3. Detection of S-nitrosoglutathione

Glutathione can spontaneously react with nitric oxide to form *S*-nitrosoglutathione (GSNO). For this reason, besides GSH and GSSG, also the quantification of this molecule, which produces NO-like biological effects and protects cells against oxidative stress [21], might be relevant when the redox state of the cell must be evaluated. We tested the possibility that our method might simultaneously detect, separate and quantify of all three metabolites. We added 0.5 mmol/l of GSNO to the GSH and GSSG standard mixture and analysed the sample. As shown in Fig. 2, the method allows the contemporaneous separation of GSNO, GSH and GSSG in less than 2 min.

## 4. Concluding remarks

In conclusion, our attempt to improve the sensibility and the separation time of the capillary electrophoresis method that we have recently proposed, indicated 25 °C, uncoated 37 cm 75 μm i.d. capillary and a 300 mmol/l borate buffer pH 7.8, as the best conditions for the separation. The CZE method, besides resolving GSH and GSSG, can contemporaneously separate and detect GSNO added to the standard mixture, allowing its quick and easy determination. By shortening to 27 cm the capillary length, the separation time of GSH and GSSG can be further decreased to less than 1 min. This rapid separation, although less efficient, is suitable for GSH detection and quantification in red blood cell samples. The comparison with the Beutler colorimetric method confirms the good analytical performance of the CZE method. In the complex, the easy and rapid preparation of samples, the short time and high reproducibility of CZE analysis, the possibility to shorten the run, the detection of GSNO, propose this method as very useful for a rapid and accurate detection of GSH, GSSG and GSNO in red blood cells in several pathological conditions.

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