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Short communication

Simultaneous detection of reduced and oxidized glutathione in tissues and mitochondria by capillary electrophoresis

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Abstract

We have developed a rapid and precise method for glutathione quantitation by capillary electrophoresis, that allows a low amount of both redox forms to be measured. Small fragments of rat heart or liver tissues (20 mg wet weight) and the corresponding mitochondria (1 mg protein) were homogenized in 1% perchloric acid and the acid-soluble phase ultrafiltered by centrifugation with a microconcentrator (M_r cut-off 3000 Da). The analysis was performed at a constant temperature (28°C) using a Beckman P/ACE System 2100, equipped with a UV absorbance detector set to 200 nm. The limit of quantitation in heart tissue was 1.8 μM for GSH and 1.2 μM for GSSG. Myocardial concentrations of GSH and GSSG were 8.1 ± 2.6 and 0.45 ± 0.15 (nmol/mg protein$\pm$S.D.), respectively. The ratio of GSH to GSSG was 17.8 ± 1.3 for heart tissue, whereas it was much higher (>100) in the mitochondria. An oxidative stress decreased the myocardial tissue GSH/GSSG ratio, indicating that the CE analysis of both glutathione forms is also a useful method to study biological redox modification.

Keywords: Heart; Liver; Mitochondria; Glutathione

1. Introduction

The determination of reduced glutathione (GSH) and oxidized glutathione (glutathione disulfide; GSSG) is useful for ascertaining the redox status in biological systems; moreover, the conversion of GSH to GSSG is widely recognized as a reliable index of oxidative stress [1,2]. GSH and GSSG concentrations are usually determined enzymatically by the glutathione reductase recycling method [3] that employs 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) as a specific reagent for thiol groups. Although this assay is selective for glutathione estimation, it does not allow the direct determination of the reduced form; the GSH value is calculated by subtracting GSSG from the total glutathione. Another disadvantage of the enzymatic method is the number of analytical steps required for preparing the neutralized biological extracts in the presence or absence of *N*-ethylmaleimide.

Both GSH and GSSG may be simultaneously determined by high-performance liquid chromatography (HPLC) [4], but increasing the sensitivity necessitates the use of fluorescent derivatizing agents [5,6] or electrochemical detection [7].

A few techniques based on capillary electropho-

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retic separation of sulfydrilic compounds, including glutathione, have been reported [8,9], but without application to biological preparations. Cappiello et al. [10] employed a free zone capillary electrophoretic method to quantitate small thiol compounds in the bovine lens, where large amounts of glutathione are present. We have developed a precise method for glutathione quantitation by capillary electrophoresis (CE), that allows the measurement of low amounts of both the reduced and the disulfide form of glutathione. This technique offers significant advantages with respect to the most commonly used procedures owing to the speed of analysis, good efficiency and resolution, and the extremely small amount of sample injected.

2. Experimental

2.1. Reagents and materials

All the reagents and solvents were obtained from Sigma (St. Louis, MO, USA). GSH and GSSG stock standard solutions were prepared by dissolving a weighed amount of each substance in HPLC-grade water. These stock solutions were stable for at least one month when stored at −20°C in the dark. Working standard solutions were obtained daily by diluting the stock standard solutions in water.

Microconcentrators (M_r cut-off 3000 Da, 12.3 mm I.D.) were purchased from Amicon (Beverly, MA, USA).

2.2. Tissue and mitochondria extraction

Male Wistar rats (300–350 g) were anaesthetized with diethyl ether and then sacrificed by decapitation. Heart and liver were excised and immediately rinsed in ice-cold saline solution. All subsequent steps were carried out at 0–4°C. Small fragments (20–50 mg wet weight) were homogenized in 10 volumes (1 volume=1 μl/mg tissue) 1% (v/v) perchloric acid (PCA) using an Ultra-Turrax and a Polytron homogenizer for heart and liver tissues, respectively. Mitochondria were prepared from both tissues as previously described [11,12] and suspended in 1% (v/v) PCA to a final concentration of 1 mg protein/ml. The pellets were dissolved in 0.3 *M* KOH in order to measure protein concentration according to Lowry et al. [13].

The acid-soluble phases were ultrafiltered by centrifugation at 14 000 *g* for 50 min (4°C) in an Amicon microconcentrator and immediately used for CE analysis.

2.3. Instrumentation and separation conditions

The analysis was performed with a Beckman P/ACE System 2100 (Palo Alto, CA, USA) equipped with a UV absorbance detector set to 200 nm. The fused-silica capillary (67 cm×75 μm I.D.) was maintained at a constant temperature of 28°C. The sample was injected by pressure (3.45 kPa) for 10 s and separated at 30 kV. Data were collected at a rate of five points per second and processed with a Beckman Gold 7.11 system for automated apparatus control and data acquisition. The background electrolyte contained 100 m*M* boric acid and 25 m*M* Tris, pH 8.2.

Rinsing between runs was necessary to return the capillary to its initial conditions and provide reproducible migration times. Rinsing was done with 0.1 *M* NaOH (1 s) and water (1 s), from the inlet to outlet and reversed. Before separation the capillary was equilibrated with the background electrolyte by flushing at high pressure (137.9 kPa) for 2 s; thus the time for a complete cycle was 12 min. At the end of a set of separations the capillary was rinsed with water for 10 min.

2.4. Recovery

To control the recovery of glutathione from heart tissue, samples were spiked with different concentrations of GSH and GSSG, ultrafiltered as described in Section 2.2, and analyzed by CE.

3. Results and discussion

Some preliminary steps have been examined and optimized with respect to existing CE procedures employed for glutathione determination [9,10]. The Tris concentration in the background electrolyte was found to be critical since more than 25 m*M* created a basal shoulder on the GSH peak, reducing the

Table 1
Recovery of GSH and GSSG from spiked rat heart tissue ($n=5$)

	Concentration added (μM)	Concentration determined ($\mu M \pm$S.D.)	Recovery (%)
GSH	0	61.4±5.5	–
	20	82.3±8.1	103
	30	90.7±2.3	97
GSSG	0	3.9±0.48	–
	20	20.3±0.57	82
	30	29.8±0.15	86

accuracy of peak integration. This effect was observed either in tissue preparations or in standard solutions, suggesting that GSH does not coelute with another species that start to be resolved as Tris concentration increases. The recovery from heart homogenates was high (>97%) for GSH and slightly lower for GSSG (Table 1).

The separation of GSH and GSSG from standard solutions or biological tissue samples was achieved in less than 8 min (Figs. 1 and 2). The time for a complete CE analysis was restricted to 12 min with respect to those of traditional high-performance

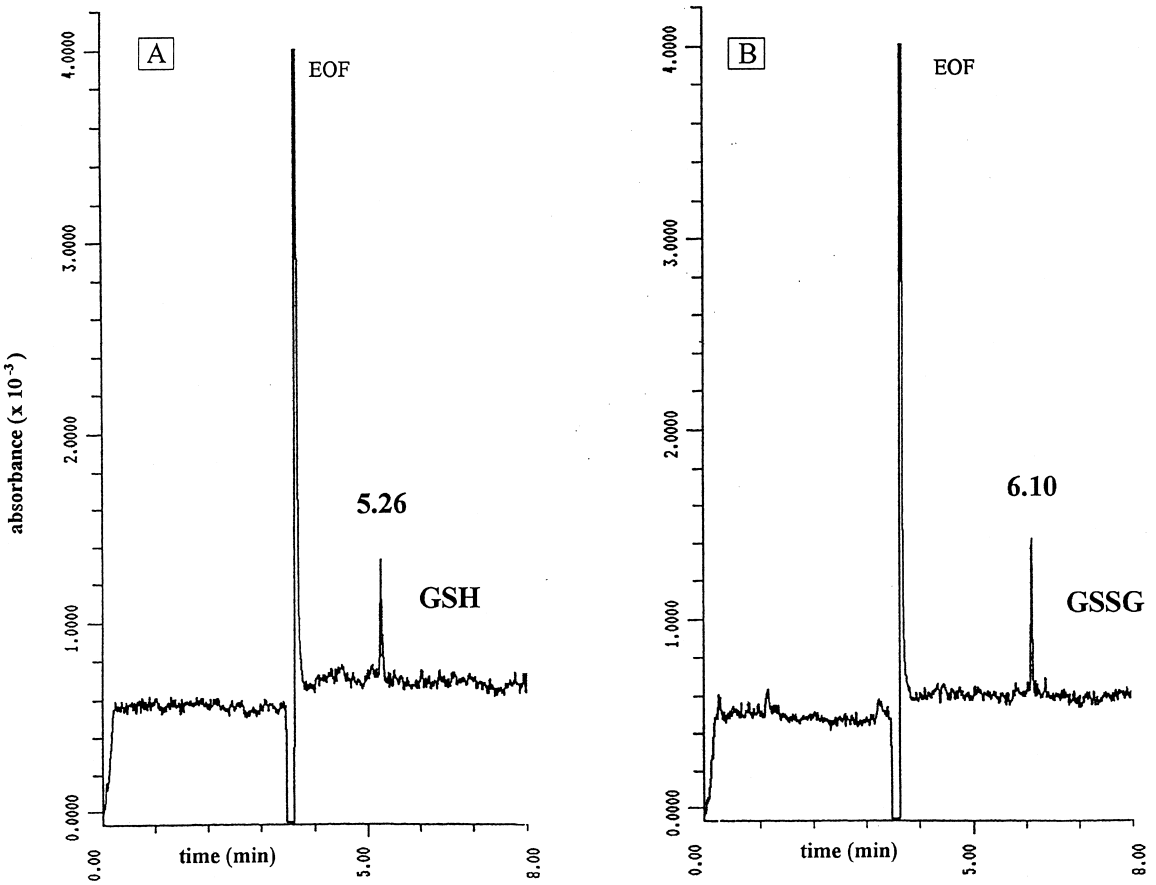

Fig. 1. Electropherograms of (A) 5 μM standard GSH and (B) 3 μM standard GSSG, dissolved in water. Separation conditions: electrolyte concentrations, 100 mM boric acid, 25 mM Tris, pH 8.2; injection pressure, 3.45 kPa; injection time, 10 s; separation voltage, 30 kV; constant temperature, 28°C.

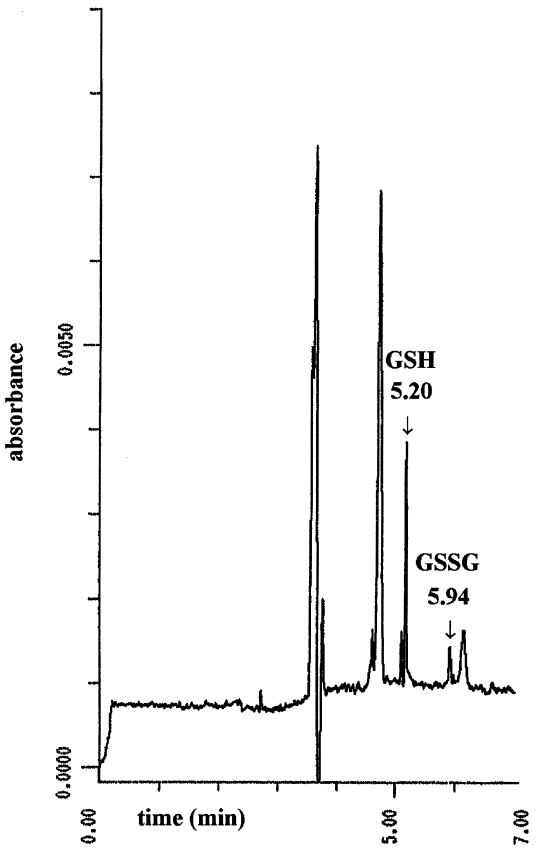

Fig. 2. Cardiac tissue extract analyzed by CE. See Fig. 1 for separation conditions.

liquid chromatographic methods, usually ranging from 20–40 min [14,15]. Also, the spectrophotometric assay for enzymatic glutathione quantitation is longer since it must be repeated twice for measuring total and oxidized glutathione. With regard to sample preparation our CE procedure is extremely rapid, requiring a few steps not longer than 60–70 min. On the contrary, the method of Tietze [3] may require several hours for sample processing.

A linear correlation between the concentration of each form of glutathione and their corresponding peak areas gave calibration curves up to 50 μ*M*. The mean calibration graphs obtained for the two compounds are described by the equations: $y=4.02x-0.005$ for GSH and $y=12.3x+0.032$ for GSSG, where y represents the peak area and x the concentration (m*M*) of the standard analyte dissolved in distilled water. Correlation coefficients (r) for the regressions were 0.99 for GSH and 0.95 for GSSG. The limit of quantification was 1.8 μ*M* for GSH and 1.2 μ*M* for GSSG in heart tissue, at a signal-to-noise ratio of 3; the coefficients of variation (C.V.s) of replicate determinations ($n=3$) were 2.54% and 1.94% for GSH and GSSG, respectively.

The mean values of migration times and migration times relative to the electroosmotic flow (EOF) signal are shown in Table 2. Relative migration times were calculated as the ratio between glutathione migration time and the corresponding EOF migration time. The within-day and day-to-day C.V. showed good repeatability.

Fig. 2 shows a representative electropherogram of a cardiac tissue extract from Wistar rats. The GSH and GSSG peaks were well shaped and resolved from other unidentified components of the sample extract. The analyte concentrations in tissue were 71.3±19.6 μ*M* and 3.90±1.0 μ*M* (mean±S.D., $n=3$), respectively, corresponding to 8.1±2.6 and 0.45±0.15 nmol/mg protein. We obtained similar values by using the glutathione reductase recycling method [16]. The ratio of GSH to GSSG was 17.8±1.3, which decreased to 12.8±3.0 (98% confidence interval) after an oxidative damage due to cardiac postischemic reperfusion (Fig. 3). Rat hearts were previously isolated and perfused according to the Langendorff technique using a glucose-enriched Krebs–Henseleit buffer solution [17]. Myocardial

Table 2
Within-day and day-to-day precision of the migration times and relative migration times (relative migration time of EOF=1) of GSH and GSSG

Parameter	Migration time (min)		Relative migration time	
	GSH	GSSG	GSH	GSSG
Within-day				
Mean ($n=6$)	5.61	6.46	1.46	1.69
C.V. (%)	0.30	0.44	1.02	0.71
Day-to-day				
Mean ($n=6$)	5.03	5.69	1.40	1.59
C.V. (%)	5.96	6.65	3.42	4.33

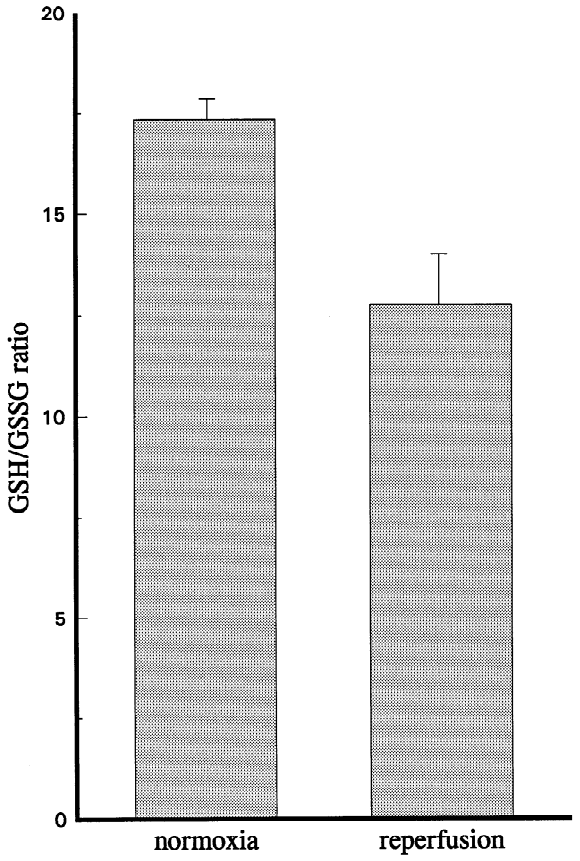

Fig. 3. Effect of myocardial ischemic perfusion on the GSH/GSSG ratio. The values, representing the mean±S.D. of five separate experiments, are significantly different (98% confidence interval).

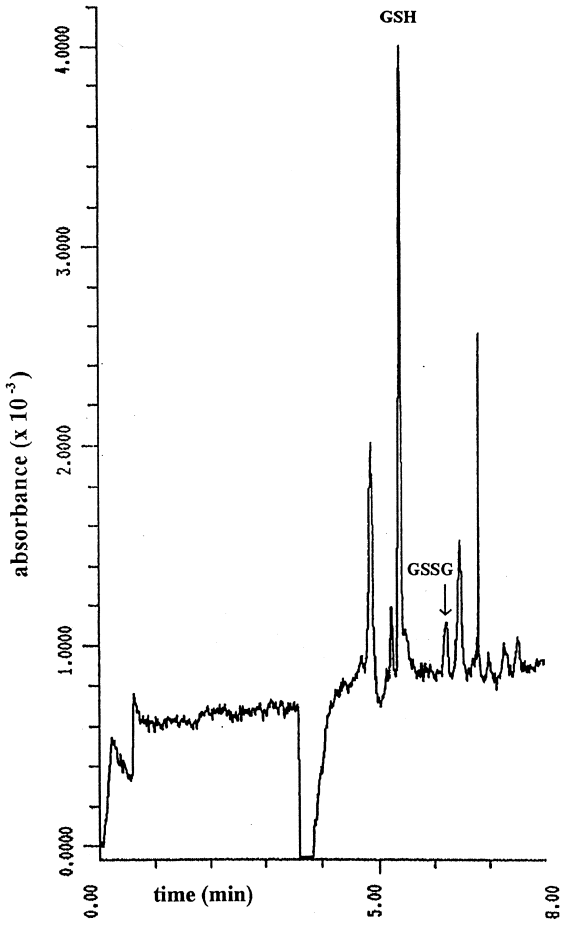

Fig. 4. Representative electropherogram of a liver tissue extract. See Fig. 1 for separation conditions.

concentrations of GSH and GSSG were determined after 30 min aerobic perfusion and 60 min reperfusion following 3 h cold (4°C) ischemic arrest; cardioplegia was induced by the infusion of a modified St. Thomas' Hospital solution at a constant flow-rate of 0.5 ml/min [18].

Well resolved profiles of both redox forms of glutathione were also obtained from liver extracts (Fig. 4) and GSH was easily determined in heart and liver mitochondria (Fig. 5); by contrast, mitochondrial GSSG was barely detectable because of its very low concentration.

In conclusion, the CE separation of reduced and oxidized glutathione offers some advantages with respect to traditional methods, such as good reproducibility, simple procedure and short run times, very low injection volumes and low cost of analysis. The small dimensions of the capillary has favourable repercussions on some important aspects of the technique. The injected volume is in the nanoliter range so that as little as 7–8 μl of the sample suffice for a set of repetitive analyses. Moreover, minute quantities of buffer (milliliter) are required for multiple runs and for rapid capillary regeneration. This technique can be used for GSH and GSSG assay in several biological systems, especially for small

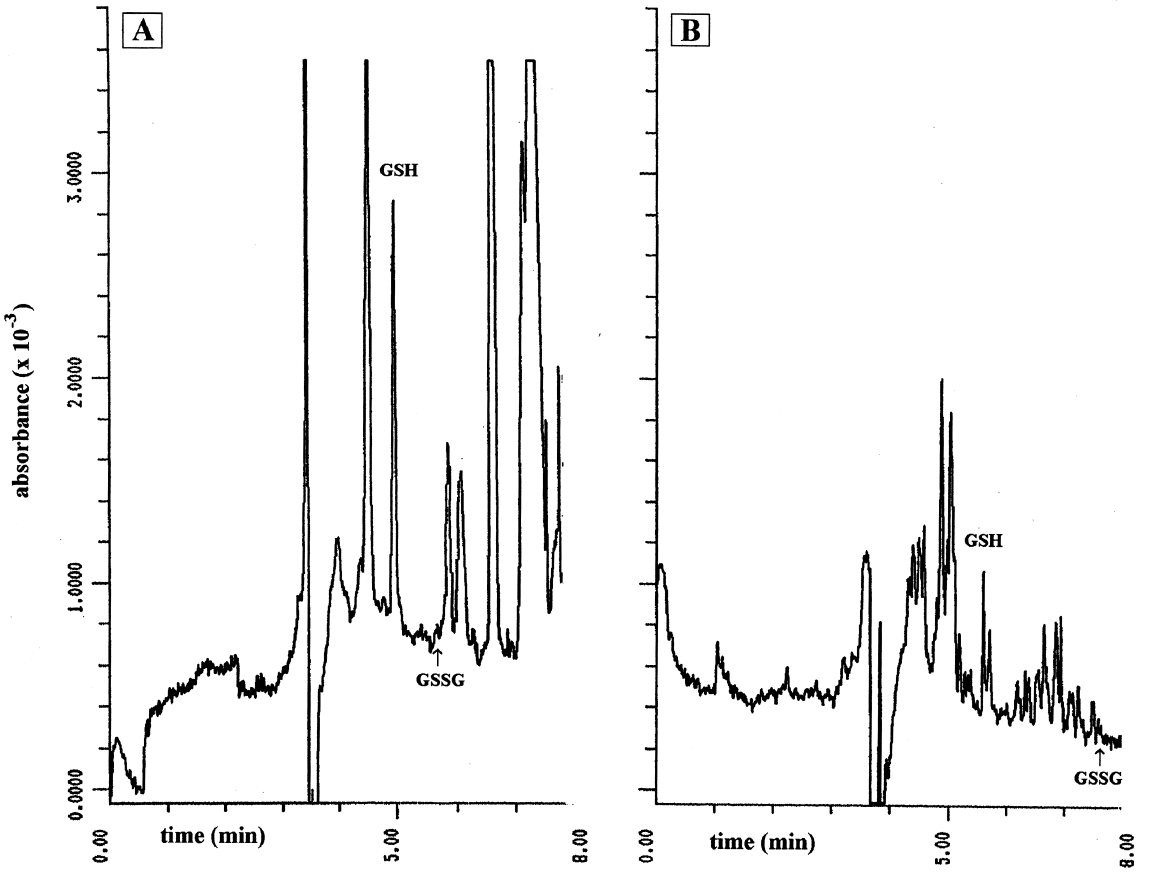

Fig. 5. Electropherograms of (A) cardiac and (B) liver mitochondria. GSSG levels are close to the limit of detection. See Fig. 1 for separation conditions.

amounts of tissue and mitochondrial preparation, as well as to reveal the conversion of the reduced to the disulfide form induced by an oxidative stress.

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