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Analysis of Thiol-Containing Compounds in Biological Samples by Capillary Zone Electrophoresis

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A new method for the determination of reduced and oxidized glutathione by using capillary zone electrophoresis was developed and compared with the *N*-(1-pyrenyl)maleimide high-performance liquid chromotography method. The tissue samples were obtained from C57BL/6 mice and homogenized in deionized water. The detection wavelength was set at 214 nm. A 50-cm effective length × 75 µm i.d. uncoated fused silica capillary with a 10 mM phosphate buffer at pH 7.5 was used for the experiment. Samples were introduced by pressure injection for 5 s. The voltage for separation was at 20 kV. Between runs, the capillary was regularly washed with 0.1 N NaOH and deionized water, followed by reconditioning with running buffer. The present method was shown to be reproducible and is convenient in that a derivatization step is not required. The method has been used to measure glutathione levels in biological samples and the results show good agreement with those obtained by high-performance liquid chromotography.

INTRODUCTION

Glutathione (GSH), γ-glutamylcysteinylglycine, is the most important non-protein thiol present in animal cells as well as in most plants and bacteria (Larsson *et al.*, 1983) and is usually the most abundant intracellular thiol (Meister and Tate, 1976; Meister and Anderson, 1983). The important role of GSH in drug biotransformation and protection from drug toxicity has long been recognized (Meister, 1981). Early work by Mitchell and associates (Mitchell *et al.*, 1973) identified GSH depletion as a critical event after tissue injury in response to chemical toxins and emphasized the function of GSH in the inactivation of accumulating reactive intermediates.

Often, the simultaneous measurement of GSH and its oxidized form glutathione disulphide (GSSG) is desired, because, in general, GSH decreases and GSSG increases in response to oxidative stress (Meister, 1983). The ratio of GSH/GSSG is frequently reported as a biological marker of oxidative stress rather than the absolute values. Thus, the determination of GSH and GSSG in biological samples has attracted great interest recently. A detailed review of methods for the measurement of GSH and GSSG in biological samples has been published (Meister and Anderson, 1983). Among currently available methods, high-performance liquid chromatography (HPLC) offers sensitivity and specificity. However, HPLC methods are generally time-consuming and require either pre-column (Takehashi *et al.*, 1979; Lankmayr *et al.*, 1979; Reeve *et al.*, 1980) or post-column (Nakamura and Tamura, 1981, 1982) derivatization with fluorescent reagents. A newly developed HPLC method for the measurement of GSH and other thiols in our lab (Winters *et al.*, 1995) offers some advantages over the currently accepted techniques, including specificity, speed, sensitivity, and ease of use. However, one drawback to this method and others like it is the dependence on indirect measurement of GSSG by using the glutathione reductase enzyme.

The present study offers a direct measurement of GSSG by capillary zone electrophoresis (CZE). CZE is a very promising separation technique which combines the resolution power of conventional electrophoresis with the instrumentation advances of modern HPLC (Jorgenson and Lukacs, 1983; Gordon *et al.*, 1988). The achievements of CZE make it "the most successful method of the last decade" (Novotny *et al.*, 1990). Between the two popular variant techniques, CZE and micellar electrokinetic capillary chromatography (MECC), CZE has proven to be of great utility in the separation of small biomolecules such as peptides (Firestone *et al.*, 1987; Deyl *et al.*, 1989). The advantages offered by CZE, such as high resolution, speed and simplicity, are valuable in measuring biological samples because they are usually complex and unstable in an aqueous environment. Cappiello and co-workers have recently developed a rapid and easy method for the measurement of GSH and other thiols using a free-zone CZE technique (Cappiello *et al.*, 1993). Based on their work and our previous HPLC technique, we have developed a new CZE method to measure GSH and GSSG directly in biological samples. The new method is a complementary technique to the current HPLC method for the measurement of GSH and GSSG in our lab, and offers some unique advantages over the HPLC method.

MATERIALS AND METHODS

Chemicals and reagents. Acetonitrile, water, methanol, acetic acid and phosphoric acid (all HPLC grade), monobasic sodium phosphate and dibasic sodium phosphate were purchased from Fisher (St. Louis, MO, USA). *N*-(1-pyrenyl)maleimide and *N*-acetyl-L-cysteine were

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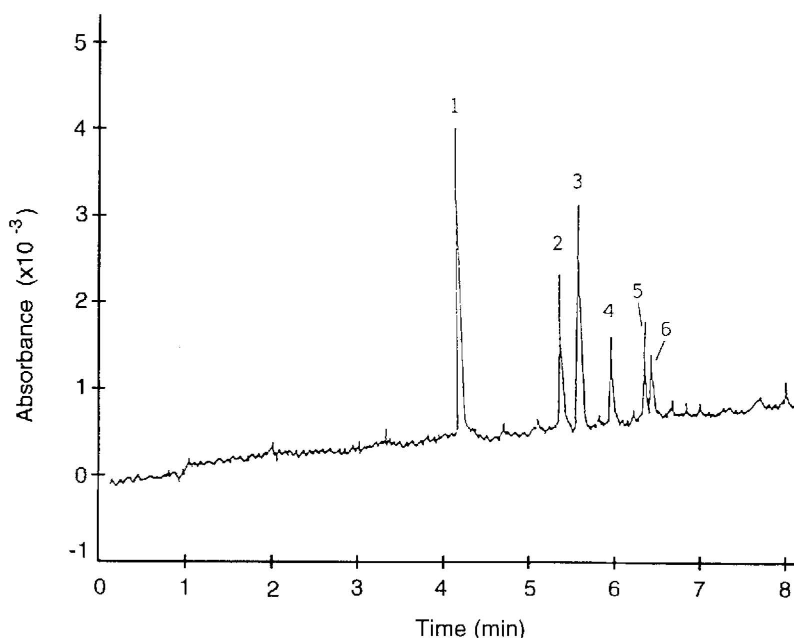

Figure 1. Electropherogram of thiol-containing standards. Labels are: (1) cysteine; (2) reduced glutathione; (3) γ-glutamylcysteine; (4) oxidized glutathione; (5) *N*-acetylcysteine; (6) cysteinylglycine. The detection wavelength was 214 nm. A 50-cm effective length × 75 μm i.d. uncoated fused silica capillary was used. The running buffer was 10 mM phosphate buffer at pH 7.5 and the voltage separation was 20 kV.

obtained from Aldrich (Milwaukee, WI, USA). GSH, GSSG, γ-glutamylcysteine, cysteine, cysteinylglycine were from Sigma (St. Louis, MO, USA). GSH and GSSG were obtained as SigmaUltra grades with purity claims of 98–100%.

HPLC method. The homogenate of tissue samples in serine borate buffer were derivatized with *N*-(1-pyrenyl)maleimide (NPM) (Winters *et al.*, 1995). This compound reacts with free sulphydryl groups to form fluorescent derivatives. 250 μL of acetronitrile was added to diluted samples to make a final volume of 500 μL. The samples were then reacted with 500 μL of 1.5 mM NPM solution in acetonitrile. This mixture was incubated at room temperature for 5 min and 10 μL of 50% (v/v) acetic acid was added to stop the reaction. After filtration through a 0.25 μm pore-size nylon filter, the derivatized samples were injected onto a 3 μm C_{18} column in a reverse phase HPLC system.

The HPLC system (Shimadzu) is comprised of a model LC-10A pump, a rheodyne injection valve with a 20 μL filling loop and a model RF 535 fluorescence spectrophotometer operating at an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The HPLC column (Astec; Whippany, NJ, USA) was 100 × 4.6 mm, and packed with 3 μm particles of C_{18} packing material. Quantitation of the peaks from the HPLC system was performed by a Chromatopac, model C-1B (Shimadzu). The mobile phase was the mixture of solvents A and B. Solvent A was 80% water and 20% acetonitrile containing 1 M acetic acid and 1 M phosphoric acid. Solvent B was 20% water and 80% acetonitrile containing 1 M acetic acid and 1 M phosphoric acid. The NPM derivatives were eluted from the column using 25% : 75% A : B at a total flow-rate of 0.5 mL/min. Peaks were quantified by comparison with standard curves prepared by plotting peak-areas versus concentration of known standards.

Table 1. Detection limits of thiol-containing standards by CZE

Compounds	Detection limit (μM)	Elution order
Cysteine	800	1
Reduced glutathione	200	2
γ-glutamylcysteine	300	3
Oxidized glutathione	25	4
N-Acetyl-cysteine	100	5
Cysteinylglycine	50	6

Biological sample preparation for CZE. Liver and lens samples were prepared in 1 mL of deionized water. After homogenization, the same amount of acetonitrile was added to precipitate proteins. The supernatant was obtained after centrifuging and filtered before being injected into the capillary. The Bradford method was used to determine the protein content of the samples (Bradford, 1970).

CZE method. CZE separations and determinations were performed with a Beckman P/ACE System 2000 equipped with System Gold (version 6.01) software. The detection wavelength was set at 214 nm and the capillary temperature was 23°C. A 50-cm effective length × 75 μm i.d. uncoated fused silica capillary with a 10 mM phosphate buffer, pH 7.5, was used for the experiment. Samples were introduced by pressure injection for 5 s. The voltage separation was 20 kV. Between runs, the capillary was washed with 0.1 N NaOH and deionized water for 2 min, followed by running buffer to equilibrate the capillary for 1 min.

RESULTS AND DISCUSSION

Separation and analysis of thiol-containing compounds were completed by CZE within a 10 min time frame. An electropherogram of six standards of those compounds is shown in Fig. 1. All of them were well resolved except *N*-acetyl-L-cysteine and cysteinylgly-

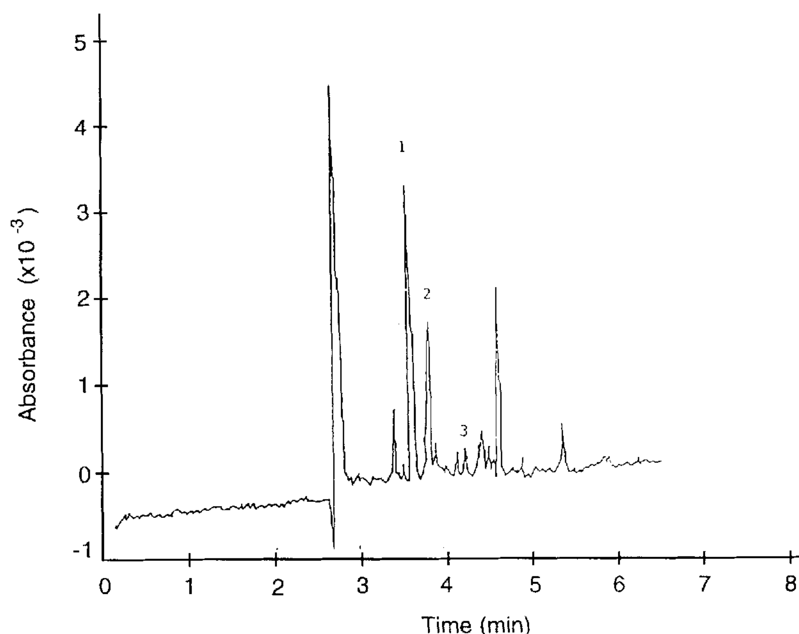

Figure 2. This electropherogram shows the separation of thiol-containing compounds in a liver sample. Labels are: (1) reduced glutathione; (2) γ-glutamylcysteine; (3) oxidized glutathione. The instrument conditions and the method were the same as described in Fig. 1.

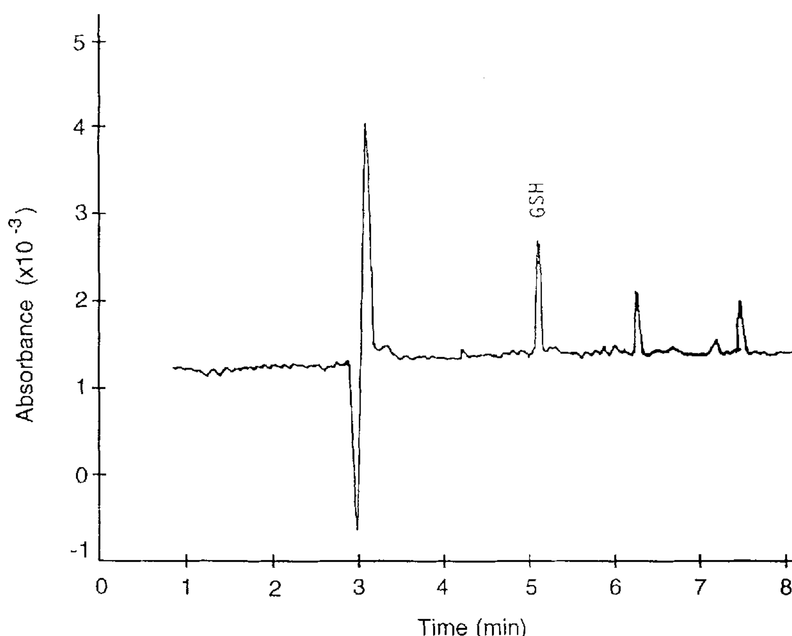

Figure 3. Electropherogram of glutathione in a cow lens sample. The detection wavelength was 214 nm. A 50-cm effective length × 75 μm i.d. uncoated fused silica capillary was used. The running buffer was 10 mM phosphate buffer, pH 7.5 and the voltage separation was 20 kV.

Table 2. Comparison of glutathione levels in biological samples measured by CZE and HPLC

Sample	Injection Number	GSH (nmol/mg protein) CZE	HPLC	Average ± SD CZE	HPLC
Liver	1	30.9	28.6	30.9 ± 1.3	28.6 ± 0.57
	2	32.5	29.3		
	3	29.2	27.9		
Lens	1	20.4	29.0	23.0 ± 2.3	27.5 ± 1.2
	2	26.0	25.9		
	3	22.7	27.6		

cine, which were partially separated. The electro-osmotic flow (μ_{eo}) was 12.82 cm/min. The detection limits for these standards are listed in Table 1. The results in Table 1 were measured with a 5 s injection time. Relatively low sensitivity was obtained compared with other methods such as HPLC, in which the detection limits were found to be in the pmol range for the thiol-containing compounds (Winters *et al.*, 1995). The detection limits for the CZE method can be increased at least three-fold by increasing injection time, because the injection time (s) vs. peak area was linear up to 15 s. The detection limit measured by our CZE method

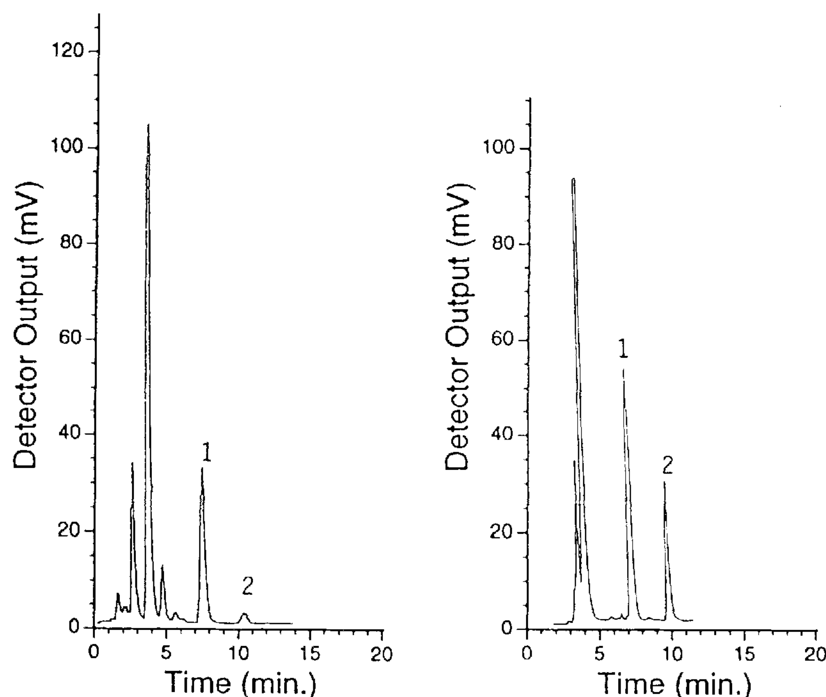

Figure 4. Chromatograms show the separation of glutathione and cysteine in (A) a lens sample and (B) a liver sample. Labels are: (1) reduced glutathione; (2) cysteine. The fluorescence detector was operating at an excitation wavelength of 300 nm and an emission wavelength of 380 nm.

for GSSG is at the level of 25 μM. The method developed in this work has satisfactory reproducibility with a 2.6% relative standard deviation (RSD) within run and 7.4% RSD between runs.

Liver and lens samples were prepared according to the method described in the experimental section. The supernatant was injected into the CZE system and electropherograms were obtained under the same experimental conditions as standards. Figures 2 and 3 are examples of electropherograms for liver and lens, respectively. The levels of GSH in those samples determined by CZE are listed in Table 2. Three injections of samples were performed for liver and lens. The same samples were prepared for HPLC measurement at the same time using the procedure as previously described. The two techniques provided comparable results as shown in Table 2. A typical chromatogram for liver and lens by HPLC is shown in Fig. 4. GSSG levels in both samples were below detection limits for CZE. This is consistent with the finding that the ratio of GSH : GSSG is approximately 250 in the absence of oxidative stress (Kaplowitz *et al.*, 1985). GSSG increases by oxidation of reduced GSH during oxidative stress. This method is best applied under oxidative stress conditions when GSSG is elevated.

The available methods for GSSG have been based on cleavage of the disulphide bond by the GSH reductase enzyme. GSSG determination was then calculated indirectly from the GSH results. CZE offers a direct approach for measurement of GSSG. One disadvantage of CZE measurement of thiol compounds is the high detection limit. However, CZE's simplicity and speed make it a viable method for the analysis of tissues that contain high concentrations of thiol compounds. Additionally, CZE allows for the measurement of γ-glutamyl cycle intermediates which provides more complete assessment of the cell's pro-oxidant/antioxidant status.

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