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Quantitation of oxidized and reduced glutathione in plasma by micellar electrokinetic capillary electrophoresis

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Abstract

A method for the separation of reduced (GSH) and oxidized (GSSG) glutathione was optimized in terms of buffer concentration, sodium dodecyl sulfate concentration, buffer pH, detection wavelength, run voltage and injection volume. The method demonstrated good linearity ($r^2 > 0.999$) and reproducibility (internal standard corrected peak area RSD$<2.3\%$) in the range of interest (16–81 μM GSH and 8–40 μM GSSG). A detection limit of less than 1 μM GSH and GSSG was obtained using a high sensitivity flow cell. When the optimized method was applied to plasma samples, concentrations of 1.6 μM GSH and 0.8 μM GSSG were easily detected without the need for derivatization. The on-capillary detection was calculated to be 38.6 fmol of GSH and 18.3 fmol of GSSG. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Reduced glutathione (GSH) is found in practically all cells. It plays a variety of important roles, such as preventing oxidation of the sulfhydryl groups of proteins within a cell, maintaining the iron of hemoglobin in the ferrous state, and as a reduced carrier for the reduction of glutaredoxin, to name a few [1]. Since evidence for GSH deficiency has been found in a variety of diseases, including diabetes [2], human immunodeficiency virus (HIV) infection [3], cystic fibrous [4], acute respiratory distress syndrome [5] and chronic renal failure [6,7], an analytical method for measuring low levels of GSH and oxidized glutathione (GSSG) in biological fluids would be desirable.

A high-performance liquid chromatographic separation for the detection of glutathione was the logical first choice for this application since the samples were aqueous in form. Unfortunately, measurement of GSH in the μM concentration range is not straightforward, since glutathione lacks a strong chromophore. Depending on the organic modifier used in the mobile phase, detection is limited to the low UV region. To obtain detection limits in the μM concentration range, researchers have resorted to chemical derivatization [8–11] or the use of electrochemical detection [12–14]. The gain in sensitivity is usually at the expense of additional sample treatment steps. These methods employ either pre- or post-column chemical derivatization or have ruggedness issues due to fouling of the electrodes used to analyze biological samples.

Capillary zone electrophoretic (CZE) methods have also been used to address this problem. Since most of the buffer systems used are optically transparent, detection at or below 200 nm [15–17] with sensitivity in the low μM range is possible. For our

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sample preparation and desired concentration range for GSH and GSSG in plasma, we felt that micellar electrokinetic capillary electrophoresis (MEKC) would be the most appropriate mode of CE to use [18,19].

We have developed and optimized a reproducible and linear method using MEKC for determining both oxidized and reduced forms of glutathione in plasma. The method employs direct UV detection of un-derivatized GSH and GSSG, using an easily prepared phosphate–borate buffer–sodium dodecyl sulfate (SDS) system and minimal sample preparation. We also show that incorporation of an internal standard (*N*-acetyl-L-cysteine) into the sample preparation procedure can reduce variability of peak area responses.

2. Experimental

2.1. Materials

N-(*N*-L-γ-Glutamyl–L-cysteinyl)–glycine (gluta-thione, reduced form), *N*-*N*′-(dithiobis{1-[(carboxy-methyl)carbamoyl]ethylene})-diglutamine (gluta-thione, oxidized form) and (L-4,4′-dithiobis[2-amino-butanoic acid] (homocystine) and potassium phos-phate monobasic, anhydrous were purchased from Sigma (St. Louis, MO, USA). The *N*-acetyl-L-cys-teine was purchased from CalBiochem (LaJolla, CA, USA). HPLC-grade acetonitrile was purchased from Burdick and Jackson (Muskegon, MI, USA), 0.1 *M* sodium hydroxide and 0.1 *M* HCl were purchased from Ricca (Arlington, TX, USA) and were used as received. The sodium phosphate monobasic, mono-hydrate and phosphoric acid (85%) were purchased from Mallinckrodt (Paris, KY, USA). The sodium tetraborate, decahydrate and sodium SDS were pur-chased from Brand-Nu (Meriden, CT, USA) and Bio-Rad (Hercules, CA, USA), respectively. The distilled deionized (DDI) water was from a Barn-stead Nanopure II water purification system.

2.2. CE instrumentation and methods

A Hewlett-Packard HP³ᴰCE equipped with a diode array detector set to 195 nm was used through-

out this study. Instrument control and data analysis were performed utilizing the Hewlett-Packard³ᴰ Chemstation software revision A.4.01. A standard running buffer of 25 m*M* NaH$_2$PO$_4$, 15 m*M* Na$_2$PO$_4$ and 50 m*M* SDS, pH 8.0, was used unless otherwise noted. The separation was performed on two types of uncoated fused-silica capillaries (Hew-lett-Packard, Palo Alto, CA, USA) with the follow-ing dimensions: (1) 64.5 cm (56 cm effective length)×50 μm I.D. extended light path and (2) 80.5 cm (72 cm effective length)×75 μm I.D. with a high sensitivity flow cell. The capillaries were prepared for initial use by treatment with 0.1 *M* NaOH, DDI water and buffer for 10, 5 and 60 min, respectively. Between injections, the capillary was conditioned for 12 min with the run buffer. The separation voltage was +224.8 V/cm for capillary 1 and +205.0 V/cm for capillary 2, with the capillary temperature set to 25°C. The samples and standards were loaded on the capillary by applying 50 mbar of pressure on the inject vial for 10 s (mbar s).

2.3. Plasma sample preparation

The plasma samples, collected using 4% sodium citrate as the anticoagulant, were prepared for in-jection by combining 100 μl of plasma with 100 μl of saline and 300 μl of 20 m*M* H$_3$PO$_4$ in acetoni-trile, in a 1.5-ml polypropylene centrifuge tube. The sample was vortex-mixed for 15 s and then cen-trifuged for 5 min at 10 000 rpm. A 200-μl aliquot of the supernatant was diluted with 800 μl of 0.1 *M* HCl and filtered through a 0.2-μm acrodisc filter.

2.4. Standard preparation

The GSH, GSSG and *N*-acetyl-L-cysteine stan-dards were prepared quantitatively using 25 m*M* KH$_2$PO$_4$, adjusted to a pH of three, as the diluent, unless otherwise noted.

3. Results and discussion

3.1. Effect of pH and buffer concentration
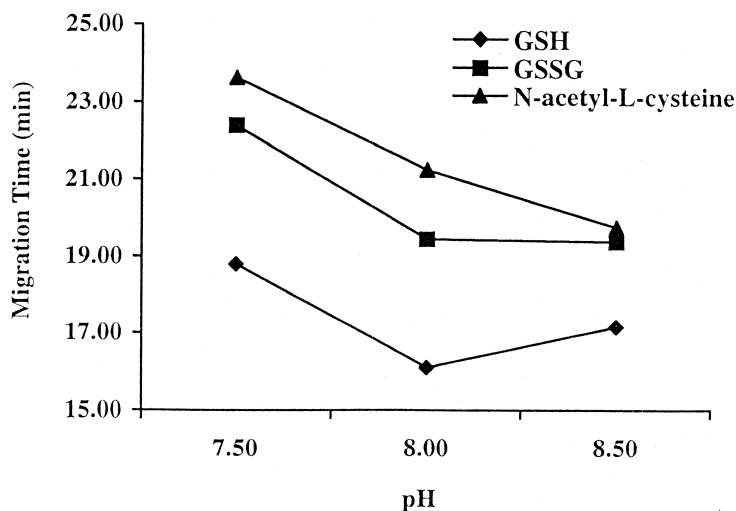
The pH of the 40 m*M* sodium phosphate–borate

Fig. 1. Effect of buffer pH on the migration times of GSH, GSSG and N-acetyl-L-cysteine.

buffer was varied between 7.5 and 8.5 (Fig. 1). The results indicate that, at a pH of 8.0, the resolution of GSH and GSSG is the greatest, with both peaks migrating off the capillary in under 20 min. The buffer concentration was adjusted between 10 and 40 mM. The electropherograms showed that, at lower buffer concentrations, peaks became broader and migration times lengthened. A buffer concentration of 40 mM was selected.

3.2. Selection of SDS concentration

The effect of SDS concentration on the separation of GSH and GSSG was evaluated using a 40-mM sodium phosphate–borate buffer, pH 8.0, at SDS concentrations of 10.1, 25.7, 51.1 and 100.1 mM. Fig. 2 illustrates the effect of SDS concentration on the resolution (R) between GSH and GSSG. A SDS concentration of 50 mM yielded the best resolution of GSH and GSSG.

3.3. Choice of internal standard

This method is intended to be used for measurement and quantitation of low levels of GSH and GSSG in plasma samples. Due to the limited amount of sample, the manipulations in the preparation of

the plasma and our past experience [19], we felt that an internal standard was necessary to enhance the quantitation of the results obtained. The internal standard had to be stable, of similar chemical structure (Fig. 3), transparently incorporated into the sample preparation process and separable from the GSH and GSSG without causing a significant increase in the overall run time of the analysis.

Cysteine, cystine and N-acetyl-L-cysteine were candidates considered to meet the above-mentioned criteria. Each was prepared to an approximate concentration of 10 µg/ml in DDI water and injected onto the CE system. The cysteine standard yielded a shoulder on the front side of the peak, while cystine was difficult to get into solution. In addition, cystine, with a pK_3 of 8.02, produced two chromatographic peaks, most likely due to two dissociation states of the molecule at the pH of the separation buffer. The N-acetyl-L-cysteine was soluble in DDI water, migrated after and was well resolved from the GSH and GSSG peaks (Fig. 4). N-Acetyl-L-cysteine was selected as the internal standard.

3.4. Selection of capillary temperature

The effect of capillary temperature on separation was explored. The capillary temperature was in-
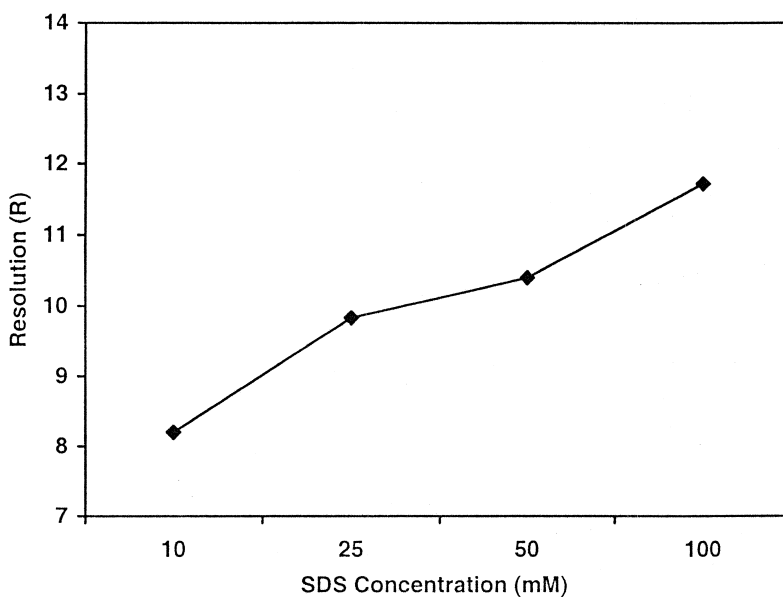
Fig. 2. Effect of SDS concentration on the resolution of the GSH and GSSG peaks.

creased by increments of 5°C between 20 and 35°C. As expected, the migration times decreased with increasing temperatures (Fig. 5) and the current increased with increasing temperature (Fig. 6). Unfortunately, the N-acetyl-L-cysteine peak began to tail as the temperature rose above 25°C. A capillary
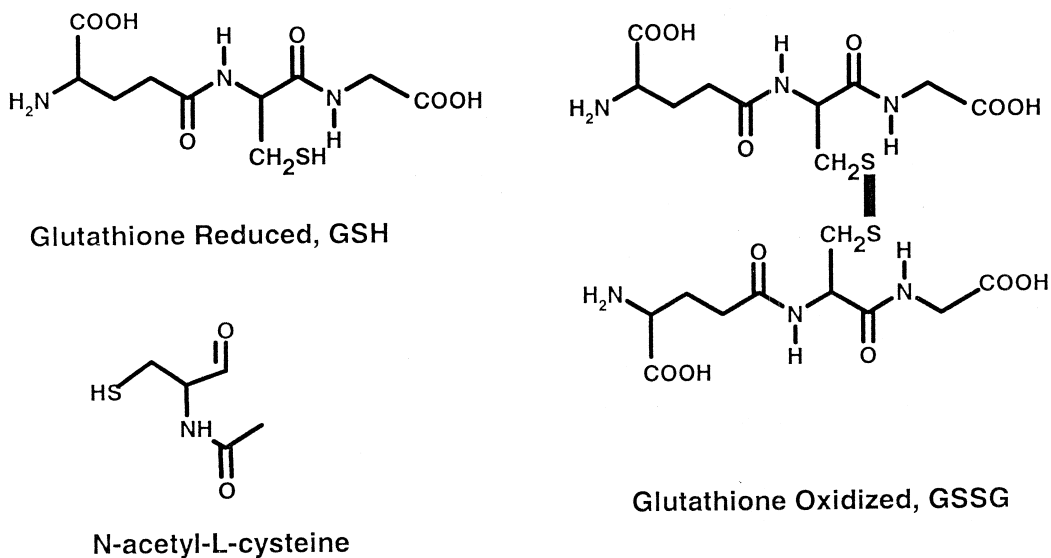

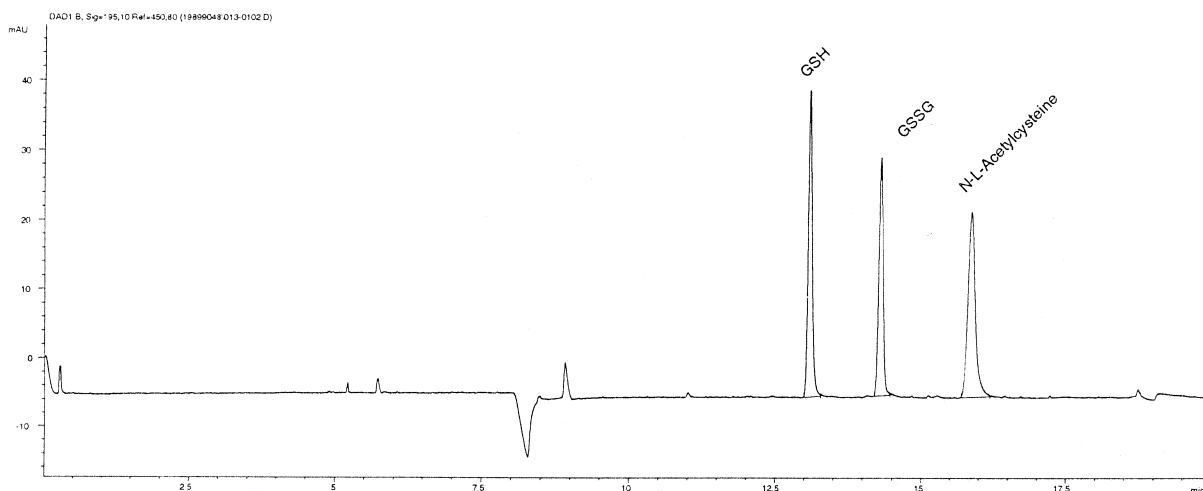Fig. 3. Structures of reduced glutathione (GSH), oxidized glutathione (GSSG) and N-acetyl-L-cysteine.

Fig. 4. Sample electropherogram of GSH, GSSG and N-acetyl-L-cysteine. Conditions: buffer, 25 mM NaH$_2$PO$_4$, 15 mM Na$_2$B$_4$O$_7$ and 50 mM SDS, pH 8.0. Capillary, 64.5 cm (56 cm effective length)×50 μm I.D. extended light path, λ=195 nm. Injection, 500 mbar·s of 81.4 μM GSH and 40.7 μM GSSG.

temperature of 25°C was selected since it yielded the shortest migration times without broadening of the N-acetyl-L-cysteine peak.

3.5. Choice of wavelength

Since the compounds of interest lack strong chromophoric properties, detection is limited to the low UV region. The buffer composition used allows for measurement of wavelengths below 210 nm without interference. Using the ability of the diode array detector to measure multiple wavelengths simultaneously, peak areas for GSH, GSSG and N-acetyl-L-cysteine were recorded at 195, 200 and 210 nm. The strongest signal was observed at 195 nm, yielding areas that were approximately 2.5- and 1.4-times greater than those obtained at wavelengths of 210 and 200 nm, respectively.
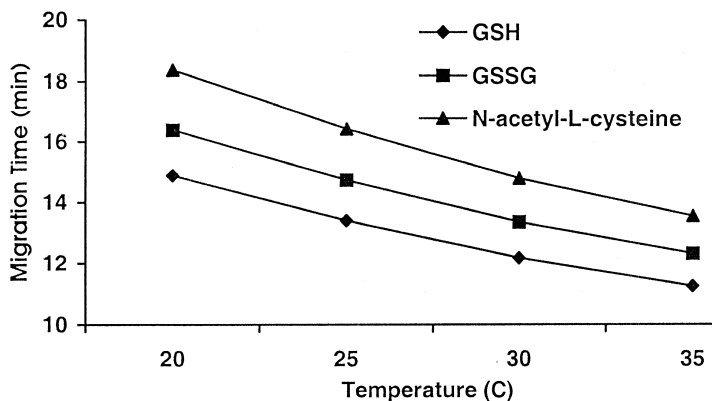

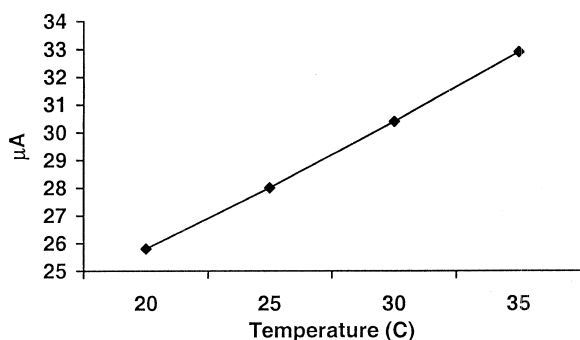Fig. 5. Effect of capillary temperature on migration times.

Fig. 6. Effect of capillary temperature on observed current.

3.6. Effect of voltage

In theory, increasing the run voltage in CE will improve the efficiency of the separation while also shortening migration times. The gains achieved, however, need to be weighed against the resultant peak-broadening and irreproducible migration times due to Joule heating effects. In this experiment, the migration times of the peaks were measured as a function of run voltage. The optimum separation that yielded narrow peaks with a run time under 20 min was between 14 – 15 kV (Fig. 7). A voltage of 14.5 kV was selected as the best run voltage.

3.7. Effect of injection volume

To determine the injection time that would yield the largest peak area while retaining good peak symmetry, injections of a 10-μg/ml GSH and GSSG standard at 250, 500, 750, 1000 and 1250 mbar·s were performed. The results indicated that, at in-

jections greater than 500 mbar·s, any gain in sensitivity was negated by poor peak shape due to band broadening. An injection of 500 mbar·s was selected.

3.8. Method reproducibility and linearity

Using the optimized instrumental and operational parameters for our sample, the reproducibility of the method was measured by replicate injections of standards. The relative standard deviations (RSDs) of the four injections of 25, 10 and 5 μg/ml GSH and GSSG standard mixtures using N-acetyl-L-cysteine as an internal standard were calculated for internal standard corrected and uncorrected peak areas (Table 1). The linearity of the method was also calculated and expressed as the correlation coefficient (r^2). For all standard concentrations, the migration time RSD was below 0.5%. The RSD values for uncorrected peak areas ranged from 2.9–5.0% and 0.47–4.6% for GSH and GSSG, respectively. When using internal standard correction, the RSD values were all below 1.3% for GSH and 2.3% for GSSG. The correlation coefficients (r^2) for the uncorrected peak areas of GSH and GSSG were 0.9998 and 0.99997. The corrected values were better for GSSG (0.99999998) and slightly lower for GSH (0.9996).

3.9. Limit of detection

The last parameter evaluated was the limit of detection. Using a 64.5-cm (56 cm effective length)×50 μm I.D. extended light path capillary, concentrations of 3.2 μM GSH and 1.6 μM GSSG were easily detected (Fig. 8). When an 80.5-cm (72

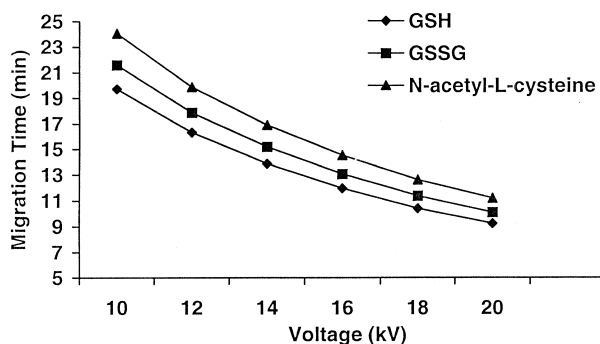Fig. 7. Effect of voltage on separation.

Table 1
Method precision calculated as the relative standard deviation over the range of 5–25 µg/ml GSH and GSSG concentrations

(%)	GSH Uncorrected	GSH Corrected	GSSG Uncorrected	GSSG Corrected
25 µg/ml	2.95	1.29	0.478	2.22
10 µg/ml	2.39	0.867	1.36	1.82
5 µg/ml	4.96	0.721	4.61	1.45

cm effective length)×75 µm I.D. capillary with a high sensitivity flow cell was used, detection of 0.8 µM GSH and 0.4 µM GSSG (Fig. 9) concentrations were possible. This method is therefore capable of detecting 38.6 fmol of GSH and 18.3 fmol of GSSG on capillary with a calculated injection volume of 48.21.

To verify the ability of the method to measure µM concentrations of GSH and GSSG in the desired matrix, GSH and GSSG were spiked into a plasma sample. The plasma samples were analyzed using the high sensitivity flow cell under the optimized conditions (Fig. 10). The GSH and GSSG were easily detected without interference at concentrations of 1.6 and 0.8 µM, respectively.

A peak present in the plasma migrates off the capillary very near the N-acetyl-L-cysteine peak (Fig. 10) and has the potential to interfere with integration of the internal standard. A solution of homocystine was prepared and injected. Although it was well resolved from the GSH and GSSG, possible interferences were noted when homocystine was spiked into a plasma sample. Additional work needs to be done in this area.

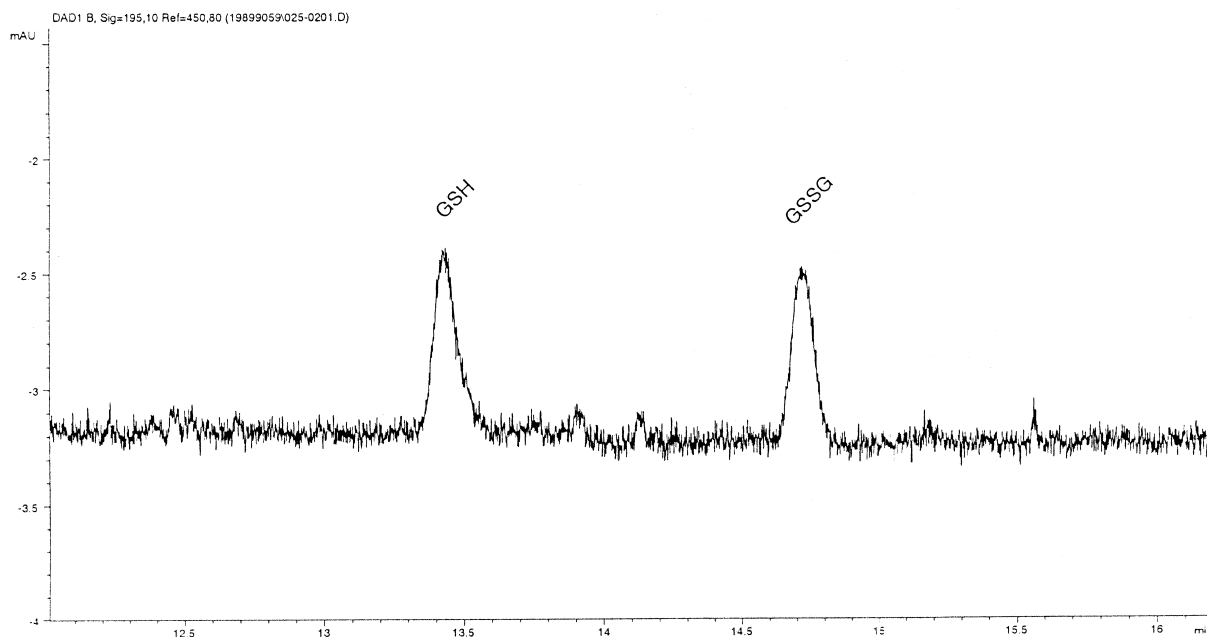## 4. Conclusions

A method for the separation of reduced and

Fig. 8. Sample electropherogram showing detection of 3.2 µM GSH and 1.6 µM GSSG. Conditions: buffer, 25 mM NaH$_2$PO$_4$, 15 mM Na$_2$B$_4$O$_7$ and 50 mM SDS, pH 8.0; capillary, 64.5 cm (56 cm effective length)×50 µm I.D. extended light path, λ=195 nm; injection, 500 mbar·s.

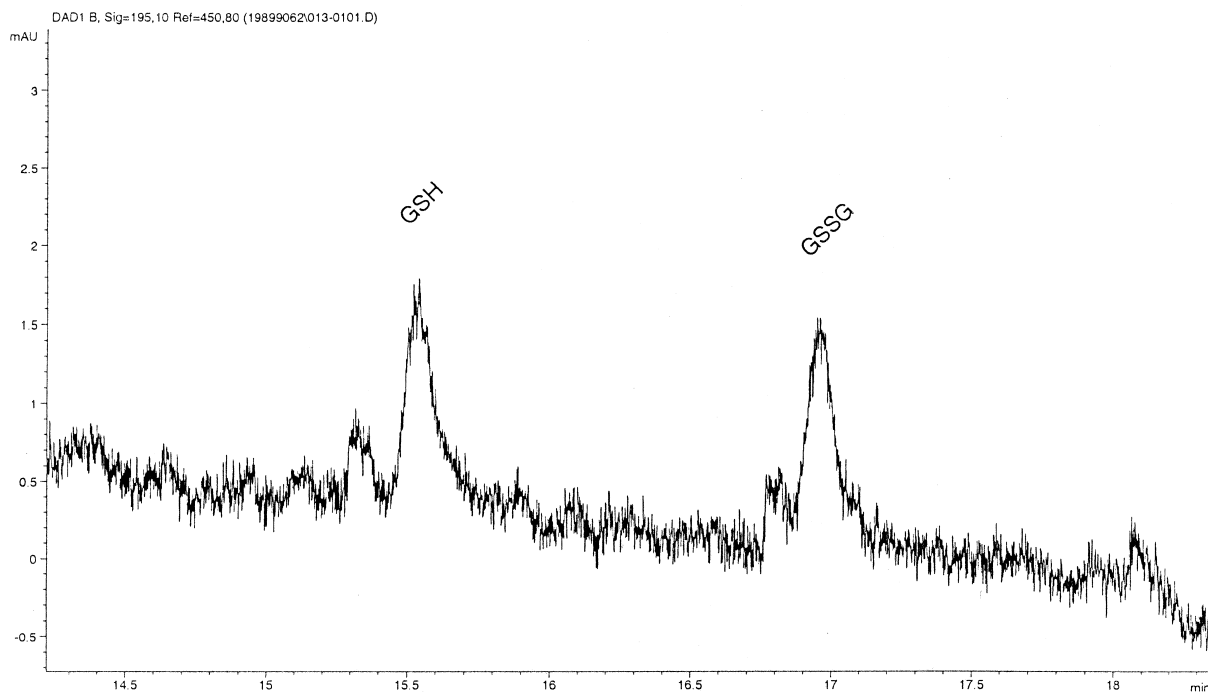Fig. 9. Sample electropherogram demonstrating detection of 0.8 μM GSH and 0.4 μM GSSG Conditions: buffer, 25 mM NaH$_2$PO$_4$, 15 mM Na$_2$B$_4$O$_7$ and 50 mM SDS, pH 8.0; capillary, 80.5 cm (72 cm effective length)$\times$75 μm I.D. with high sensitivity flow cell, λ=195 nm; injection, 500 mbar·s.
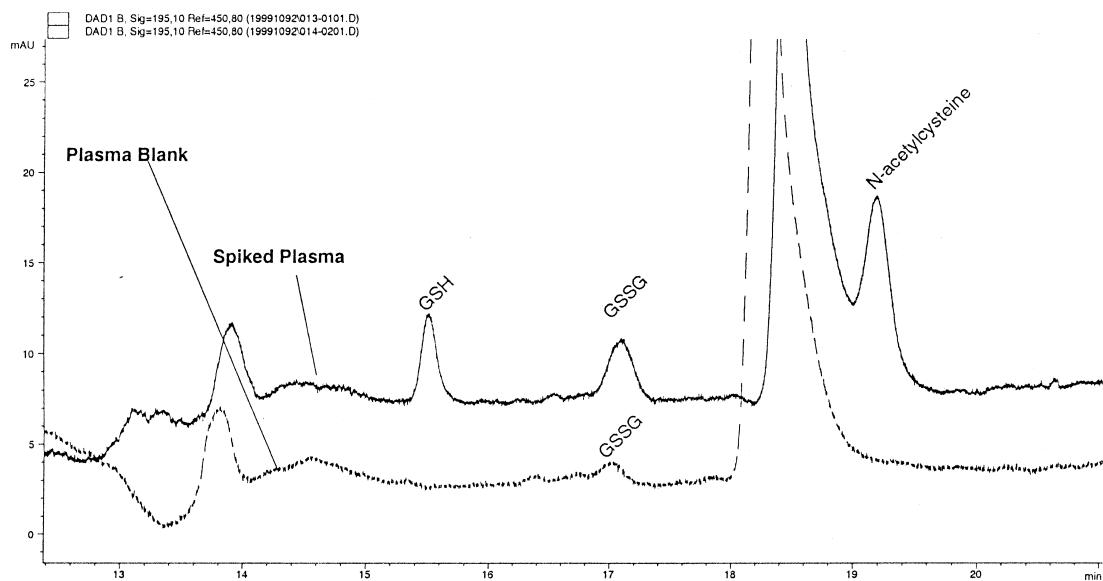

Fig. 10. Sample electropherogram of spiked and unspiked plasma samples showing that GSH and GSSG are detectable at concentrations of 1.6 μM GSH and 0.8 μM GSSG in plasma. Conditions: buffer, 25 mM NaH$_2$PO$_4$, 15 mM Na$_2$B$_4$O$_7$ and 50 mM SDS, pH 8.0; capillary, 80.5 cm (72 cm effective length)$\times$75 μm I.D. with high sensitivity flow cell, λ=195 nm; injection: 500 mbar·s.

oxidized glutathione was optimized in terms of buffer concentration, SDS concentration, buffer pH, detection wavelength, run voltage and injection volume. The method demonstrated good linearity ($r^2 > 0.999$) and reproducibility (internal standard corrected peak area RSD <2.3%) in the range of interest (16–81 μM GSH and 8–40 μM GSSG). A detection limit of less than 1 μM GSH and GSSG was obtained by using the high sensitivity flow cell. When the optimized method was applied to plasma samples, 1.6 μM GSH and 0.8 μM GSSG were easily detected without interference and without the need for derivatization. The on-capillary detection was calculated to be 38.6 fmol of GSH and 18.3 fmol of GSSG.

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