

Capillary Zone Electrophoresis with Electrochemical Detection for the Determination of Glutathione in Human Red Blood Cells without Preseparation of Hemoglobin

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Abstract

Interference is studied for the determination of glutathione (GSH) in human red blood cells by using capillary zone electrophoresis with end-column amperometric detection at a gold–mercury amalgam microelectrode. It is found that when interference substances such as hemoglobin (Hb) in the hemolysate flow off from the end of the separation capillary, they can be adsorbed on the surface of the electrode and interfere with the signal of GSH. If the concentration of hemolysate is lower than 0.5% (v/v), this phenomenon can be overcome because they are adsorbed on the surface of the capillary wall and do not flow off from the capillary. A method is developed for the determination of GSH in human erythrocytes without the preseparation of Hb.

Introduction

The reduced glutathione γ -glutamylcysteinylglycine (GSH) is a physiologically important tripeptide known to be involved in many biological processes (1–4). GSH in human red blood cells has been determined with high sensitivity using reversed-phase liquid chromatography with amperometric detection (5) and capillary electrophoresis with fluorescence detection (6,7) or amperometric detection (8). Because of the hemoglobin (Hb) in red blood cells, it has to be separated in the methods stated using precipitating agents such as orthophosphoric acid (5), trichloroacetic acid (6), and perchloric acid (7,8) before injection and detection. This step can increase the analysis error for the determined results except when inconvenient.

Of the many separation systems available in chemical analysis, capillary zone electrophoresis (CZE) has experienced the most significant growth in recent years. Among the advantages of the technique are the high-separation efficiencies achieved,

short analysis times, low consumption of solvents and samples, simplicity, and wide applicability to a variety of charged analytes. Recently, we have demonstrated that CZE using end-column amperometric detection with a gold–mercury amalgam microelectrode at a constant potential of 0.02 to 0.06 V versus the saturated calomel electrode (SCE) can be employed to the analysis of GSH in human red blood cells. When a capillary of 25- μ m i.d. was used, the linear range was 1×10^{-3} to 0.1mM and the limit of detection 6.3×10^{-4} mM (or 126 amol) (8). However, the Hb in the red blood cells must be separated before injection and detection. In this study, we will describe the voltammetric characteristics of GSH in red blood cells and the effect of the hemolysate and Hb on the determination of GSH by using CZE. It was found that the interference of the substances such as Hb in the hemolysates could be minimized by their adsorption on the wall of the fused-silica capillary. A procedure for the determination of GSH in human red blood cells was developed by using CZE with end-column amperometric detection without preseparation of Hb. The results shown in this study clearly indicate that this procedure is suitable for the determination of GSH in red blood cells with high efficiency, low cost, and a fast rate.

Experimental

Apparatus

Cyclic voltammetry

A voltammetric analyzer (Model 83-2.5, Ningde Analytical Instruments, Ningde, China) coupled with a X-Y recorder (Model 3086-11, Yokogawa Hokuskin, Tokyo, Japan) was used. It was in connection with a cell using potentiostatic control of the electrode potential by means of a three-electrode system that consisted of a gold–mercury amalgam microelectrode as the working

electrode, a Pt wire as the auxiliary electrode, and an SCE as the reference electrode. The reference electrode was connected to the analyte by a salt bridge filled with the same supporting electrolyte as the cell.

CZE

The CZE system used was similar to that described by Jin and Wang (8). Briefly, the apparatus consisted of a fused-silica capillary with dimensions of 10- μm i.d. and 330- μm o.d. (Yongnian Optical Conductive Fiber Plant, Yongnian, China) that was cut to a length of 30 cm and placed between two buffer reservoirs. A high voltage was applied at the injection end when the reservoir containing the electrochemical detection cell was held at ground potential. Separations were carried out at an applied voltage of 25 kV with a high-voltage power supply (Model GDY, Shandong Institute of Chemical Engineering and School of Chemistry, Shandong University, Jinan, China). In this study, a capillary of 10- μm i.d. was selected instead of the 25- μm i.d. that was used in a previous study (8) in order to enhance the sensitivity of the method. In this case, a linear relationship was held between the peak current detected and the concentration in the range of 2×10^{-4} to 2×10^{-2} mM GSH. The limit of detection was 1×10^{-4} mM (26 amol).

The electrochemical detection at a constant potential with CZE was performed using an end-column amperometric approach with a microcurrent voltammeter (Model 901-pA, Ningde Analytical Instruments, Ningde, China). The detection cell and detector were housed in a Faraday cage in order to minimize interference from external sources of noise. Electrochemical detection was carried out with a three-electrode system. It consisted of a gold-mercury amalgam microelectrode as the working electrode, a coiled Pt wire as the auxiliary electrode (which also served as the ground for the high potential drop mentioned previously across the capillary), and a SCE as the reference electrode. The arrangement of the electrochemical detection cell has been illustrated elsewhere in detail (9).

Gold-mercury amalgam microelectrode

The gold-mercury amalgam microelectrodes were constructed using a 100- μm diameter, 7-cm long gold wire, which was inserted

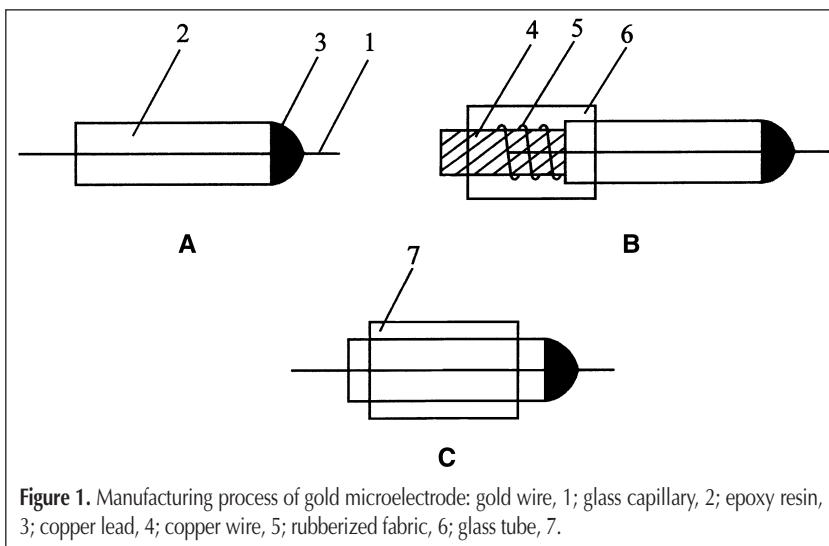
carefully through a 4-cm \times 0.3-mm-i.d. and 0.8-mm-o.d. glass capillary until it protruded approximately 0.5 cm from the end. Epoxy resin was then applied to the junction of the glass capillary in order to seal the fine gold wire to it (Figure 1A). The approximate 2.5-cm gold wire at the other end was wound onto a copper lead. Then the gold wire and the copper lead were bound together with a copper wire of approximately 400 μm . Finally, the copper lead and the glass capillary were bound together using a piece of rubberized fabric in order to protect the electrical junction (Figure 1B). Before use, the gold wire was cut to approximately 1 mm under a microscope. The electrodes were directly used in CZE after dip-coating. For linear sweep voltammetric experiments, a glass tube (1-mm i.d. \times 1-cm o.d.) was put outside the glass capillary with the gold wire and copper lead, which was bonded to the glass tube at its two ends using epoxy resin (Figure 1C) in order to protect the glass capillary. All gold-mercury amalgam microelectrodes were prepared by dip-coating. First, the gold wires were washed with ethanol and water. After drying with filter paper, the gold wires were dipped into pure mercury for approximately 2 min. A fresh gold-mercury amalgam electrode was used for each experiment. The procedure of renewing the electrodes has been described elsewhere (10).

Reagents and solutions

A 0.0100M stock solution of GSH was prepared by dissolving an appropriate amount of GSH (content >98%) (Shanghai Dongfeng Biochemical Technology Company, Shanghai, China) in 0.02M $\text{Na}_2\text{H}_2\text{EDTA}$ of pH 3.0 and stored in a refrigerator at 4°C. Dilute solutions were obtained by serial dilution of the stock solution with water. A 3.13×10^{-2} mM Hb was prepared by dissolving an appropriate amount of Hb (biochemical reagent, crystal pure, Xinjiang Chemistry Institute, Wulumuqi, China) in 6.1mM Na_2HPO_4 -3.9mM NaH_2PO_4 of pH 7.0 and stored in a refrigerator at 4°C. The physiological buffer saline (PBS) consisted of 0.135M NaCl and 0.02M NaH_2PO_4 -NaOH (pH 7.4). All reagents were of analytical grade except for GSH and Hb. All solutions were prepared with double distilled water.

Procedure

For linear sweep voltammetry after the solutions were de-aerated with pure nitrogen, the voltammograms were recorded. In CZE, the procedure was as described elsewhere (8). The gold-mercury amalgam electrode that is cemented onto a microscope slide has to be aligned with the detection end of the capillary under a microscope. Before each run, the capillaries were flushed with 1 mol/L NaOH for 20 min and then with water and the corresponding separation buffer for 20 min each by means of a syringe. In addition, the buffer at the electrochemical cell was also replaced before each run. During the experiments, the separation voltage was applied across the capillary, and the detection potential was applied to the working electrode. After the electro-osmotic current reached a constant value (after approximately 10 to 20 min), the electromigration injection was carried out and the electropherogram was recorded.



Preparation of human red blood cell samples

Human red blood from a normal adult male was collected in a 5-mL centrifuge tube and centrifuged at 1000 rpm for 5 min in order to separate red blood cells. The supernatant liquid was removed. In order to wash the red blood cells, a four-fold of PBS was added into the centrifuge tube. After vibrating lightly, the mixture was centrifuged again, and the resulting supernatant liquid was removed. The step was repeated over six times until the supernatant liquid was clear and transparent. After the supernatant liquid was removed, the red blood cells were stored in a refrigerator at 4°C. Before use, the red blood cells were

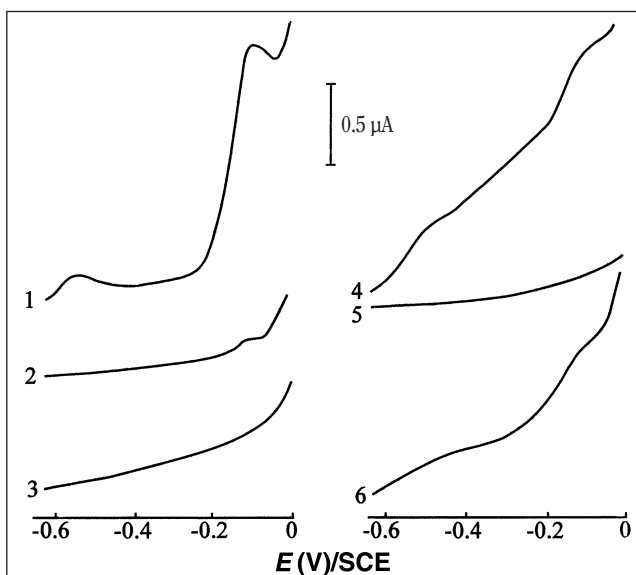


Figure 2. Linear sweep voltammograms of GSH, hemolysate, and Hb: 0.1 mM GSH, 1; 0.2% (v/v) hemolysate, 2; 0.4% (v/v) hemolysate, 3; 0.4% (v/v) hemolysate + 0.500 mM GSH, 4; 1.56×10^{-2} mM Hb, 5; 1.56×10^{-2} mM Hb + 0.500 mM GSH, 6. Gold-mercury amalgam electrode: 2×0.1 mm, $v = 40$ mV/s.

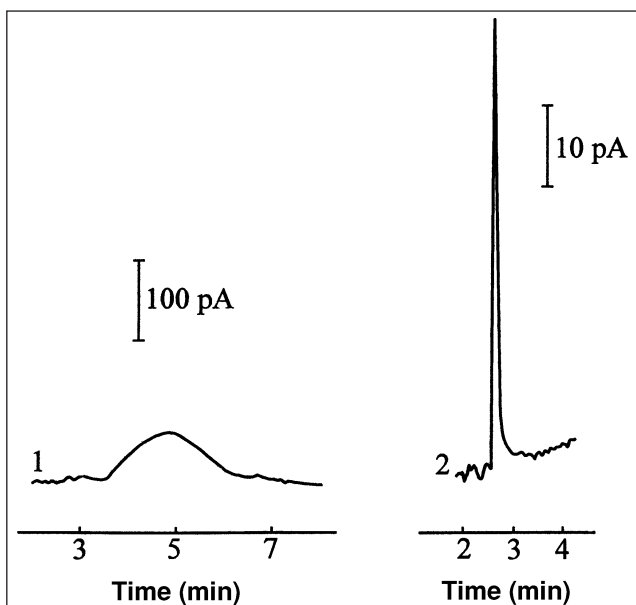


Figure 3. Electropherograms of hemolysate at different concentrations: 20% (1) and 0.5% (2) 6.1 mM Na_2HPO_4 –3.9 mM NaH_2PO_4 (pH 7.0). Capillary used: 42.5-cm \times 10- μm i.d. (1) and 30-cm \times 10- μm i.d. (2).

hemolyzed and diluted by 6.1×10^{-3} mol/L Na_2HPO_4 – 3.9×10^{-3} mol/L NaH_2PO_4 of pH 7.0 (electrophoresis buffer).

Results and Discussion

Voltammograms of GSH in hemolysate

Typical linear sweep voltammograms of hemolysate from human red blood cells for different concentrations at the gold-mercury amalgam electrode in 6.1 mM Na_2HPO_4 –3.9 mM NaH_2PO_4 (pH 7.0) are shown in Figure 2. In the electrolyte solution, GSH had an anodic peak (curve 1). When the concentrations of hemolysate were lower than 0.2% (v/v), an anodic peak appeared at the same potential as that of GSH (curve 2). When the concentrations of hemolysate were higher than 0.4% (v/v), the peak disappeared (curve 3). When GSH was added into the 0.4% (v/v) hemolysate, the peak of GSH appeared again (curve 4), but its peak current was much lower than that of GSH without hemolysate (compared with curve 1). This means that the substances in hemolysate can interfere with the peak of GSH. From the linear sweep voltammograms of Hb (which is the most abundant substance in red blood cells) shown in Figure 2 (curve 5 and 6), it can be seen that the voltammograms of Hb with and without GSH are similar to that of hemolysate, which means that Hb in hemolysate can interfere with the peak of GSH.

Interference of hemolysate with the electrophoresis curve of GSH

Figure 3 shows the electropherograms of hemolysate at different concentrations. It can be found that when the hemolysate concentration was 20% (v/v), a very wide peak was obtained with a peak half-width of 98 s (curve 1). If the hemolysate concentration decreased to 0.5% (v/v), a very fine peak of GSH could be observed with a half-width peak of 1.4 s (curve 2). After 20% (v/v)

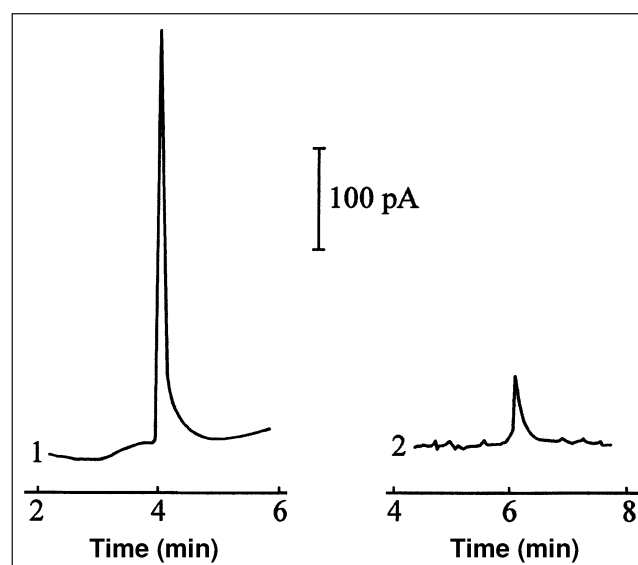


Figure 4. Electropherograms of GSH with a fresh capillary (1) and the capillary after the injection of hemolysate twice with an injection voltage of 5.0 kV and an injection time of 10 s (2). Concentration of GSH, 0.100 mM; capillary length, 42.5 cm.

hemolysate was injected through the separation capillary two times under 5.0 kV for 10 s, the electrophoretic peak current of standard GSH (Figure 4, curve 2) was much lower than that obtained using a fresh capillary (Figure 4, curve 1), and its migration time of the electrophoretic peak was longer than that obtained using the fresh capillary shown in Figure 4, curve 1. These facts indicate that: (a) higher hemolysate concentrations can affect the electrophoretic peak of GSH; (b) it is possible that the substances in hemolysate are adsorbed on the inner wall of the capillary, which explains the prolonged migration time of GSH (compare Figure 4, curve 1 with curve 2); and (c) the interference substances in hemolysate flowing off from the end of the capillary affects the electrochemical detection (see Figure 3, curve 1 and Figure 4, curve 2), because they are probably adsorbed on the surface of the electrode. The volume of

hemolysate or the amount of Hb injected by electromigration (M_i) can be determined using the following equation:

$$M_i = \pi r^2 c L V_i t_i / V_s t_m \quad \text{Eq. 1}$$

where r is the radius of the capillary; c is the concentration of hemolysate (v/v) or Hb (mol/L); L is the length of the capillary; V_i and t_i are the injection voltage and the injection time, respectively; V_s is the separation voltage; and t_m is the migration time.

The dependence of the electrophoretic peak current of standard GSH added to the hemolysate after deducting the peak current of hemolysate and the migration time of standard GSH on the volume of hemolysate injected (V_h) is shown in Figure 5A. The migration time of GSH increased when the volume of hemolysate increased. When the volume of hemolysate injected into the capillary

was smaller than 500 pL, the peak current of GSH was almost a constant. When the volume of hemolysate injected into the capillary was larger than 500 pL, the peak current of GSH obviously decreased. Figure 5B shows the dependence of the electrophoretic peak current and the migration time of standard GSH on the amount of Hb injected into the capillary. It can be found that Figure 5B is very similar to Figure 5A, which means that the substances such as Hb in the hemolysate interfere with the electrophoretic peak of GSH. This conclusion is the same as that of the linear sweep voltammetry previously mentioned. As is well-known, proteins can be adsorbed on the wall of the capillary (12). Therefore, when the volume of hemolysate injected increases, the prolongation of the migration time of GSH results from the adsorption of substances such as Hb on the inner wall of the capillary, which changes the interfacial construction of the capillary.

From Figure 5A, it can be seen that when the volume of hemolysate injected is lower than 500 pL for the capillary with a 10- μm i.d. and 30-cm length used in this study, the peak current of GSH is not changed. This means that when the volume of hemolysate injected is lower than this value, the interference substances adsorbed on the wall of the capillary do not reach the "saturation value". In this case, interference substances cannot flow off from the end of the capillary and reach to the surface of the electrode, hence they cannot interfere with the peak current of GSH. In our experiments, the area of the inner wall of the capillary is 9.42 mm^2 for a capillary with a 10- μm i.d. and 30 cm in length. Thus, the "saturation volume" of hemolysate injected is 53 pL for the inner surface area of the capillary of 1 mm^2 . According to this analysis, if the volume of hemolysate injected into the inner surface area ratio is smaller than 53 pL/ mm^2 , the substances in hemolysate do not interfere with the determination of GSH. This provides a method to solve the interference problem for the determination of GSH in hemolysates.

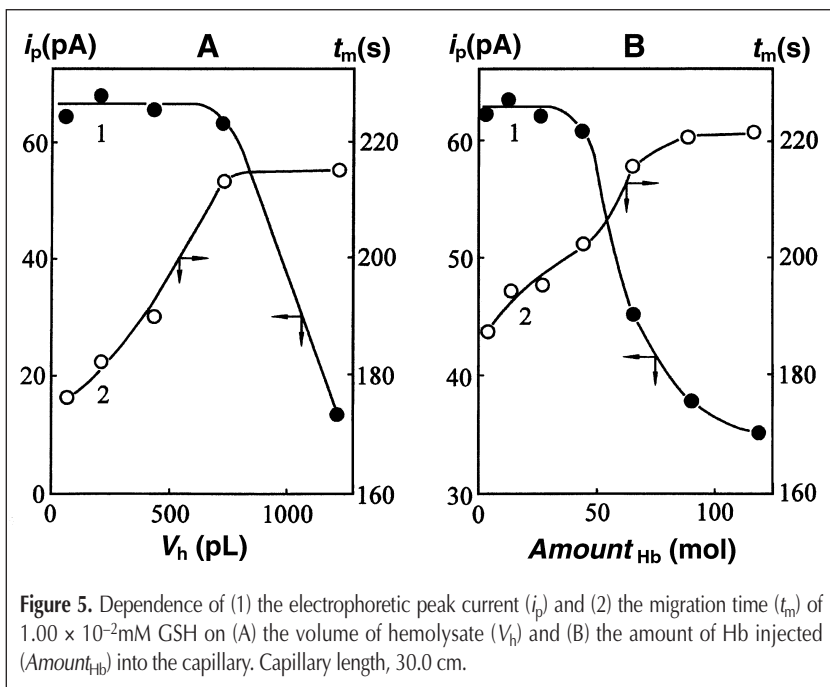


Figure 5. Dependence of (1) the electrophoretic peak current (i_p) and (2) the migration time (t_m) of 1.00×10^{-2} mM GSH on (A) the volume of hemolysate (V_h) and (B) the amount of Hb injected ($\text{Amount}_{\text{Hb}}$) into the capillary. Capillary length, 30.0 cm.

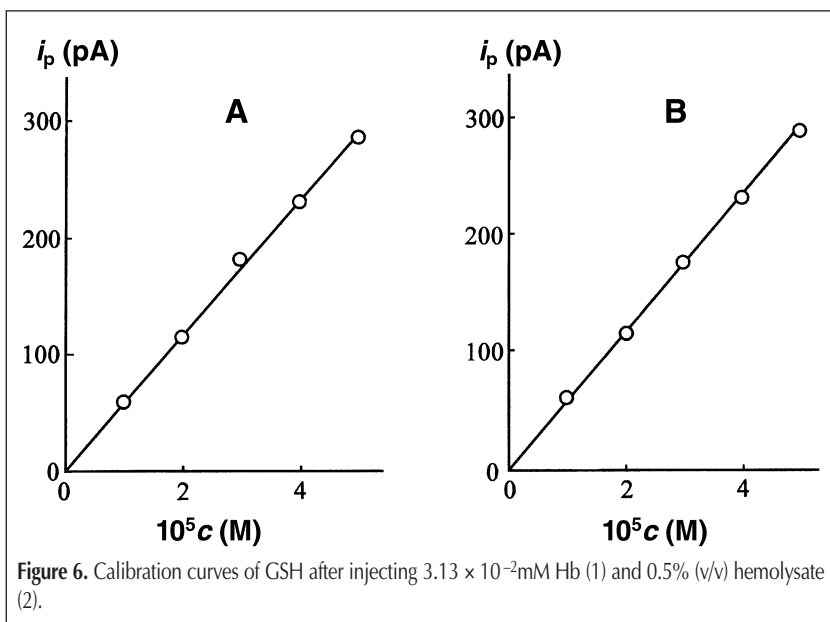


Figure 6. Calibration curves of GSH after injecting 3.13×10^{-2} mM Hb (1) and 0.5% (v/v) hemolysate (2).

Based on the adsorption characteristics of the capillary for interference substances such as Hb in hemolysates, the capillary can serve as the separation medium as long as the volume of the hemolysate injected into the capillary is lower than the 53 pL/mm² inner area of the capillary. Therefore, in our experiments less than 0.5% (v/v) hemolysate, a 5.0-kV injection voltage, and a 10-s injection time were used. Under these conditions, the requirements mentioned previously can be satisfied.

Quantitation of GSH

In order to tally with the actual situation, the calibration curve of GSH should be done in the presence of hemolysate or Hb. Figure 6A is the calibration line of GSH after injecting 3.13×10^{-2} mM Hb for 10 s at 5.0 kV. The calibration line GSH in the presence of hemolysate can be obtained by the following procedure. First, a 0.5% (v/v) hemolysate was injected for 10 s at 5.0 kV, and a peak current of 47 pA for GSH was obtained. Then, the standard GSH of different concentrations was injected and the corresponding electropherograms were recorded. After subtracting the peak current of GSH in the hemolysate (47 pA), the calibration line of GSH in the presence of hemolysate shown in Figure 6B was obtained. The slope and intercept of the straight line were almost the same as that of the calibration straight line shown in Figure 6A. A synthetic red blood cell sample containing 1.00×10^{-2} mM standard GSH and 3.13×10^{-3} mM Hb was used to verify the possibility of the standard addition method. The concentration of GSH in the sample determined was 1.05×10^{-2} mM by using the standard addition method, which agrees with the value in the synthetic sample. The results demonstrate that both the calibration curve method and the standard addition method can be used for the determination of GSH in hemolysates. The results obtained by using the calibration curve method and the standard addition method are listed in Table I for three human red blood cell samples taken from a healthy adult. The concentration of GSH obtained by the calibration curve method is in agreement with the value obtained by the standard addition method. The values of GSH in the three samples are 1.42, 1.55, and 1.61 mM, respectively, which agree with the literature value 1.59 ± 0.08 mM (5). In order to prove the reliability of the method, a certain amount of standard GSH with the concentration C_{add} was added to the hemolysate sample with the concentration C_{sample} . Then, the hemolysate sample with the standard GSH added was measured, and its concentration was found to be C_{found} . Thus, we can obtain the recovery defined by the following equation:

$$\text{Recovery (\%)} = [(C_{\text{found}} - C_{\text{sample}}) / C_{\text{add}}] \times 100\% \quad \text{Eq. 2}$$

The recovery of the method was between 95% and 102% by

| Sample | Concentration (mM) | | Average value (mM) | Recovery (%) |
|--------|--------------------------|--------------------------|--------------------|--------------|
| | Calibration curve method | Standard addition method | | |
| A | 1.40 | 1.43 | 1.42 | 102 |
| B | 1.54 | 1.56 | 1.55 | 95 |
| C | 1.61 | 1.60 | 1.61 | 97 |

using both the calibration curve method and the standard addition method.

Reproducibility

The response for a series of 18 injections of 0.25% hemolysate was measured. After 15 runs, the peak current decreased and the migration time prolonged noticeably. In addition, the noise increased enormously. In this case, the capillary must be washed and the electrode renewed. The relative standard deviations (RSD) of the migration time and the peak current are 1.3% and 3.0%, respectively, for the first 15 values. If 0.5% hemolysate was used, the response for a series of six injections resulted in an RSD of 2.4 to 3.4% for the migration time and 3.5 to 4.9% for the peak current.

Conclusion

In CZE with amperometric detection, the interference of the substances in human red blood cells for the determination of GSH can be overcome using their adsorption characteristics on the surface of the capillary wall. Therefore, the concentrations of hemolysate lower than 0.5% (v/v) are recommended for a 10- μ m i.d. \times 30-cm capillary. If the length of the capillary is longer or the internal diameter of the capillary is larger, higher concentrations of hemolysate can be allowed. In this case, GSH in human red blood cells can be determined by using CZE with amperometric detection without the pre-separation of Hb.

Acknowledgments

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