

Quantifying Reduced Glutathione by Square-wave Voltammetry

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Abstract

This study examines the use of square wave voltammetry (SWV) to quantify reduced glutathione (GSH) dissolved in phosphate buffer (pH 7.5) using a static mercury drop electrode (working), Ag/AgCl electrode (standard) and a platinum wire (auxiliary). The applied voltage ranged from -0.7 to -0.2 V. Increasing concentrations of GSH (13-188 $\mu\text{mol/L}$) correlated with the voltammogram peak area ($R^2=0.99$) and with the current at peak potential (I_p) ($R=0.99$). The reaction of GSH with diamide was monitored for validation of the method. Addition of increasing concentrations of diamide (13.3-50.8 $\mu\text{mol/L}$) to a fixed concentration of GSH (120 $\mu\text{mol/L}$) decreased the I_p , and the results obtained presented a relative deviation (RD) $\leq 14.5\%$ (compared with expected concentrations by stoichiometry) for GSH concentrations above 33.8 $\mu\text{mol/L}$, whereas the spectrophotometric method (Elman's reagent) presented RD $\leq 25.6\%$. These data indicate that SWV method is more accurate and presented equal precision ($SD < 8\%$) as compared to the commonly used spectrophotometric method. This method seems suitable for measuring GSH concentrations at room temperature and pH 7.5 (near biological conditions). Other advantages of this method that make it highly desirable for rapid diagnostic purposes include low cost, simplicity, sensitivity, rapid response and no prior sample preparation.

Keywords: Square-wave voltammetry; Static mercury drop electrode; Reduced glutathione; Oxidative stress

Introduction

The reduced glutathione/glutathione disulfide ratio (GSH/GSSG) is crucially important for maintaining an appropriate redox state necessary for cellular metabolic functions. Many proteins, including enzymes of cell metabolism and regulation [1-6], transcription factors and regulators of cellular cycle [7-9] are potentially influenced by the formation of glutathione adducts [6]. In addition to regulating these processes, GSH participates in the antioxidant system as a preferred target of radical species. It chelates metal ions and is a substrate for the enzyme glutathione peroxidase, which maintains adequate levels of hydroperoxides (H_2O_2 or ROOH) [10-12]. GSH also acts as a substrate for transferase enzymes, linking these compounds to electrophilic xenobiotics to form S-conjugates that are exported from the cell to the blood or from the body to the environment [12,13].

Even small changes in GSH concentrations are related to oxidative stress, which, depending on its intensity, has been associated with the onset or progression of many diseases such as atherosclerosis, cancer, psoriasis, Alzheimer's, hypertension, heart and liver disease and with important physiological processes such as aging and physical exercise [12,14-21].

Therefore, it is of great importance to develop or improve methods to detect and quantify GSH and other thiols under biological conditions. Several techniques for the quantification of these species are described in the literature. Among these techniques is high performance liquid chromatography coupled to various detection methods [22-32], capillary electrophoresis, mass spectrometry, nuclear magnetic resonance [33] and the spectrophotometric method based on the Elman's reagent [34-36]. Furthermore, Wang et al. [37] have proposed an electrochemical method (using a gold electrode) to quantify GSH that uses the decrease in the voltammogram of piarselenole induced by the reaction with GSH dissolved in Britton-Robinson buffer at pH (2.0).

Because the redox pair GSH/GSSG can donate electrons in negative potentials (using the Ag/AgCl electrode as a reference), voltammetric

techniques, especially those based on mercury electrodes, appear to be able to detect and quantify GSH and other thiols in biological samples [13,38].

The electrode reaction of GSH at the static mercury drop has been studied by means of square-wave voltammetry (SWV) [13]. At potentials more positive than -0.350 V (vs. Ag/AgCl (3 mol/L KCl)), the oxidation of the mercury electrode in the presence of GSH produces a sparingly soluble mercury-GSH complex that deposits onto the electrode surface. Under cathodic potential scan, the deposited complex acts as a reducible reactant resulting in a well-defined cathodic stripping reversible SW response. In this study, quantitative results were not obtained. SWV appears to be an appropriate method for this type of analysis; its main advantages are speed of acquisition and good signal definition [39].

The objective of this study was to quantify GSH by SWV under near-physiological conditions using a static mercury drop electrode as the working electrode.

Materials and Methods

All chemicals were of analytical reagent grade. GSH is a product of Sigma, whereas all of the other chemicals were purchased from Merck. Double distilled water was used in all experiments. A stock solution of GSH was prepared by dissolving GSH in double distilled

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Received February 19, 2013; Accepted March 27, 2013; Published March 29, 2013

Citation: Corrêa-da-Silva MV, Pigoso AA, Ribeiro BF, Barbosa LO, Rosado Miloch CA, et al. (2013) Quantifying Reduced Glutathione by Square-wave Voltammetry. J Biosens Bioelectron 4: 133. doi:10.4172/2155-6210.1000133

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water. Phosphate buffer (0.10 mol/L), pH 7.5, prepared from Na_2HPO_4 and NaH_2PO_4 , was used as supporting electrolytes. Pure nitrogen was used to purge the electrolyte solutions for 5 min followed by 2 min of rest prior to each measurement. Thereafter, a nitrogen blanket was maintained over the electrolyte solution.

All voltammograms were recorded using an Autolab multimode potentiostat/galvanostat (PGSTAT) 101 (Metrohm, Netherlands) connected to a static mercury drop electrode (SMDE), Model 663 VA (Metrohm, Netherlands). A platinum wire was used as an auxiliary electrode and Ag/AgCl (3 mol/L KCl) (MME-Metrohm, Netherlands) was used as the reference electrode. To minimize noise, the cell was kept inside a grounded Faraday cage. Origin 6.0 was used for statistical analyses and to generate the graphs.

The formation of thionitrobenzoic acid (TNB) by the reaction between GSH and Elman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)-DTNB] was monitored at 412 nm [35]. The reaction between GSH and diamide was monitored by the change in absorbance at 298 nm [40]. Measurements were performed 7 min after the addition of reagents.

Results and Discussion

SW voltammetric responses to increasing concentrations of reduced glutathione

A typical SW voltammetric response of GSH (61.5 $\mu\text{mol/L}$) at a static mercury drop electrode recorded in phosphate buffer at pH 7.5 is shown in figure 1. The net SW component, calculated as the difference between the cathodic and anodic currents, provides information on both the reduction and oxidation half electrode reactions and can be observed as a single bell-shaped curve with a peak potential of $E_p = -0.506$ V. This E_p is less than that reported by Mladenov et al. [13], possibly because those authors used slightly different experimental parameters, including different GSH concentrations and phosphate buffer at pH 7.0.

Mladenov et al. [13] showed that the electrochemical activity of -SH groups is related more to the complexation reaction between GSH and mercury than its redox transformation; however, because the objective of this study was to quantify GSH levels, only the changes in the analyte concentration (GSH) that are directly correlated with variations in the signal (voltammogram) are relevant.

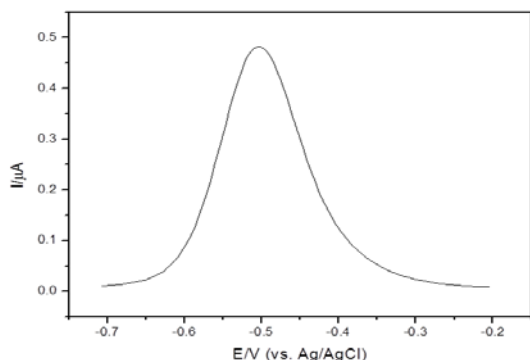


Figure 1: SW voltammogram (δ current) of GSH (62.5 $\mu\text{mol/L}$) dissolved in 0.1 mol/L phosphate buffer, pH 7.5. The experimental conditions were: SW frequency $f=50$ Hz; SW amplitude $E_{sw}=0.02$ V; step potential $dE=0.005$ V; deposition potential $E_{dep}=1.1$ V and deposition time $t_{dep}=5$ s.

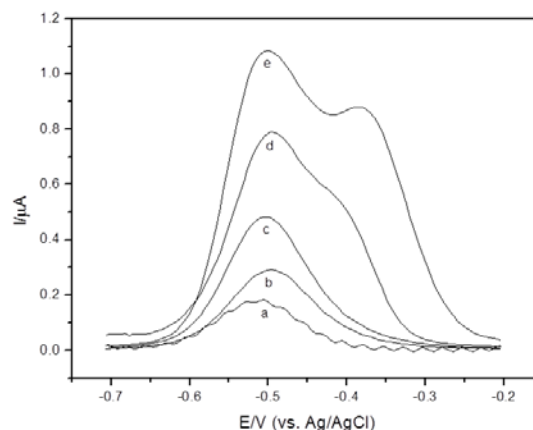


Figure 2: SW voltammograms (δ current) of increasing concentrations of GSH dissolved in 0.1 mol/L phosphate buffer (pH 7.5). a=12.5; b=37.5; c=62.5; d=125; e=187.5 $\mu\text{mol/L}$. Each voltammogram represents the mean of five independent experiments. Other experimental conditions were the same as those described in figure. 1.

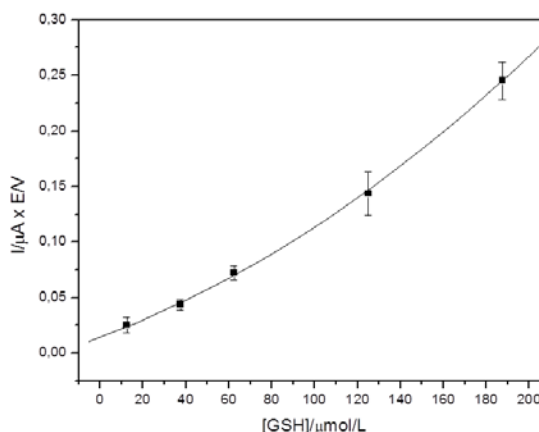


Figure 3: Plot of the value of the Integral of the SW-voltammograms curves (described in figure 2) as a function of the increasing concentrations of GSH (12.5-187.5 $\mu\text{mol/L}$) dissolved in 0.1 mol/L phosphate buffer (pH 7.5). The regression equation is described in the text. Error bars denote standard deviation from 5 independent experiments.

Figure 2 shows the means of the SW voltammograms obtained with increasing concentrations of GSH (from 12.5 to 187.5 $\mu\text{mol/L}$) for five independent experiments ($n=5$).

The concentration of GSH directly correlates with the peak area ($R^2=0.99$) obtained by integrating the curves of the voltammograms and the results are presented in figure 3. The equation that fits the curve is:

$$x = \frac{[-b + (b^2 - 4a(c - y))^{1/2}]}{2a} \quad (1)$$

where:

$$x = [\text{GSH}] (\mu\text{mol/L})$$

$$y = \text{Int. I(E).dE} (\mu\text{A.V})$$

$$a = 2.7 \cdot 10^{-6} (\pm 5.4 \cdot 10^{-7}) (\mu\text{A.V.L}^2/\mu\text{mol}^2)$$

$$b=7.2 \cdot 10^{-4} (\pm 1.0 \cdot 10^{-4}) (\mu\text{A.V/L}/\mu\text{mol})$$

$$c=f(0)=0.02 (\pm 0.003) (\mu\text{A.V})$$

The concentration of GSH, also correlates with the current intensity of the peak ($I_p/\mu\text{A}$) ($R=0.99$), and the data was described in figure 4. I_p was defined as the point of maximum current in the voltammograms. The equation that fits the curve is:

$$x = \frac{(y-b)}{a} \quad (2)$$

where:

$$x=[\text{GSH}] (\mu\text{mol/L})$$

$$y=I_p (\mu\text{A})$$

$$a=\text{slope}=0.005 (\pm 0.0003) (\mu\text{mol/L}/\mu\text{A})$$

$$b=f(0)=0.13 (\pm 0.02) (\mu\text{A})$$

It should be noted that at lowest concentrations of GSH, the standard deviation between experiments is smaller, and therefore, the measurement is more precise in these conditions. The concentrations greater than 200 $\mu\text{mol/L}$, the mercury drop could not be sustained long enough to perform the measurement. Because the concentrations of GSH in mammalian cells range from 0.5 to 10 mmol/L [12,41,42], such samples must be diluted to precisely quantify GSH by this method. The dilution of biological samples is advantageous because it enables the use of smaller quantities of biological material conveniently dissolved in a suitable buffer.

Reaction between GSH and diamide

To validate the proposed method, the reaction between GSH and diamide, a specific ligand, was studied. Diamide [diazenedicarboxylic acid bis(N,N-dimethylamide)] has been shown to produce a rapid decrease in GSH concentration in rat erythrocytes [40] both *in vitro* and *in vivo* [43]; this process can be reversed by treatment with glucose [40] and is reversed more quickly with reduced cysteine [22,43]. In mitochondria from the rat liver, diamide induced a rapid efflux of Ca^{2+} associated with increased respiratory state 4, collapse of the membrane potential and large mitochondrial swelling; these effects were related to the decrease in GSH and NADPH [44,45]. These studies indicate

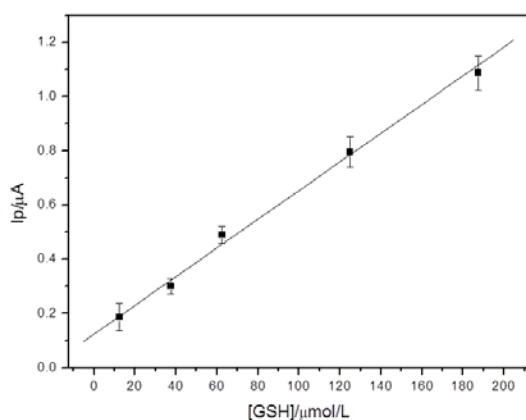


Figure 4: Plot of the value I_p (peak current intensity) of the SW-voltammograms curves (described in figure 2) as a function of the increasing concentrations of GSH (12.5-187.5 $\mu\text{mol/L}$) dissolved in 0.1 mol/L phosphate buffer (pH 7.5). The linear regression is described in the text. Error bars denote standard deviation from 5 independent experiments.

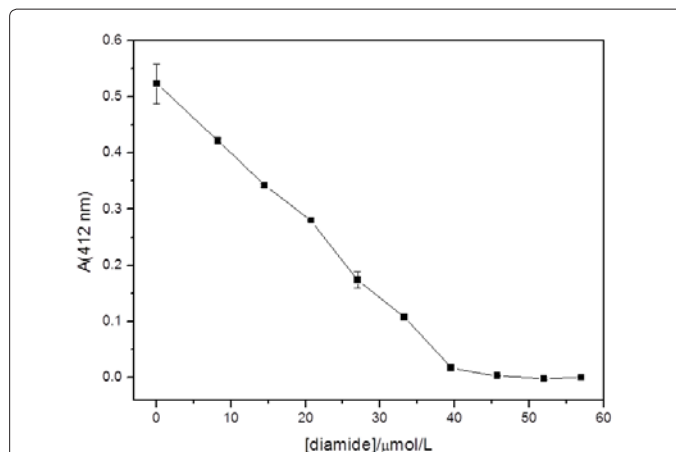


Figure 5: Effect of addition of increasing concentrations of diamide (8.25-57 $\mu\text{mol/L}$) to a fixed GSH concentration (100 $\mu\text{mol/L}$). The TNB, that absorbs 412 nm was produced when the remaining GSH were reduced by DTNB. All reagents were dissolved in 0.1 mol/L phosphate buffer (pH 7.5). Error bars denote standard deviation from 5 independent experiments.

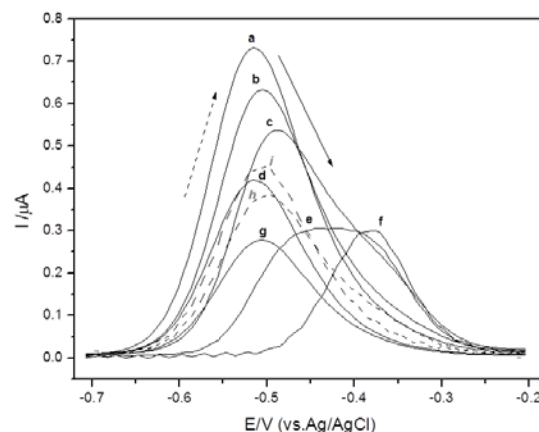


Figure 6: Effect of increasing concentrations of diamide (13.25-63.2 $\mu\text{mol/L}$) on the SW voltammograms (δ current) of reduced glutathione (120 $\mu\text{mol/L}$) dissolved in 0.1 mol/L phosphate buffer (pH 7.5). Voltammograms that decreases as a function of the concentration of diamide are described with solid line and those that increase in dashed line. Each voltammogram represents the mean of five independent experiments. Other experimental conditions were the same as those described in figure 1.

that diamide could be used to induce controlled decreases of GSH in biological samples *in vitro* and *in vivo*. The stoichiometric diamide/GSH ratio is 0.5 (1 mol of diamide to 2 mol of GSH) [40,46].

GSH can be quantified by the Elman's reagent (DTNB), which reacts with GSH to produce TNB, which absorbs light at 412 nm [35]. In the presence of excess of DTNB, the stoichiometric ratio between GSH and TNB is 1:1 [34-36]. Figure 5 shows the effect of increasing concentrations of diamide (8.25-77 $\mu\text{mol/L}$) on the absorbance of TNB (412 nm) produced from the remaining GSH. At the first point, the GSH concentration is 100 $\mu\text{mol/L}$; upon addition of diamide, the concentration of GSH decreases. The curve shows two distinct behaviors: for the first seven points, the absorbance decreases as a function of GSH concentration; for all other points, the absorbance is constant, tending toward zero. Each point represents the mean of five independent experiments ($n=5$) and the measurements were performed at least 7 minutes after the addition of GSH.

This reaction was also performed using the SWV, the voltammograms are described in figure 6 and the I_p values from these voltammograms are shown in figure 7. In this experiment, increasing concentrations of diamide (13.25-63.2 $\mu\text{mol/L}$) were added to a fixed concentration of GSH (120 $\mu\text{mol/L}$). Each point represents the mean of five independent experiments ($n=5$) and the measurements were performed at least 7 minutes after the addition of GSH as described for figure 5. The curve shows two distinct behaviors for the first seven points, the I_p decreases as a function of the diamide concentration and for the last two points, the I_p increases. Changes in the integral of the voltammograms area indicate a high standard deviation.

It should be noted that for the last two points, both in figures 5 and 7, the diamide/GSH ratio exceeded the stoichiometric ratio (0.5), but the behaviors were different. The spectrophotometry results (Figure 5) behaved as predicted by the rate constant of the reaction, which is quite high (300 mol/L in aqueous buffer pH 7.3) [40].

To explain the increase in I_p above the stoichiometric ratio, several

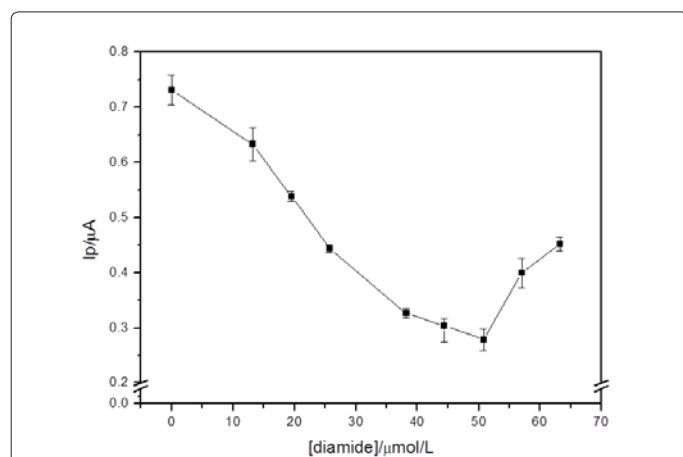


Figure 7: Effect of increasing concentrations of diamide (13.25-63.2 $\mu\text{mol/L}$) on the SW voltammogram peak potential current (I_p) of reduced glutathione (120 $\mu\text{mol/L}$) dissolved in 0.1 mol/L phosphate buffer (pH 7.5). The voltammograms are presented in figure 6. Error bars denote standard deviation from 5 independent experiments.

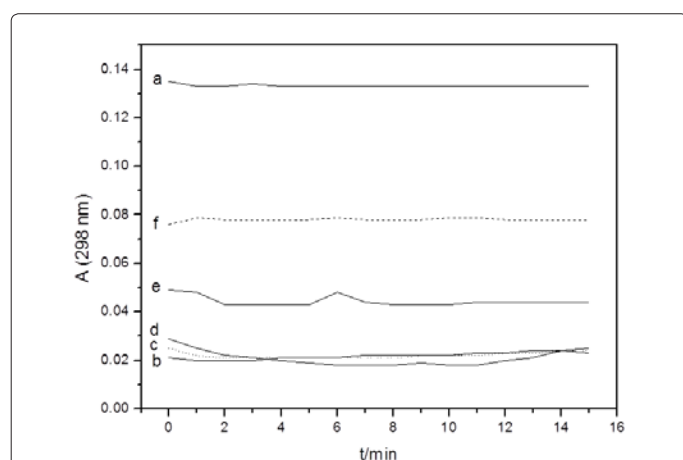


Figure 8: Variation of diamide induced absorbance during 15 min reacting with GSH (100 $\mu\text{mol/L}$) dissolved in 0.1 mol/L phosphate buffer (pH 7.5). (a) Only diamide (90 $\mu\text{mol/L}$); (b) Only GSH; (c) diamide (6 $\mu\text{mol/L}$) plus GSH; (d) diamide (30 $\mu\text{mol/L}$) plus GSH; (e) diamide (60 $\mu\text{mol/L}$) plus GSH; (f) diamide (90 $\mu\text{mol/L}$) plus GSH.

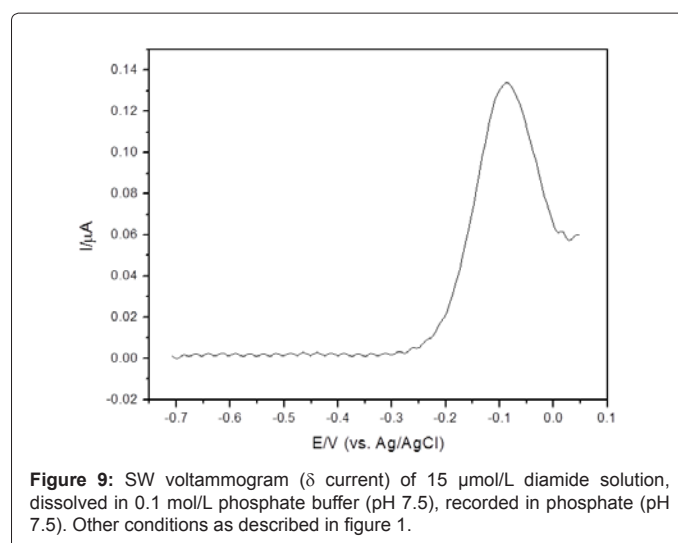


Figure 9: SW voltammogram (δ current) of 15 $\mu\text{mol/L}$ diamide solution, dissolved in 0.1 mol/L phosphate buffer (pH 7.5), recorded in phosphate (pH 7.5). Other conditions as described in figure 1.

control experiments were performed, as shown in figure 8. First, the absorbance of 90 $\mu\text{mol/L}$ of diamide (298 nm) [40] was monitored for 15 minutes (Figure 8, line a), and no change in absorbance was observed during this time. Next, the absorbance of GSH alone was monitored; this absorbance remained near zero (Figure 8, line b). Upon addition of a fixed concentration of GSH (100 $\mu\text{mol/L}$) and varying concentrations of diamide (Figure 8, lines c, d, e, f), the diamide absorbance remained constant. When the diamide/GSH ratio was lower than 0.5, the diamide was completely consumed, and the absorbance decreased to a level similar to that observed with only GSH (Figure 8, lines c and d compared with line b). However, when the ratio was greater than 0.5, diamide became the excess reagent, and its absorbance could be detected (Figure 8, lines e, f), as predicted by the reaction mechanism. Notably, the absorbance of the diamide did not change during the period monitored, indicating that the reaction occurred before the start of measurements. This experiment demonstrates that the spectrophotometric (Figure 5) and voltammetric measurements (Figure 7), which were performed 7 minutes after initiating the reaction, represent the endpoint of the reaction, which is not reversible, as predicted by the rate constant of this reaction, which is high [40]. These findings rule out the possibility of increased I_p values, for points at which the diamide/GSH ratio is greater than 0.5 (Figure 7) due to the presence of GSH, because this compound would be completely consumed prior to signal acquisition and the reaction is not reversible.

Another explanation for the increase in I_p is that diamide could be interacting with the electrodes to generate a voltammogram. Figure 9 depicts the SW voltammogram for 15 $\mu\text{mol.L}^{-1}$ of diamide in phosphate buffer at pH 7.5. One current peak is observed near -0.1 V. Because diamide also interacts with the mercury electrode, it is possible that this compound may be responsible for the increase in I_p for the points at which the diamide/GSH ratio is greater than 0.5 (Figure 5). No experiments were performed to investigate whether hydrazine (other reaction product) interacts with the electrodes, although this reaction does not appear to occur at the first seven diamide concentrations.

Precision and accuracy

To demonstrate the precision and accuracy of the proposed method, the GSH concentrations for the first seven points of figure 7 (diamide concentration versus I_p) were compared with the values expected by the

reaction stoichiometry. These data are shown in table 1. The values for percent standard deviation (SD%) between the voltammograms are all below 8%, indicating an acceptable precision (low dispersal data). When the GSH concentrations obtained from the voltammograms (Obtained) were compared with those expected by the reaction stoichiometry, the percent relative deviation values (RD%) were equal or less than 14.5% for GSH concentrations greater than or equal to 33.8 $\mu\text{mol/L}$.

Similarly, GSH concentrations obtained for the first eight points of figure 7 (diamide concentration versus absorbance at 412 nm) were compared with the values provided by the reaction stoichiometry. The data in table 2 shows acceptable precision at all GSH concentrations used ($\text{SD} \leq 12.8$). Accuracy was acceptable only at GSH concentrations greater than or equal to 57.5 $\mu\text{mol.L}^{-1}$ GSH ($\text{RD} \leq 5.8\%$).

The results in tables 1 and 2 indicate that the data obtained for the parameter I_p of the SW voltammograms, under the conditions described herein, are more accurate than and show precision equivalent to those obtained by the spectrophotometric method described by Favier [35]. It can be seen that the lowest concentration of GSH quantified with acceptable accuracy by the Elman's method was 57.5 $\mu\text{mol/L}$ comparing with 33.8 $\mu\text{mol/L}$ obtained by the voltammetric method (58.8% less). Thus, the voltammetric method showed higher accuracy compared to the spectrophotometric method.

The experiments performed in the present study cannot determine whether reactions between diamide and GSH or between GSH and the

electrode occur under ideal conditions, i.e., at 100% yield. However, the data were obtained at room temperature and pH 7.5, which is similar to biological conditions, thus indicating its use in biological samples.

Conclusion

This work demonstrates that it is possible to quantify GSH using both the area as the peak current (I_p) of the SWV voltammograms recorded using the static mercury drop electrode and the Ag/AgCl as standard electrode. These results were validated by monitoring the specific reaction with diamide that presented equal precision and better accuracy when compared to the results obtained by the spectrophotometric method that uses the Elman's reagent.

Our results indicating the use of this method for use in samples containing cells, extracellular fluids, tissue homogenates or isolated proteins under physiological and pathological conditions, where monitoring alterations in the antioxidant capacity is a relevant factor.

The main advantage of using the mercury drop electrode is that a new drop is used for each reading. Therefore, there much less interference from other molecules, particularly proteins that encrust solid electrodes and reduce the signal or even passivate the electrode, thereby decreasing its selectivity and sensitivity [47-49].

The concentration of GSH and other related thiol groups is very sensitive to levels of oxidative stress. Because SWV proved to be a rapid and precise technique, it may be useful for monitoring medical treatments, especially those based on chemotherapy, radiation and oxygen therapies [12,47]. It may also be useful for monitoring the oxidative stress induced by physical training related to both increased performance and injuries [50-52]. All of these situations require rapid intervention to address radical species or to increase the antioxidant capacity of the individual through proper supplementation [12,15,16].

The advantages of this method include the low cost of instrumentation, operational simplicity, speed of response, high sensitivity [37] and simple preparation (only suspension and dilution in buffer); these properties are desirable for rapid diagnostic methods. These properties indicate the importance of future applications of this method using biological samples.

Acknowledgements

This research was supported by the Brazilian Foundation-FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) (Proc. 2010/02791-7). The authors thank Dr. Denise Vaz de Macedo (Labex, IB-UNICAMP, Brazil) and Dr. Raquel Gianolla Miranda (UNIARARAS-Brazil), for help in writing the manuscript.

References

- Ravichandran V, Seres T, Moriguchi T, Thomas JA, Johnston RB Jr (1994) S-thiolation of glyceraldehyde-3-phosphate dehydrogenase induced by the phagocytosis-associated respiratory burst in blood monocytes. J Biol Chem 269: 25010-25015.
- Ward NE, Pierce DS, Chung SE, Graviitt KR, O'Brian CA (1998) Irreversible inactivation of protein kinase C by glutathione. J Biol Chem 273: 12558-12566.
- Brandwein HJ, Lewicki JA, Murad F (1981) Reversible inactivation of guanylate cyclase by mixed disulfide formation. J Biol Chem 256: 2958-2962.
- Kil IS, Park JW (2005) Regulation of mitochondrial NADP+-dependent isocitrate dehydrogenase activity by glutathionylation. J Biol Chem 280: 10846-10854.
- Silva CM, Cidlowski JA (1989) Direct evidence for intra- and intermolecular disulfide bond formation in the human glucocorticoid receptor. Inhibition of DNA binding and identification of a new receptor-associated protein. J Biol Chem 264: 6638-6647.
- Kil IS, Kim SY, Park JW (2008) Glutathionylation regulates I κ B. Biochem Biophys Res Commun 373: 169-173.

[Diamide] $\mu\text{mol/L}^{(a)}$	[GSH] $\mu\text{mol/L}$		Precision (SD %) ^(d)	Accuracy (RD %) ^(e)
	Obtained ^(b)	Expected ^(c)		
0	114	120	4.50	6.50
13.3	96.2	93.5	6.00	6.70
19.5	78.2	81.0	2.10	4.40
25.8	60.3	68.5	2.10	14.5
38.2	38.1	43.6	4.40	14.5
44.4	33.8	31.2	7.90	13.6
50.8	25.9	18.4	5.20	*47.8

Table 1: Precision and accuracy of the voltammetric method proposed.

To a fixed concentration of GSH (120 $\mu\text{mol/L}$) was added increasing concentrations of diamide^(a). The final GSH concentrations obtained^(b) presented acceptable precision ($\text{SD} < 7.9\%$)^(d). When compared to the expected by the reaction stoichiometry^(c), the accuracy was acceptable below 33.8 $\mu\text{mol/L}$ of GSH^(e) ($\text{RD} \leq 14.5$). The GSH concentrations^(b) were obtained using the I_p of the first seven voltammograms described in figure 6 and the standard curve described in figure 4. *RD above 14.5%.

[Diamide] $\mu\text{mol/L}^{(a)}$	[GSH] $\mu\text{mol/L}$		Precision (SD %) ^(d)	Accuracy (RD %) ^(e)
	Obtained ^(b)	Expected ^(c)		
0	104	100	3.50	5.80
8.25	86.4	83.5	1.10	4.10
14.5	70.2	71.0	0.80	2.70
20.8	57.5	58.5	0.50	2.10
27.0	36.1	46.0	8.10	*25.6
33.3	22.7	33.5	5.10	*37.3
39.5	4.29	21.0	12.8	*91.9
45.5	1.51	8.50	15.2	*94.9

Table 2: Precision and accuracy of the Elman's method in this work conditions.

To a fixed concentration of GSH (100 $\mu\text{mol/L}$) was added increasing concentrations of diamide^(a). The final GSH concentrations^(b) presented acceptable precision ($\text{SD} \leq 12.8\%$)^(d). When compared with the expected by the reaction stoichiometry (1GSH:2diamide)^(c) the accuracy^(e) was acceptable below 57.5 $\mu\text{mol/L}$ of GSH ($\text{RD} \leq 5.80\%$) The GSH concentrations^(b) were obtained with the absorbance of the first eight points described in figure 5 and a suitable standard curve. *RD above 5.80%.

7. Klatt P, Molina EP, Lamas S (1999) Nitric oxide inhibits c-Jun DNA binding by specifically targeted S-glutathionylation. *J Biol Chem* 274: 15857-15864.
8. Klatt P, Molina EP, De Lacoba MG, Padilla CA, Martinez-Galesteo E, et al. (1999) Redox regulation of c-Jun DNA binding by reversible S-glutathiolation. *FASEB J* 13: 1481-1490.
9. Jahngen-Hodge J, Obin MS, Gong X, Shang F, Nowell TR Jr, et al. (1997) Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. *J Biol Chem* 272: 28218-28226.
10. Dringen R, Gutterer JM, Hirrlinger J (2000) Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur J Biochem* 267: 4912-4916.
11. Griffith OW (1999) Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic Biol Med* 27: 922-935.
12. Halliwell B, Gutteridge JMC (2007) *Free Radicals in Biology and Medicine*. (4th Ed), Oxford University Press.
13. Mladenov M, Mirceski V, Gjorgoski I, Jordanoski B (2004) Redox kinetic measurements of glutathione at the mercury electrode by means of square-wave voltammetry. The role of copper, cadmium and zinc ions. *Bioelectrochemistry* 65: 69-76.
14. Magalhães LM, Santos M, Segundo MA, Reis S, Lima JL (2009) Flow injection based methods for fast screening of antioxidant capacity. *Talanta* 77: 1559-1566.
15. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, et al. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39: 44-84.
16. Finaud J, Lac G, Filaire E (2006) Oxidative stress : relationship with exercise and training. *Sports Med* 36: 327-358.
17. Dröge W (2002) Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47-95.
18. Gandra PG, Alves AA, Macedo DV, Kubota LT (2004) Determinação eletroquímica da capacidade antioxidante para avaliação do exercício físico. *Química Nova* 27: 980-985.
19. Reid MB (2001) Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. *J Appl Physiol* 90: 724-731.
20. Simonian NA, Coyle JT (1996) Oxidative stress in neurodegenerative diseases. *Annu Rev Pharmacol Toxicol* 36: 83-106.
21. Alessio HM (1993) Exercise-induced oxidative stress. *Med Sci Sports Exerc* 25: 218-224.
22. Priora R, Coppo L, Margaritis A, Di Giuseppe D, Frosali S, et al. (2010) The control of S-thiolation by cysteine via gamma-glutamyltranspeptidase and thiol exchanges in erythrocytes and plasma of diamide-treated rats. *Toxicol Appl Pharmacol* 242: 333-343.
23. Chen SJ, Chang HT (2004) Nile red-adsorbed gold nanoparticles for selective determination of thiols based on energy transfer and aggregation. *Anal Chem* 76: 3727-3734.
24. Kawakami SK, Gledhill M, Achterberg EP (2006) Determination of phytochelatins and glutathione in phytoplankton from natural waters using HPLC with fluorescence detection. *TrAC-Trends Anal Chem* 25: 133-134.
25. Liu J, Roussel C, Lagger G, Tacchini P, Girault HH (2005) Antioxidant sensors based on DNA-modified electrodes. *Anal Chem* 77: 7687-7694.
26. Wang S, Ma H, Li J, Chen X, Bao Z, et al. (2006) Direct determination of reduced glutathione in biological fluids by Ce(IV)-quinine chemiluminescence. *Talanta* 70: 518-521.
27. Jiang H, Ju H (2007) Electrochemiluminescence sensors for scavengers of hydroxyl radical based on its annihilation in CdSe quantum dots film/peroxide system. *Anal Chem* 79: 6690-6696.
28. Ivanov AR, Nazimov IV, Baratova L (2000) A Determination of biologically active low-molecular-mass thiols in human blood: 1. Fast qualitative and quantitative gradient and isocratic reversed-phase high-performance liquid chromatography with photometric and fluorescence detection. *J Chromatogr* 895: 157-166.
29. Haj-Yehia AI, Assaf P, Nassar T, Katzhendler J (2000) Determination of lipoic acid and dihydrolipoic acid in human plasma and urine by high-performance liquid chromatography with fluorimetric detection. *J Chromatogr A* 870: 381-388.
30. Amarnath K, Amarnath V, Amarnath K, Valentine HL, Valentine WM (2003) A specific HPLC-UV method for the determination of cysteine and related aminothiols in biological samples. *Talanta* 60: 1229-1238.
31. MacCoss MJ, Fukagawa NK, Matthews DE (1999) Measurement of homocysteine concentrations and stable isotope tracer enrichments in human plasma. *Anal Chem* 71: 4527-4533.
32. Guan X, Hoffman B, Dwivedi C, Matthees DP (2003) A simultaneous liquid chromatography/mass spectrometric assay of glutathione, cysteine, homocysteine and their disulfides in biological samples. *J Pharm Biomed Anal* 31: 251-261.
33. Pastore A, Federici G, Bertini E, Piemonte F (2003) Analysis of glutathione: implication in redox and detoxification. *Clin Chim Acta* 333: 19-39.
34. Smith IK, Vierheller TL, Thorne CA (1988) Assay of glutathione reductase in crude tissue homogenates using 5,5'-dithiobis(2-nitrobenzoic acid). *Anal Biochem* 175: 408-413.
35. Favier AE (1995) In: *Analyses of free radicals in biological systems*. Verlag Boston.
36. Alves AA, Pereira da Silva L, Macedo DV, Kubota LT (2003) Amperometric sensor for glutathione reductase activity determination in erythrocyte hemolysate. *Anal Biochem* 323: 33-38.
37. Wang W, Li L, Liu S, Ma C, Zhang S (2008) Determination of physiological thiols by electrochemical detection with piasselenole and its application in rat breast cancer cells 4T-1. *J Am Chem Soc* 130: 10846-10847.
38. Kohen R, Beit-Yannai E, Berry EM, Tirosh O (1999) Overall low molecular weight antioxidant activity of biological fluids and tissues by cyclic voltammetry. *Methods Enzymol* 300: 285-296.
39. Sholz F (2002) *Square wave voltammetry, Electroanalytical Methods, guide to experiments and applications*. Springer-Verlag, Berlin.
40. Kosower NS, Kosower EM (1995) Diamide: an oxidant probe for thiols. *Methods Enzymol* 251: 123-133.
41. Alberich A, Serrano N, Ariño C, Díaz-Cruz JM, Esteban M (2009) Bismuth film electrodes for the study of metal thiolate complexation: an alternative to mercury electrodes. *Talanta* 78: 1017-1022.
42. Meister A, Anderson ME (1983) Glutathione. *Annu Rev Biochem* 52: 711-760.
43. Di Simplicio P, Giannerini F, Giustarini D, Lusini L, Rossi R (1998) The role of cysteine in the regulation of blood glutathione-protein mixed disulfides in rats treated with diamide. *Toxicol Appl Pharmacol* 148: 56-64.
44. Zago EB, Castilho RF, Vercesi AE (2000) The redox state of endogenous pyridine nucleotides can determine both the degree of mitochondrial oxidative stress and the solute selectivity of the permeability transition pore. *FEBS Lett* 478: 29-33.
45. Trendeleva T, Sukhanova E, Ural'skaya L, Saris NE, Zvyagil'skaya R (2011) Effect of prooxidants on yeast mitochondria. *J Bioenerg Biomembr* 43: 633-644.
46. Kosower EM, Kanety-Londner H (1976) Glutathione. 13. Mechanism of thiol oxidation by diazenedicarboxylic acid derivatives. *J Am Chem Soc* 98: 3001-3007.
47. Speit G, Dennog C, Radermacher P, Rothfuss A (2002) Genotoxicity of hyperbaric oxygen. *Mutat Res* 512: 111-119.
48. Smolka MB, Zoppi CC, Alves AA, Silveira LR, Marangoni S, et al. (2000) HSP72 as a complementary protection against oxidative stress induced by exercise in the soleus muscle of rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1539-1545.
49. Essig DA, Nosek TM (1997) Muscle fatigue and induction of stress protein genes: a dual function of reactive oxygen species? *Can J Appl Physiol* 22: 409-428.
50. Hoyer B, Jensen N (2006) Stabilization of the voltammetric serotonin signal by surfactants. *Electrochem Commun* 8: 323-328.
51. Hoyer B, Jensen N, Blusch LP (2001) Effect of the pretreatment of recast nafion membranes on their rejection of the albumin interference in anodic stripping voltammetry. *Electroanalysis* 13: 843-848.
52. Raj CR, Tokuda K, Ohsaka T (2001) Electroanalytical applications of cationic self-assembled monolayers: square-wave voltammetric determination of dopamine and ascorbate. *Bioelectrochemistry* 53: 183-191.