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Biochimica et Biophysica Acta 1570 (2002) 47–52

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Determination of intracellular glutathione and thiols by high performance liquid chromatography with a gold electrode at the femtomole level: comparison with a spectroscopic assay

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Received 18 June 2001; received in revised form 11 December 2001; accepted 18 December 2001

Abstract

Glutathione (GSH) is an important thiol, which has multiple functions in human metabolism, including the detoxification of xenobiotics, radioprotection and antioxidant defense. Here we provide a sensitive and specific method to quantify intracellular GSH and other thiols using an electrochemical detector coupled to a high performance liquid chromatograph (HPLC-ECD). This HPLC-ECD system includes a specially devised gold electrode with a large surface area and a thin gasket to provide an extremely high sensitivity to thiols. The standard curve for GSH showed a good linear relationship at low femtomole levels ($r = 0.970$). We could simultaneously detect GSH, cysteine, *N*-acetylcysteine, γ-glutamyl-cysteine and cysteinyl-glycine by this method. We compared the specificity and sensitivity of this method with those of the conventional spectroscopic method by measuring the amounts of GSH in HL-60 cell extracts. Although the values obtained from these methods were closely correlated ($r = 0.984$), the electrochemical method was much more specific for GSH. This method could detect 2 fmol of GSH and was 6 orders and 2–3 orders of magnitude more sensitive than the spectroscopic method and previous methods using HPLC, respectively. As an example of the application of this method, we demonstrated that the time-dependent alteration in intracellular GSH and cysteine levels could be easily measured using buthionine sulfoximine, an inhibitor of GSH synthesis. On the basis of these results, the advantage of this electrochemical method is extremely sensitive and specific to detect femtomole levels of GSH and other various thiols.

Keywords: Glutathione; Thiol; High performance liquid chromatography; Electrochemical analysis; Gold electrode

1. Introduction

Biological thiols, such as glutathione (GSH) and cysteine, occur widely in living tissues. Particularly, GSH is the most abundant low molecular mass thiol found in cells. GSH is synthesized in two steps catalyzed by γ-glutamyl-cysteine (Glu-Cys) synthetase and GSH synthetase, and then degraded into cysteinyl-glycine (Cys-Gly) by γ-glutamyltranspeptidase [1]. GSH plays a central role in human metabolism, including the detoxification of xenobiotics, cell homeostasis, radioprotection and antioxidant defense [1]. Recently, it has been reported that GSH is incorporated into certain proteins in response to oxidative stress and may participate in posttranslational protein modification [2]. GSH is incorporated into a certain enzyme in metabolic process [3]. Depletion of GSH is associated with a variety of human diseases including diabetes [4], AIDS [5] and neurodegenerative diseases [6]. Accurate measurement of a trace amount of GSH bound to macromolecules and contained in organelles may be helpful to clarify the pathogenesis of various human diseases. To measure an extremely small amount of GSH, the development of the methods with higher sensitivity and specificity is required.

Various methods, such as enzymatic [7–9] and fluorimetric [10] assays, have been available for determination of GSH. The recycling enzymatic method using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) has frequently been used to measure the total amount of GSH [7,8]. To improve the specificity and sensitivity, various methods using high per-

Abbreviations: GSH, glutathione; Glu-Cys, γ-glutamyl-cysteine; Cys-Gly, cysteinyl-glycine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HPLC, high performance liquid chromatograph(y); ECD, electrochemical detector; NAC, *N*-acetylcysteine; BSO, buthionine sulfoximine; SOS, sodium 1-octanesulfonate; TCA, trichloroacetic acid; FBS, fetal bovine serum; CFQ, 4-chloro-7-trifluoromethyl-1-methyl quinolinium methylsulfate; SBD-F, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate

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0304-4165/02/$ – see front matter
PII: S0304-4165(02)00152-6

formance liquid chromatography (HPLC) coupled to a fluorometer [11–15] or an electrochemical detector (ECD) [16–18] have been developed. However, these methods still have insufficient sensitivity and specificity. Thus, the development of methods with higher sensitivity and specificity is required.

In this study, we performed the determination of femtomole levels of GSH using an HPLC-ECD system including a gold electrode with a large surface area and a thin gasket to provide an extremely high sensitivity to GSH. In addition to GSH, simultaneous detection of various authentic thiols, Glu-Cys, Cys-Gly, cysteine, and *N*-acetylcysteine (NAC) was demonstrated. We measured the GSH content in cell extracts obtained from a human promyelocytic leukemia cell line, HL-60, to compare this electrochemical method with a spectroscopic method using a commercial kit. As an example of the application of this method, we measured the alteration in GSH and cysteine levels in HL-60 cells treated with buthionine sulfoximine (BSO), an inhibitor of Glu-Cys synthetase. Furthermore, we discuss the sensitivity and specificity of our method in comparison with previously reported methods.

2. Materials and methods

2.1. Materials

GSH and Glu-Cys were purchased from Kohjin (Tokyo, Japan). BSO, NAC and Cys-Gly were obtained from Sigma (St. Louis, MO, USA). Trichloroacetic acid (TCA) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Cysteine and sodium 1-octanesulfonate (SOS) were obtained from Nacalai Tesque (Kyoto, Japan). Bicinchoninic acid (BCA) was obtained from Dojin Chemical (Kumamoto, Japan).

2.2. Electrochemical analysis of thiol compounds

To measure the amounts of thiol compounds, an HPLC system consisting of an EP-300 pump and an ECD-300 electrochemical detector (Eicom, Kyoto, Japan) equipped with an Eicompak SC-5ODS column (i.d. 3.0 mm×150 mm, Eicom), a WE-AU gold electrode (Eicom) and a 50 μm GS-50 gasket (Eicom) was used. The mobile phase consisted of 99 mM phosphate buffer (pH 2.5), 1% methanol, 200 mg/l SOS and 5 mg/l EDTA. SOS was added to delay the elution of amino compounds, including GSH. The analysis was carried out at a column temperature of 25°C and a flow rate of 0.5 ml/min. The voltage of the gold electrode was set at +600 mV against the Ag/AgCl reference electrode. Authentic thiol compounds (GSH, cysteine, NAC, Glu-Cys and Cys-Gly) dissolved in 0.1 N HCl were simultaneously measured under these conditions. The chromatograms were analyzed and the amounts of thiol compounds were calculated with a PowerChrom v2.1.3J software (AD Instruments). The detection limit for the analysis of GSH was determined by injecting serial dilutions of GSH with 0.1 N HCl onto the HPLC column. The sample injection volume was 5 μl. It is recommended that GSH standards are kept on ice before the measurement. The lower limit of detection was defined by a peak height to baseline noise ratio of 3:1 or greater.

2.3. Measurement of thiols in HL-60 cells

HL-60 cells were grown in RPMI 1640 supplemented with 6% fetal bovine serum (FBS, Gibco, NY, USA) at 37°C. In certain experiments, HL-60 cells (1×10^6 cells/ml) were treated with 0.5 mM BSO in RPMI 1640 with 6% FBS for indicated durations at 37°C. The cells were washed twice with PBS, followed by addition of 100 μl/10^6 cells of 5% (w/v) TCA to the cells to precipitate proteins. Then, the cells were homogenized for 5 s with a microhomogenizer with a Teflon-coated pestle, and centrifuged at $18\,500\times g$ for 10 min at 4°C. The supernatant was diluted with 0.1 N HCl and the amounts of GSH and cysteine were measured with an HPLC-ECD. The sample injection volume was 5 μl. GSH is relatively stable in acidic solution, but it is recommended that the samples are kept on ice throughout the preparation.

2.4. Spectroscopic analysis of intracellular GSH

The amounts of GSH in protein-precipitated cell extracts obtained as described above were measured using a commercial kit (Bioxytech GSH-400, Oxis International, Portland, OR, USA). GSH in the cell extract was derivatized with 4-chloro-7-trifluoromethyl-1-methyl quinolinium methylsulfate (CFQ) to form GSH-thioether [19]. Then, NaOH was added to form thione, which has a maximal absorbance at 400 nm, through β-elimination of GSH-thioether under alkali conditions. The absorbance at 400 nm of the reaction mixture was measured and the amount of GSH was calculated using the standard curve of GSH.

2.5. Measurement of protein content

After HL-60 cells were treated with BSO as described above, the cells were washed twice with PBS. The cells were homogenized in PBS for 5 s with a microhomogenizer with a Teflon-coated pestle, and then centrifuged at $18\,500\times g$ for 10 min at 4°C. The amount of proteins in the supernatant was measured utilizing a modification of the method described by Smith et al. [20]. The absorbance of the reaction mixture at 570 nm was measured with a microplate reader (Model 550, Bio-Rad), and the amount of proteins was calculated using bovine serum albumin as the internal standard.

2.6. Statistical analysis

We compared the results of intracellular GSH contents obtained from electrochemical and spectroscopic assays. For linear regressions, Pearson correlation coefficients were given with accompanying P values. The correlation coefficient was calculated with Microsoft Excel software. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Detection limit and standard curve of GSH

We determined the detection limit of GSH analyzed with an HPLC-ECD by a signal-to-noise ratio of 3:1. The minimal detectable amount of GSH was 2 fmol. The standard curve for GSH at a range of 2–20 fmol is shown in Fig. 1. This standard curve showed a good linear relationship ($y = 0.323x + 0.042$) with a correlation coefficient of 0.970. The intraassay coefficient of variation was 8.2% at 10 fmol GSH. In the case of intact HL-60 cells, 97.4% of GSH was recovered.

3.2. HPLC chromatogram of various thiol compounds

We performed simultaneous analysis of various authentic thiol compounds with an HPLC-ECD. Fig. 2A shows the HPLC chromatogram of the same amount (5 pmol) of thiol compounds. Cysteine, NAC, GSH, Cys-Gly and Glu-Cys were eluted at 4.5 min, 6.1 min, 12.3 min, 14.0 min and 15.9 min, respectively. Fig. 2B shows the chromatogram of the cell extract obtained from HL-60 cells. In HL-60 cells, large amounts of cysteine (3.8 nmol/mg protein) and GSH (11.6 nmol/mg protein) and a relatively small amount of Cys-Gly (0.22 nmol/mg protein) were detected. In addition, a very small amount of Glu-Cys (0.025 nmol/mg protein) could be detected (Fig. 2B, inset).

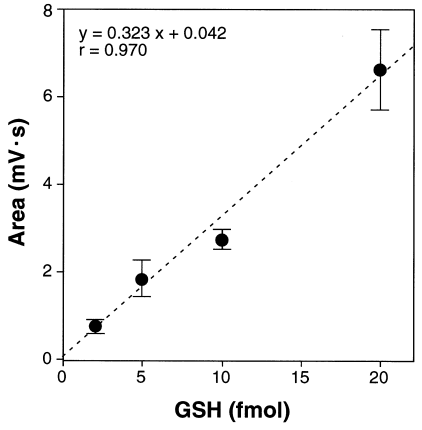

Fig. 1. Standard curve of GSH at low femtomole levels. 5 μl of various concentrations (0.4–4 nM) of GSH (2–20 fmol of GSH) dissolved in 0.1 N HCl were analyzed with an HPLC-ECD under the condition described in Section 2. Values represent means ± S.D. of four or five independent experiments.

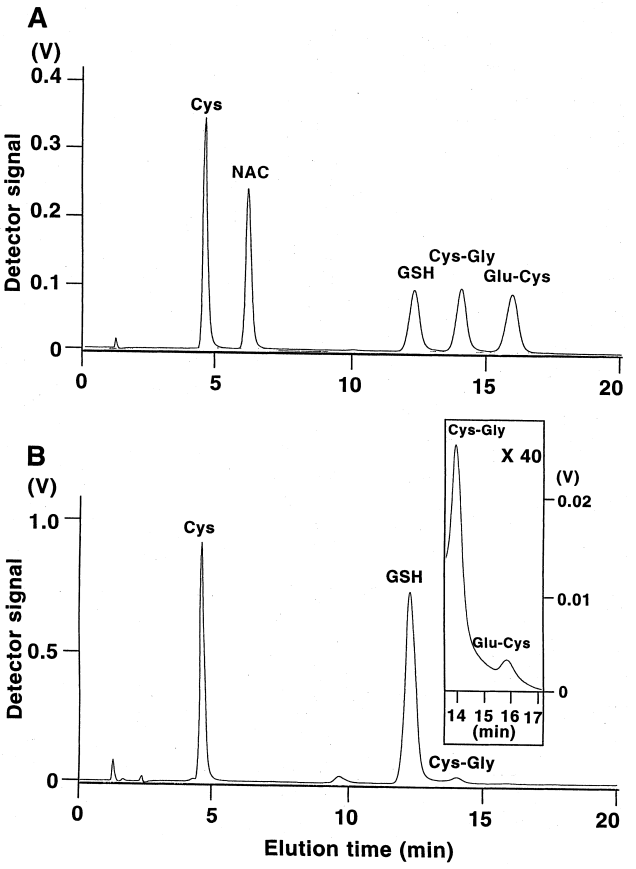

Fig. 2. HPLC chromatograms of various thiol compounds. (A) Chromatogram of authentic thiols. 5 μl of the mixture containing 1 μM thiol compounds (GSH, cysteine, NAC, Glu-Cys and Cys-Gly) in 0.1 N HCl were analyzed with an HPLC-ECD as described in Section 2. (B) Chromatogram of the HL-60 cell extract. The deproteinized HL-60 cell extract was obtained, and then analyzed with an HPLC-ECD as described in Section 2. (Inset) The chromatogram magnified vertically (40-fold).

3.3. Comparison of electrochemical and spectrophotometric assays

We compared the specificity and sensitivity of electrochemical and spectroscopic assays. The GSH values obtained from these assays showed a good correlation ($y = 1.32x + 6.56$, $r = 0.984$, $P < 0.001$), but the values obtained from the spectrophotometric assay were 1.3–2.1-fold larger than those from the electrochemical assay (Fig. 3). The electrochemical and spectroscopic assays revealed that the detection limits of GSH were 2 fmol (as described above) and 2.7 nmol, respectively.

3.4. Time course of GSH and cysteine contents in HL-60 cells treated with BSO

Fig. 4 shows the time course of GSH and cysteine levels

Table 1
Comparison of methods for GSH determination

	Ref.	Detection limit
Enzymatic recycling assay		
Griffith (1980)	[9]	1.475 nmol
HPLC fluorometry		
Monobromobimane		
Newton et al. (1981)	[11]	2 pmol
Velury and Howell (1988)	[12]	1 pmol
SBD-F		
Toyo'oka and Imai (1983)	[13]	0.07–1.4 pmol
Dansyl chloride		
Martin and White (1991)	[14]	1 pmol
Maleimide derivative		
Nakashima et al. (1987)	[15]	17 fmol
HPLC-ECD		
Carbon electrode containing cobalt phthalocyanine		
Halbert and Baldwin (1985)	[29]	4 pmol
Platinum electrode		
Kuninori and Nishiyama (1991)	[31]	1 pmol
Graphite electrode		
Krien et al. (1992)	[18]	0.125 pmol
Gold-mercury electrode		
Kleinman and Richie (1995)	[17]	0.4 pmol
Gold electrode		
Honegger et al. (1989)	[16]	10 pmol
This study		2 fmol

in HL-60 cells treated with BSO, an inhibitor of GSH synthesis. BSO induced a time-dependent decrease in the GSH level. The GSH level was significantly decreased by BSO at 4 and 6 h ($P < 0.001$), whereas the cysteine level was not.

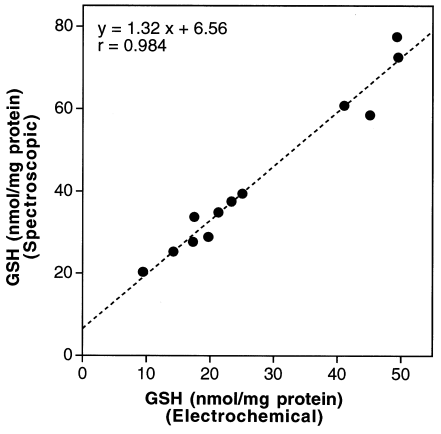

Fig. 3. Correlation of the GSH content analyzed by electrochemical and spectrophotometric assays. HL-60 cells were treated with 0.5 mM BSO in RPMI 1640 with 6% FBS for 0–6 h at 37°C. The cell extracts were obtained as described in Section 2, and then the amounts of GSH were determined with an HPLC-ECD and a commercial kit (Bioxytech GSH-400, Oxis International). Statistical analysis was done as described in Section 2.

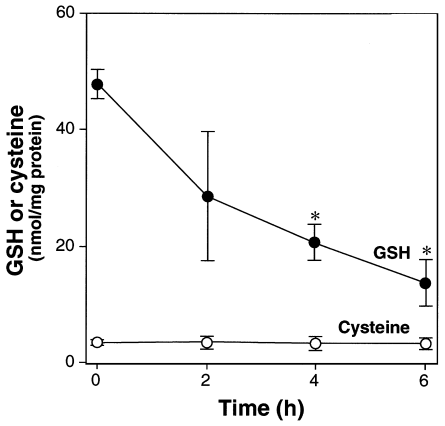

Fig. 4. Time course of GSH and cysteine contents in HL-60 cells treated with BSO. HL-60 cells were treated with 0.5 mM BSO in RPMI 1640 with 6% FBS for 0–6 h at 37°C. Then, the cell extracts were obtained and the amounts of GSH (●) and cysteine (○) were determined with an HPLC-ECD as described in Section 2. Values represent means ± S.D. of three independent experiments. *$P < 0.001$, significantly decreased compared with the control.

4. Discussion

In this study, we provided an extremely sensitive and specific method to detect GSH and other biological thiols using an HPLC-ECD equipped with a gold electrode. The standard curve for GSH showed a good linear relationship at low femtomole levels. In addition, this method has shown that various thiol compounds, GSH, Glu-Cys, Cys-Gly, cysteine and NAC, could be simultaneously detected. The values of intracellular GSH obtained from the electrochemical and the spectroscopic assays showed a good correlation ($r = 0.984$), but the values of the spectroscopic assay were 1.3–2.1-fold larger than those of the electrochemical assay. This result suggests that in addition to GSH, other thiols, including protein-bound thiols, are falsely detected by the spectroscopic assay, and therefore, the electrochemical assay is much more specific for GSH. The electrochemical assay could detect 2 fmol of GSH, whereas the spectroscopic assay was available at 2.7 nmol of GSH, indicating the electrochemical assay is 6 orders of magnitude more sensitive. As an example of the application of this method, we measured the levels of GSH and cysteine in BSO-treated cells. The GSH level was significantly decreased by BSO in a time-dependent manner in agreement with previous studies [21,22], whereas the cysteine level was not. Thus, the alteration in intracellular thiol levels can be easily measured by this method.

Several methods, such as a recycling enzymatic method using DTNB [7,8] and the fluorimetric [10] method, are currently available to measure GSH amount in biological tissues. In this study, a spectroscopic assay was carried out by measuring the products generated through GSH derivatization with a chromogenic reagent, CFQ. However, these methods have disadvantages of low sensitivity and specificity. Liquid chromatography allows specific and sensitive measurement of various thiols. Although low pico-

mole or subpicomole levels of GSH could be detected by HPLC with a fluorescence detector, some methods require precolumn or postcolumn derivatization of GSH with fluorogenic reagents, such as monobromobimane [11,12] and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [13]. Electrochemical detection has been attempted to detect GSH directly without derivatization [23–25], and increased the analytical sensitivity by 1000-fold compared to fluorescence detectors [26,27].

HPLC-ECD has been demonstrated using various electrodes, such as carbon [16], graphite [18], mercury [28], carbon paste containing cobalt phthalocyanine [29], gold-mercury amalgam [30], platinum [31] and gold [32] electrodes. Carbon (1.8 V) [16] and graphite electrodes (above 0.6 V) [18] require high potentials to oxidize GSH, and thus, other electrochemically active compounds may be measured. A gold-mercury electrode needs a low oxidation potential (0.1 V) to detect GSH specifically. However, this electrode is very fragile in the presence of electrochemically active contaminants [27,30] and becomes less sensitive to GSH after measurement of 200–300 samples [27]. Although a gold electrode needs a higher oxidation potential for determination of GSH (0.5 V in [16]; 0.6 V in this study), this electrode appears to be more stable for physiological samples [16]. The gold electrode is highly specific for thiols [16], because thiols are chemisorbed on gold with high affinity. The most important point is that in this study, a specially devised gold electrode and a thin gasket were used. The large surface area of the electrode led to high sensitivity to thiols. The surface of this gold electrode, devised to promote detachment of thiols, resulted in an increase in the electrochemical signals. In addition, the use of a thin gasket contributed to efficient measurement of thiols. Therefore, extremely sensitive and specific analysis of thiols could be performed using this system. The comparison of detection limits of the methods for GSH determination is shown in Table 1. Determination of GSH using GSH reductase detected nanomole levels of GSH [9]. Low picomole or subpicomole levels of GSH have been determined by HPLC fluorometry and HPLC-ECD. Attempts have been made to detect extremely small amounts of GSH using various electrodes. Approx. 10 pmol of GSH has been detected with a gold electrode [16], whereas a recent study revealed that a dual gold-mercury electrode showed higher sensitivity to detect 0.4 pmol of GSH [17]. The present study has demonstrated that the electrochemical assay with a gold electrode is capable of detecting as low as 2 fmol of GSH, and is 2 or 3 orders of magnitude more sensitive to GSH than previous studies using glassy carbon, graphite and gold-mercury electrodes.

On the basis of our findings, it is concluded that the advantages of this electrochemical analysis are: (a) extremely small amounts of GSH and other thiols can be detected; (b) a variety of thiol compounds can be simultaneously quantified. HPLC-ECD with a gold electrode could make it possible to measure a trace amount of thiols in not only cells but also organelles. Recently, it has been reported that GSH is incorporated into certain proteins (protein glutathiolation) [2], and thus, this method may be used for measurement of thiols bound to various macromolecules. Accurate measurement of an extremely small amount of thiols can provide insight into alteration in intracellular distribution of various thiols and their redox status associated with the pathogenesis of various human diseases.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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