Optimization of Determination of Reduced and Oxidized Glutathione in Rat Striatum by HPLC Method with Fluorescence Detection and Pre-column Derivatization

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Summary. A high-performance liquid chromatography (HPLC) method was used to assess the concentration of reduced and oxidized glutathione (GSH and GSSG) in rat striatum. Following decapitation, striatum was isolated from the male Wistar rat brain and immediately homogenized with double distilled water. GSH level was determined after pre-column derivatization with o-phthalaldehyde (OPA). The optimal incubation time with OPA was tested. The concentration of GSSG was determined after blockage the thiol groups of GSH by N-ethylmaleimide (NEM). The useful time for incubation with NEM was optimized. Next, disulfide bounds of GSSG were reduced by dithiothreitol (DTT), and released GSH is derivatized with OPA. The total glutathione, tGSH (sum of free and bound GSH, GSSG, and other low-molecular-mass aminothiols), was determined after reduction with DTT and then derivatization with OPA. The level of GSSG was calculated of the difference in concentrations of tGSH and GSH, but we showed that the calculated concentration of GSSG was within the range of standard deviation of the mean concentration of tGSH or GSH. Finally, the concentration of GSH was determined after 5-min incubation with OPA and the concentration of GSSG after 30-s incubation with NEM and 5-min incubation with DTT and OPA. The relative standard deviation (RSD) values obtained for the assay of GSH and GSSG were lower than 10%. The values obtained for accuracy for GSH (50–500 nM) and GSSG (0.5–5 nM) were within limits regarded as acceptable for analysis of biological samples (percent of recovery: 95–105%). Mean absolute recovery of GSH and GSSG was ranged from 97.1% to 99%. Limit of detection for GSH was 2.7 nM, and limit of quantification was 8.2 nM. Limit of detection (LOD) for GSSG is twice the value for GSH. Described method allows to determine GSH and GSSG levels in isolated rat brain structures with high level of reliability.

Key Words: reduced glutathione, oxidized glutathione, HPLC method, rat striatum

Introduction

Glutathione (GSH) plays a crucial role in maintaining the balance between oxidation and reduction processes in cells. Glutathione in mammalian tissues exists as reduced form (GSH) and oxidized form, the glutathione disulfide (GSSG).
In human and animal tissues, in the normal physiological conditions, the molar ratio of GSSG/GSH is low (from ca. 1/100 to 1/1000) [1, 2]. This ratio may increase during oxidative stress in many pathological conditions and detoxification reactions.

The brain of mammals, in comparison to other tissues, consumes large amounts of oxygen, and reactive oxygen species (ROS) are continuously produced [3]. GSH in the brain plays a crucial role in maintaining the redox homeostasis in both glial and neuronal cell types [4]. The assessment of the level of GSH in the brain may be useful in studies of the role of glutathione in brain tumor drug resistance [5] or in studies of the possible role of antioxidants as neuroprotective therapeutic strategies for some mental illness [6]. A number of factors including neurodegenerative diseases may affect the changes in GSH and GSSG levels in the brain [7–9].

Therefore, determination of the actual proportion of oxidized to reduced glutathione form seems to be critical. However, in practice, this procedure faces difficulties related to two main factors: first, free thiol groups easily undergo oxidation processes, which leads to conversion of reduced to oxidized form; second, GSH concentration in tissues is much higher than the concentration of GSSG, and the difference between the concentrations may be in the error limits applicable to certain methods.

There are many analytical methods (i.e., colorimetric, spectrofluorometric, and chromatographic) used to determine GSH and GSSG in various biological samples [10–13].

Frequently, high-performance liquid chromatography (HPLC) methods are used for the determination of these compounds [14, 15].

GSH assay based on HPLC method is associated with derivatization. In many cases, o-phthalaldehyde (OPA) as a reagent for a fluorometric detection of glutathione is used, but it does not allow the detection of GSSG [16, 17]. One of the GSSG quantification methods is the reduction of GSSG to GSH with dithiothreitol (DTT) after blockage of thiol groups with N-ethylmaleimide (NEM). In this case, NEM prevents reoxidation of GSH. Then the free thiol groups of released GSH are derivatized with OPA [18, 19].

Another GSSG assay requires determination of two parameters: the total glutathione level (sum of free and bound GSH, GSSG, and other low-molecular-mass aminothiols) and a reduced glutathione level (GSH). A total GSH amount (tGSH) is estimated after reduction of free glutathione disulfide and glutathione protein-bound with DTT and then derivatization with OPA. Determination of reduced glutathione (GSH) also requires derivatization with OPA, but without reduction step. A GSSG level is estimated after subtraction of GSH content from tGSH and by dividing the difference by two [20–22].
Any method for the analysis of GSH and GSSG in tissues requires a very careful elaboration. System GSH/GSSG is very dynamic and subject to significant fluctuations. In addition, the amplitude of the concentration ratio is large and is influenced by many different factors. This applies especially to the brain and its lipid-rich structures in which the pro-/antioxidative balance often requires intensive antioxidation processes [23]. For these reasons, obtaining the correct value of concentrations of GSH and GSG is affected by several factors; among them are pH, temperature, and reaction time. For the determination of these compounds in the individual structures of rat brain, the method additionally must ensure a satisfactory level of precision and accuracy with a very small amount of the material [14]. It appears that, in this range, there is no satisfactory solution until now. The aim of the present study was the optimization of conditions for analytical procedure of GSH and GSSG determination in two independent assays in rat striatum by HPLC method with fluorescence detection and pre-column derivatization. Using less complicated and easier to operate fluorescence detector seems to be a good alternative to the electrochemical and mass detectors more usual in the literature. Two analytical procedures for the determination of GSSG were compared: (a) after blocking the thiol group of GSH with NEM, reduction of disulfide bounds with DTT and derivatization the free thiol groups of released GSH by OPA; (b) by estimating a GSSG level based on the difference in a level of tGSH (estimated after reduction and derivatization) and GSH (estimated after derivatization).

In our opinion, efficiency of each method for glutathione determination depends on the particular interaction time of reagents. Thus, in the present study, we additionally examined the incubation time required for optimal derivatization with OPA and the time necessary to the most effective block thiol groups with NEM.

**Experimental**

**Chemicals**

Reduced glutathione (GSH) and oxidized glutathione (GSSG), DL-dithiothreitol (DTT), and N-ethylmaleimide (NEM) were purchased from Sigma-Aldrich (Germany). Sodium tetraborate and trichloroacetic acid were from POCh (Poland). Boric acid, o-phthalaldehyde (OPA), and acetonitrile were purchased from Merck (Germany).
Preparation of Stock Solution and Working Standard Solutions

Stock solution of GSH or GSSG was prepared daily by dissolving these compounds in double distilled water at concentrations of 100 mM or 10 mM, respectively. Working solutions were prepared by diluting stock solution to final concentrations in the ranges of 5–50 mM and 0.5–5 mM for GSH and GSSG, respectively.

Striatum Homogenate Preparations

Striata were isolated from brains of male Wistar rats and individually weighted and homogenized with double distilled water (1:20) in Eppendorf tubes for 30 s on ice. Homogenates were next proceeded or frozen in −80°C.

Standard Curves

Striatum calibration standards for validation were prepared by adding of each working solutions of GSH or GSSG to 50 μL of homogenate. We received the concentration in striatum homogenate in the ranges of 50–500 nM and 0.5–5 nM for GSH and GSSG, respectively.

Analytical Procedures

Reduction and Derivatization to Obtain Total GSH (tGSH)

Striatum homogenates (50 μL) were pipetted into Eppendorf tubes containing double distilled water (10 μL) and 1% TCA–trichloroacetic acid (20 μL). Vortexed specimens were centrifuged at 20,000g for 5 min. Then supernatants (60 μL) were moved into the tubes containing: 10 μL DTT solution (12 mM in 0.72% sodium tetraborate), borate buffer (pH = 8.5; 20 μL), and 10 μL OPA (38 mM obtained by dissolving in methanol and 0.72% sodium tetraborate in proportion 0.05/L). Vortexed specimens were incubated for 5 min at room temperature and then assayed or frozen at −80°C.

Derivatization of GSH

Double distilled water (10 μL) and 1% TCA (20 μL) were added to striatum homogenates (50 μL) in Eppendorf tubes. Next, samples were vortexed and centrifuged at 20,000g for 5 min. Supernatants (60 μL) were transferred into clean Eppendorf tubes, and the following reagents were added: water (10 μL), borate buffer (pH = 8.5; 20 μL), and 38 mM OPA (10 μL). Samples
were mixed using vortex and incubated for 5, 15, and 30 min at room temperature. Next, specimens were examined or frozen at −80°C. Borate buffer was added because the final pH of the solution should be higher than 8, which is important for fluorescence intensity and stability of the fluorescent derivatives [24, 25].

**Determination of GSSG**

Two different procedures (A and B) were employed:

(A) After blocked thiol groups of GSH by N-ethylmaleimide (NEM) and reduction disulfide bounds with DTT and derivatization by OPA.

Water solution of NEM (freshly prepared, 10 mM, 10 μL) and double distilled water (10 μL) were added to striatum homogenates (50 μL) in Eppendorf tubes. Then samples were gently mixed for 10, 20, 30, 60, 300, 900, or 1800 s, and then 1% TCA was added (20 μL). Samples were immediately stirred using vortex and centrifuged at 20,000g for 5 min. Supernatants (60 μL) were transferred into clean tubes, which contained: DTT solution (12 mM, 10 μL), borate buffer (20 μL), and OPA (38 mM, 10 μL). Samples were vortexed and incubated for 5 min at room temperature. Specimens were examined or frozen at −80°C.

(B) The concentration of tGSH and GSH were measured as described above, and the concentration of GSSG was calculated as a difference between tGSH and GSH.

**Determination of Protein**

In the striatum samples, the concentration of protein was determined by the Lowry method [26].

**Chromatography**

HPLC was performed with the following equipment: Merck-Hitachi (Germany) L-7000 LaChrome pump, Shimadzu (Japan) RF-551 spectrofluorometric detector equipped with Merck-Hitachi D-7500 programmable integrator, and Rheodyne 77251 sample injector fitted with 20 μL sample loop (Rheodyne, USA). Sample components were separated on Ultrasphere ODS-DABS 250 mm × 5 mm × 3 μm analytical column (Beckman, USA) by isocratic elution with 95:5 (v/v) water-acetonitrile, at flow rate 1.2 mL min⁻¹. Mobile phase mixture was degassed by sonication for 5 min. Fluorometric detection was carried out at the wavelengths $\lambda_{ex} = 350$ nm and $\lambda_{em} = 420$, with high sensitivity.
Validation of the Method

The analytical methods for evaluation of GSH levels (after derivatization with OPA) and GSSG (with NEM, thiol-masking agents and after reduction and derivatization) in rat striatum were validated by determination of precision, accuracy, absolute recovery (in the range of concentration using for standard curves), and limit of detection and quantification.

The precision of the assay for GSH and GSSG was evaluated by analyzing six replicate GSH or GSSG spiked striatum samples and comparing the glutathione concentration calculated from the peak areas of these six replicates. The coefficient of variation (RSD %) was calculated.

The accuracy was determined by analyzing six replicates of striatum samples spiked with GSH or GSSG. The accuracy was expressed as the mean amount (%) analyte recovered in the assay.

The mean absolute recovery of the method was calculated in the range of the calibration plots for both compounds. The areas of peaks obtained for native samples were subtracted from values obtained for samples containing glutathione standards. The absolute recovery was expressed as a percentage of the area of the peak obtained for the pure standard.

Limit of detection for GSH was calculated as the concentration corresponding to a peak three times the noise level on the chromatogram. Quantification limit was calculated by multiplying the limit of detection by three.

Statistics

Data are presented as means ± SD. Comparisons between samples were made using the paired t-test. Statistics were performed using the STATISTICA programme (ver. 6.0 StatSoft).

Results and Discussion

Selectivity

The selectivity of the method was determined by comparison of chromatograms obtained from native striatum samples and samples spiked with GSH or GSSG. As shown in Fig. 1, good separation of GSH derivatized with OPA was achieved by use of the chromatographic conditions described.
Optimization of Determination by HPLC Method

Fig. 1. Chromatogram of striatum sample after derivatization with OPA

**Linearity**

Calibration curve was plotted using integrated peak areas vs. standard GSH concentrations (Fig. 2). Because GSSG is reduced to two molecules of GSH in the presence of DDT, the area under the peak of GSSG was two times higher in comparison to the corresponding GSH area.

Optimization of conditions for the determination of GSH and GSSG in rat striatum is described below:

The effect of incubation time with OPA on the concentration of GSH-OPA:

- The commonly used methods require 15 min of homogenate incubation with OPA [18, 24]. In the present study, we used
different incubation time for studying the product of reaction of o-phthalaldehyde with GSH contained in brain tissue; the concentration of GSH-OPA corresponding to the result of the concentration of GSH in striatum was measured after 5, 15, and 30 min at room temperature.

We observed that the measured level of GSH significantly decreased after 15- and 30-min incubation time compared to 5-min incubation (Fig. 3). Thus, for further analysis, we used the 5-min incubation.

- Effect of time incubation with NEM on the measured concentration of GSSG.
- Some authors described that incubation time with NEM takes from a few seconds [20] to 30 min [18]. We studied the influence of various times of incubation (10–1800 s) on the measured concentration of GSSG in rat striatum (Fig. 4).

Values are means ± SD for five different samples.
*The statistically significant decrease comparing to 5-min incubation ($p < 0.05$).

**Fig. 3.** The effect of incubation time with OPA on the measured concentration of GSH

Our results indicate that the effect of NEM in the time interval 10–30 s did not change the GSSG peak values after reduction and derivatization via DTT and OPA, respectively.
Values are means ± SD for five different samples
* The statistically significant increase comparing to 10, 20 and 30 sec incubation $(p < 0.05)$

Fig. 4. The effect of incubation time with NEM

Further prolonging the incubation with NEM results in the increase of the area under the peak. After a 15-min (900 s) sample incubation with NEM, the area under the peak is comparable to the peak field value for the procedure used for the determination of total GSH after reduction and derivatization. This may indicate instability of the thiol groups–NEM compounds.

For further analysis with NEM, we chose the 30-s incubation time.

According to procedure A and B, we determined the level of GSSG in rat striatum.

According to procedure B, the level of tGSH and GSH in striatum brain samples obtained from Wistar rats was $75.8 \pm 1$ nmol mg$^{-1}$ protein and $75.2 \pm 3.1$ nmol mg$^{-1}$ protein, respectively. The level of GSSG as a result of tGSH–GSH was $0.6$ nmol mg$^{-1}$ protein. We can observe that the difference between the concentration of tGSH and GSH is within the range of standard deviation of the mean concentration of tGSH or GSH. Because the level of GSSG is very low, it appears that the determination of concentrations of GSSG following procedure B is of little use.

According to procedure A, we indicated that the level of GSSG in rat striatum was $1.01 \pm 0.07$ nmol mg$^{-1}$ protein.
In the literature, there are few data on the concentrations of GSH and GSSG in the brain of rats. Ates et al. [22] in brain samples obtained from Sprague-Dawley rats determined the concentration of GSSG at 0.3–0.4 nmol mg⁻¹ protein.

However, the used methods of determination of GSSG and GSH in different tissues described by different authors are varied, and it is difficult to compare results [27].

In our study, the validation was made for the determination of GSH after derivatization by OPA. We used 5-min incubation with OPA. For determination of GSSG, procedure A was validation. We used 30-s incubation with NEM and 5-min incubation with DTT and OPA.

**Precision and Accuracy**

The mean RSD values obtained for the assay of GSH and GSSG were 6.4 and 9.1%, respectively. Acceptable limit value in such studies is 15%.

Mean values obtained for accuracy for GSH (50–500 nM) and GSSG (0.5–5 nM) were 97% and 101%, respectively, and that were within limits regarded as acceptable for analysis of biological samples (95–105%).

Mean absolute recovery of GSH and GSSG was ranged from 97.1 to 99.0%.

Limit of detection was 2.7 nM for GSH, and limit of quantification was 8.2 nM. LOD for GSSG is twice the value for GSH.

**Conclusions**

Data presented above indicate that the described method can be effectively applied for the determination of GSH and GSSG in rat brain. Moreover, the use of a small amount of tissue allows the determination of these compounds in the isolated structures of this organ. As shown in a few previous studies, occurring concentrations of GSH and GSSG in the rat brain can be determined by HPLC method with fluorescence detection [22]. Our research confirms that this thesis also indicates the greater degree of reliability in the independent determination of GSSG after blocking thiol groups by NEM. The optimized method for the determination of reduced (GSH) and oxidized glutathione (GSSG) in rat brain striatum met the established criteria. The difficulty that consists in low GSSG level relative to GSH in the examined tissue, derived in very small quantities from a single individual, was overcome by using the variant GSSG determination after prior blockade of thiol groups by NEM. In addition, we pointed out that the incubation time both with derivatizing factor (OPA) and blocking thiol groups (NEM) is important for the efficiency and reliability of the method.
References


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