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# A Practical HPLC Method to Measure Reduced (GSH) and Oxidized (GSSG) Glutathione Concentrations in Animal Tissues

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**Abstract:** Glutathione is a tripeptide ubiquitously distributed in living cells, which play an important role in the intracellular protective mechanism against oxidative stress. There are many methods for glutathione analysis including spectrophotometric, fluorometric and bioluminometric assays, often applied to HPLC. In this study, an improved and modified method to measure reduced and oxidized glutathione (GSH and GSSG, respectively) concentrations in animal tissues has been developed. In the study, quail and rat tissue samples were homogenized with the mixture of 10 mM ETDA and 50 mM $NaClO_4$ 0.1% $H_3PO_4$ buffer. Proteins were precipitated by the addition 5% metaphosphoric acid. 50 mM $NaClO_4$ 0.1% $H_3PO_4$ was used as mobile phase and Discovery RP-Amide C16 was used as HPLC column. Glutathione molecules were separated at 215 nm by the UV detector. GSH molecule was eluded between 2.3 and 3 min and GSSG molecule was eluted between 3.5 and 4.5 min from the column. It was observed that in low level of glutathione molecules was detected by this process. Recovery rates of GSH and GSSG were 99.32 and 90.29%, respectively. In conclusion, this method is speed, sensitive and achieved since, it doesn't need forward derivatization process in contrast to many HPLC methods used for glutathione analysis.

**Key words:** GSH, GSSG, HPLC method, liver, wistar rat

## INTRODUCTION

Glutathione (L-g-glutamyl-L-cysteinyl-glycine) is an important intracellular cysteine-containing tripeptide that plays a key role in maintaining cellular homeostasis, as well as protecting the cell against reactive electrophiles and oxidative stress (Lu, 1999). Glutathione (GSH) is the most abundant low molecular mass thiol, widely distributed in living cells and involved in there many biological reactions (Sies, 1999). Measurements of GSH and glutathione disulphide (GSSG) concentrations in tissues or cells are a sensitive indicator of their redox status. GSSG is an especially sensitive means of assessing redox status because its concentration is normally maintained at very low levels relative to GSH (Reed *et al*., 1980; Ross *et al*., 1986; Bellomo *et al*., 1987). The conversion of GSH to GSSG is widely recognized as a reliable index of oxidative stress and is known to be implicated in a wide range of cellular and physiological roles including antioxidant defense, detoxification of electrophilic xenobiotics, cysteine-reservoir functions and modulation of redox-regulated cellular signal transduction and regulation of cell proliferation (Sen, 1997).

GSH and GSSG concentrations are traditionally determined by an enzymatic method (Fiorentino *et al*., 1989) that employs Ellman's reagent (5, 5-dithio-bis-(2-nitrobenzoic acid) is widely used for the analysis of thiols in biological samples via the determination of the liberated anion (Pastore *et al*., 2003). Although, this assay selectively estimates cellular glutathione, it does not allow the direct determination of GSH, which is calculated by subtracting the GSSG value from total glutathione. Both GSH and GSSG can be simultaneously determined by High Performance Liquid Chromatography (HPLC), but increasing the sensitivity of this method necessitates the use of fluorescent derivatizing agents (Mosmann, 1991;

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Macatonia *et al*., 1993). Numerous derivatization procedures for HPLC have been developed for the analysis of fluorescent derivatives of amino acids (Fiorentino *et al*., 1991; Martin and White, 1991; Gardiner and Reed, 1994; Romagnani, 1995; Jones *et al*., 1998). Of these, derivatization with 1-dimethylaminon-aphthalene-5-sulphonyl chloride (dansyl chloride, or dansyl-Cl) gives the highest yields with sulfur-containing amino acids such as cysteine. Among HPLC methods, the amino group (Sereda *et al*., 1997). Derivatization with 2, 4-dinitrofluorobenzene (FDNB) provides the advantage to evaluate both GSH and GSSG with a single run. A number of groups have described HPLC detection procedures for GSH and GSSG using dansyl-Cl (Macatonia *et al*., 1993; Fiorentino *et al*., 1991; Gardiner and Reed, 1994). In this research, a practical HPLC method was developed, molecules of glutathione in animal tissues both reduced (GSH) and oxidized (GSSG) were directly separated without further derivatization.

## MATERIALS AND METHODS

**Chemicals:** Reduced glutathione (GSH), oxidized glutathione (GSSG), Metaphosphoric acid (MPA), ETDA, $NaClO_4$, Phosphoric acid ($H_3PO_4$) were acquired from Sigma (St. Louis MO, USA).

**Biological materials:** In the study, quail liver and muscle samples and rat (Wistar) erythrocytes, liver and kidney tissues were used. Tissue samples were taken from the control group animals at the end of a study and stored in -85°C until being used for biochemical analyses.

**Standard solution:** Working standard solutions (10 μM) of GSH and GSSG molecules were daily prepared in 50 mM $NaClO_4$ 0.1% $H_3PO_4$ buffer.

**Determination of glutathione species tissues:** For the analysis and separation GSH and GSSG molecules were used the fully automatic High Performance Liquid Chromatography equipment (HPLC). The equipment for HPLC consisted of a pump (LC-10 ADVP), a UV-visible detector (SPD-10AVP), a column oven (CTO-10ASVP), an autosampler (SIL-10ADVP) a degasser unit (DGU-14A) and a computer system with class VP software (Shimadzu, Kyoto Japan). Discovery RP-Amide 16 column (150×4.6 mm, 5 μm; Sigma, USA) was used as the HPLC column, 50 mM $NaClO_4$ 0.1% $H_3PO_4$ as the mobile phase whose flow rate was adjusted to as 1 mL $min^{-1}$. Detection was performed at 215 nm by UV detector and column owen temperature was set to 40°C.

In order to analyze GSH and GSSG molecules in animal tissue, 100 mg tissue sample was homogenized with the mixture of 3 mL 10 mM EDTA and 50 mM $NaClO_4$ 0.1% $H_3PO_4$ buffer. Proteins were precipitated by adding 0.5 mL metaphosphoric acid (5% w $v^{-1}$) (Yoshida, 1996). The samples were vortexed for 20 sec and centrifuged at 8000×g for 10 min at 4°C. Supernatants were transferred to autosampler vials of the HPLC instrument. The analysis of glutathione molecules resulted in 5-7 min. Quantification was carried out by external standardization using class VP software. The results were expressed as μmol $g^{-1}$ tissue weight.

**Recovery studies:** For recovery studies, standard solution contenting 2.7 μM $mL^{-1}$ GSH and 6.6 μM $mL^{-1}$ GSSG was prepared. Then, 200 mg rat liver tissue was homogenized in 3 mL 10 mM EDTA and 50 mM $NaClO_4$ (pH = 3.5) and deproteinized with the 5% MPA solution. Fallowing centrifugation, 1 mL supernatant was transferred to auto sampler vials. Once HPLC equipment was ready, 10 μL standard and tissue supernatant were injected and analyzed. In the end of analysis, the peak areas and concentrations of GSH and GSSG molecules were calculated using the HPLC package program. Then, 100 μL standard solutions were added to supernatant solution of the liver tissue and 10 μL mixtures was again injected to HPLC equipment and analyzed.

## RESULTS

During the analysis, it was observed that GSH was eluted between 2.8-3.2 min and GSSG was eluted between 4.3-4.8 min from the HPLC column. GSH level in quail muscle tissue was found to higher than its liver tissue (Table 1). In Wistar rats, the content of GSH level in liver tissue was higher than the lung, kidney and erythrocytes (Table 2).

In recovery studies, the peak areas of GSH and GSSG were found as 1126821 and 1104860, respectively. The peak areas of GSH and GSSG in the tissue supernatant were found 73612±7.07 and 180479±5.83, respectively. The concentration of GSH in the tissue supernatant was found 0.269±0.01 μg and GSSG was 0.608±0.02. The peak area of GSH in the combination of tissue supernatant plus standard solution was found to be 102636±6.34 and GSSG 319031±7.07. The concentration of GSH in the combination of tissue supernatant plus standard solution was 0.418±0.02 and GSSG was 1.144±0.03 (Table 3). When the results calculate, the recovery ratio of GSH was found as 99.32 and GSSG as 90.29%.

Table 1: Levels of the GSH and GSSG molecules in tissues of quail (μmoL $g^{-1}$ tissue)

| Tissue samples | GSH (μmoL $g^{-1}$ tissue) | GSSG (μmoL $g^{-1}$ g tissue) |
|---|---|---|
| Liver | 2.12±0.22 | 0.70±0.02 |
| Muscle | 2.65±0.33 | 1.76±0.16 |

Table 2: Levels of the GSH and GSSG molecules in tissues of rat (μmoL $g^{-1}$ tissue)

| Tissue samples | GSH (μmoL $g^{-1}$ tissue) | GSSG (μmoL $g^{-1}$ tissue) |
|---|---|---|
| Liver | 2.59±0.46 | 0.19±0.02 |
| Kidney | 0.73±0.06 | 0.30±0.02 |
| Erythrocytes | 0.76±0.08 | 0.15±0.03 |
| Lung | 0.41±0.03 | 0.20±0.01 |

Table 3: The results of recovery studies for GSH and GSSG

| Parameters | GSH | GSSG |
|---|---|---|
| Standard concentration (μM/10 uL) | 0.270 | 0.660 |
| Standard pik area (for 10 uL) | 1126821 | 1104860 |
| Tissue samples concentration (ug/10 uL) | 0.269±0.01 | 0.608±0.02 |
| Tissue samples area (ug/10 uL) | 73612±7.07 | 180479±5.83 |
| Standard plus tissue sample conc. (ug/10 uL) | 0.418±0.02 | 1.144±0.03 |
| Standard plus tissue samples area (ug/10 uL) | 102636±6.34 | 319031±7.07 |
| The ratio of recovery (%) | 99.32 | 90.29 |

## DISCUSSION

Glutathione plays an essential role in cellular metabolism and has important functions in many biological processes, particularly in protecting cells from free radicals and reactive oxygen species (Pastore *et al*., 2003). Under normal conditions, glutathione is mainly found as the reduced form (GSH) and in much smaller amounts, as the oxidized form (GSSG) (Cereser *et al*., 2001). GSH and GSSG levels can significantly change upon oxidative stress or pathological conditions, so determination of the GSH/GSSG ratio provides useful information about the redox status of cells (Cereser *et al*., 2001). Therefore, assays for glutathione determination should be able to measure both GSH and GSSG and be sensitive to small amounts of GSSG. Correct measurement of glutathione difficult is cause of the instability of GSH in aqueous solution. GSH auto-oxidation can easily occur during sample preparation and derivatization process, resulting in an overestimation of the GSSG level. Consequently, assays must be rapid, specific and sensitive enough in order to ensure accurate measurements.

In the current method, 100 or 200 mg tissue samples were homogenized with 10 mM EDTA and 50 mM $NaClO_4$ (pH = 2.5) and were deproteinized by 5% MPA. After centrifugation process, the supernatant was injected to HPLC equipment. During analysis, GSH molecule was eluted between 2.7-3.2 min and GSSG molecule was eluted between 3.8-4.55 min (Fig. 1-4). In this separation process,

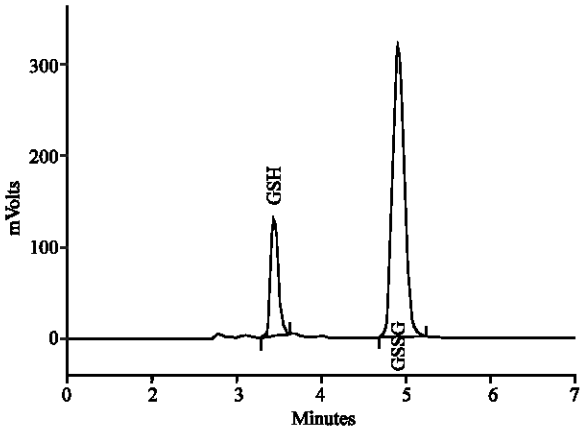


Fig. 1: The chromatogram of GSH and GSSG standard molecules

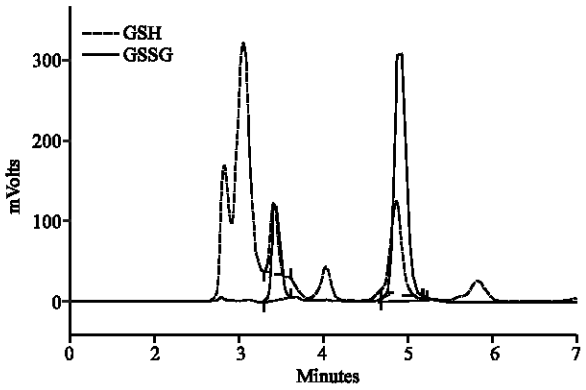


Fig. 2: Comparisons of the standard glutathione molecules chromatogram with the quail muscle chromatogram Red color chromatogram is the quail muscle chromatogram. Blue color chromatogram is the Standard chromatogram

50 mM $NaClO_4$ 0.1% $H_3PO_4$ buffer was used as the mobile phase and for analysis of GSH and GSSG molecules doesn't need further derivization processing. However many HPLC methods were based on derivatization process (Cereser *et al*., 2001; Abukhalaf *et al*., 2002; Lenton *et al*., 1999). Finally, the supernatant of tissues were directly injected to HPLC equipment. Once Discovery RP Amide16 was used as HPLC column and UV detector or Diode Array Detector (DAD) was used, the separation of glutathione molecules was observed in the short time (Fig. 1-4). In many HPLC methods, the different buffers as mobile phase and HPLC columns have been used; in addition fluorescence detector has been used for detection (Abukhalaf *et al*., 2002; Hiraku *et al*., 2002).

It is important for the biological and medical researches that the time of analysis of molecules to be

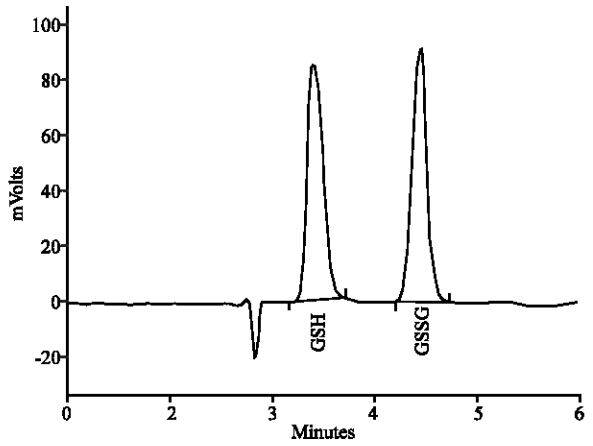


Fig. 3: The chromatogram of GSH and GSSG standard molecules

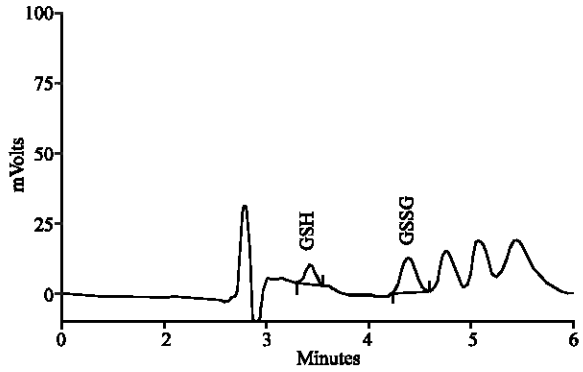


Fig. 4: The GSH and GSSG chromatogram of rat erythrocytes

short. In present method, the same positive results have been reached in examined analysis (Liver, muscle, kidney, lung and erythrocytes) (Fig. 1-4). In recovery studies, it was found that the ratios of GSH and GSSG as 99.32 and 90.29, respectively. When a different mobile phase (i.e. Acetonitrile) is added to the 50 mM $NaClO_4$ it was observed that the glutathione molecules do not separate from the column. In addition, when the pH of mobile phase was not adjusted to 2.5-3.5 with the $H_3PO_4$, the separation of glutathione molecules did not occur. The results of GSH and GSSG were summarized in Table 1 and 2. As a result of this method, when 50 mM $NaClO_4$ 0.1% $H_3PO_4$ is used as mobile phase and Discovery RP Amide 16 is used as HPLC column (or the same properties different HPLC columns), the separation and analysis of glutathione molecules of biological tissue samples can be performed accurately in shorter time. We conclude that the method is practical, fast and sensitive for the analysis of glutathione molecules.

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