Article *NIMHANS Journal*

Spectrophotometric Assay of Oxidized Glutathione Using Iodoacetic Acid and Enzymatic Recycling Method

Volume: 09 Issue: 01 January 1991 Page: 35-38

B R Shivakumar, Vijayalakshmi Ravindranath, - *Department of Neurochemistry, National Institute of Mental Health & Neuro Sciences, Bangalore 560 029, India*

Abstract

Reduced glutathione (GSH) is the most abundant non-protein thiol in the cell and is involved in the maintenance of thiol homoestasis in the cell and protects the tissue from oxidative stress. Oxidation of GSH results in its conversion to the disulfide, GSSG. Estimation of GSSG is often indicative of the oxidative stress within the cell. Analysis of GSSG is difficult due to the low levels of GSSG present in the cell and facile oxidation of GSH to GSSG during analysis. Thus, it is necessary to derivatize the thiol group in GSH prior to estimation of GSSG. We describe here the rapid and easy derivatization of GSH by iodoacetic acid, following which GSSG is estimated by the enzymatic recycling method using glutathione reducatase, NADPH and 5,5 dithiobis (2-nitronbenzene acid). The excess iodoacetic acid present does not interfere with the assay and the recovery of added GSSG is 102%. This method has been used for the assay of GSSG levels in brain and the values obtained are comparable with that obtained by earlier methods.

Key words -

Glutathione,
Oxidised glutathione,
Spectrophotometric assay,
Brain

The tripeptide GSH is the major non-protein thiol in the cell and plays an important role in the maintenance of protein-thiol homeostasis in the cell and in protecting the cell against oxidative stress [1], [2]. In the cell, a substantial portion of total GSH is in the form of reduced glutathione (>99%).During oxidative stress, GSH is converted to its disulfide, GSSG. The GSH / GSSG homeostasis is maintained by conversion of GSSG to GSH by glutathione reductase, utilizing reducing equivalents of NADPH [3]. Thus, the GSSG concentration in the cell is only a fraction of the GSH level, and is often indicative of the oxidative stress in the cell. Due to the facile oxidation of GSH to GSSG, estimation of cellular GSSG is difficult.

One of the most commonly used methods of total GSH (GSH-GSSG) estimation is the spectrophotometric assay involving the enzymatic recycling of GSH using glutathione reductase, NADPH and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) [4]. In order to estimate the concentration of GSSG alone, it is necessary to derivatize the thiol group in GSH, to prevent the oxidation of GSH to GSSG during the assay. N-ethyl this purpose. However, excess N-ethyl maleimide inhibits glutathione reductase and thus, interferes with the assay of GSSG by the enzymatic recycling method [5]. Therefore, excess N-ethyl maleimide is removed prior to the determination of GSSG. The various methods employed for this purpose include extraction with ether [4], chromatographic separation [6] or hydrolysis under alkaline conditions [7].

2-Vinyl pyridine has also been used for the derivatization of GSH. 2-Vinyl pyridine does not interfere with the enzymatic recycling assay [8]. However, the use of 2-vinyl pyridine involves accurate adjustment of pH with triethanolamine (as 2-vinyl pyridine reacts with GSH in neutral pH), thus introducing variable volume changes in the sample which have to be carefully adjusted.

Iodoacetic acid has been used to block the thiol group of GSH, prior to derivatization with fluorodinitrobenzene and analysis of GSH and GSSG to HPLC [9]. Iodoacetic acid has also been used to block the thiol group of GSH prior to analysis of GSH and GSSG by ion-exchange chromatography [10]. The derivatization of GSH with iodoacetic acid in the above two procedures proved to be facile and effective. The present study was conducted to determine if iodoacetic acid could be used as a blocking agent for GSH and subsequent assay of GSSG by the commonly used enzymatic recycling assay. The brain was used as a tissue of choice, since GSH/GSSG determination in brain has proven to be difficult [11].

Material and Methods

Reduced GSH, GSSG, NADPH, 5,5' dithiobis 2-nitrobenzene acid, 2-vinyl pyridine, triethanolamine, glutathione reductase, iodoacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Male adult Swiss Albino mice (3 months old) were obtained from the stock colony of the Institute. Animals had free access to pelletted diet (Hindustan Lever, Bombay, India) and water ad libitum. Prior to decapitation, animals were anaesthesized with ether and perfused transcaridally with saline (0.9% w/v), to prevent contamination of the brain tissue with blood. The brain was removed and frozen in liquid nitrogen immediately.

The tissue was weighed in frozen condition and homogenized in two volumes of 5% perchloric acid containing 1 mM EDTA. The homogenate was centrifuged at 500mg for 2 min. One aliquot of the supernatant was used for the assay of total GSH (GSH + GSSG), while another aliquot (100µ l) was transferred to a test tube containing solid sodium bicarbonate (tilde 50 mg). Immediately 10µ l of iodoacetic acid (0.2 M in water) was added to the tube and it was kept in the dark at room temperature for 1 hr. The contents of the tube was centrifuged briefly and an aliquot was taken for analysis of GSSG by the enzymatic recycling method [4].

The standard consisted of GSSG (10µ g/ml) in 100mM phosphate buffer (pH 7.4). A parallel standard containing a similar concentration of iodoacetic acid that was used for derivatization of brain GSH, was also run. The assay was linear for GSSG concentration from 0.05µ g to 0.4µ g. To determine the recovery of GSSG. 10µ l (1µ g) of standard GSSG was added to the perchloric acid supernatant of the tissue homogenate along with iodoacetic acid, as described above, and analysed for GSSG [4]. 2-Vinyl pyridine was also used for the derivatization of GSH and the unreacted GSSG was estimated as described [8].

Results

The standard curve for GSSG using the enzymatic recycling assay is depicted in Figure 1A. The reaction was linear upto 0.4µ g of GSSG. The linearity of the assay upto 0.2µ g of GSSG is shown in Figure 1A. The effect of addition of iodoacetic acid on the standard curve is shown in Figure 1B. The addition of iodoacetic acid does not seem to affect the slope of the standard curve for GSSG.

Standard curve for GSSG (A) without iodoacetic acid and (B) in presence of iodoacetic acid. Values are mean of triplicate estimations. Assay was carried out as described in 'Methods'

Recovery studies were carried out by addition of known amount of GSSG (1.0μ g) to the brain homogenate before processing the samples for GSSG assay. This recovery was 102 ± 6.8%.

Concentrationof GSSG in the brain was estimated after blocking the GSH group with 2-vinyl pyridine or iodoacetic acid as described in Methods. The results are depicted in Table I. The GSSG concentration in the brain was estimated as 0.022μ moles/gm tissue or 1.07% of total GSH+GSSG, using iodoacetic acid for derivatization of GSH. The GSSG concentration in the brain was estimated as 0.0415μ moles/gm tissue or 1.93% of total GSG+GSSG, following derivatization of GSH with 2-vinyl pyridine. The total brain GSH concentration was determined to be 2.05 ± 0.06μ moles/gm tissue.

Table I - Comparison of GSSG concentration in brain estimated by two different methods

Table I - Comparison of GSSG concentration in brain estimated by two different methods

GSH was derivated using (I) iodoacetic acid or (II) 2-vinyl pyridine, as described in Methods. Following derivatization GSSG was estimated by enzymatic recycling method [4]. The values are expressed as mean ± SEM (n=3-7)

Discussion

The spectrophotometric assay for the determination of GSH and GSSG, using the enzymatic recycling method [4] is one of the most widely used methods. Although several HPLC methods are now available for the estimation of GSH and GSSG [9], [10], [12], [13], the enzymatic recycling assay is the method of choice, when rapid determination of GSH is essential. This is particularly so, when a large number of samples need to be analysed in a short time.

The GSSG concentration in brain as estimated by the iodoacetic acid method is significantly lower than that determined following 2-vinyl pyridine derivatization.. This indicates that the oxidation of GSH to GSSG during the sample processing is substantially minimized in the present method.

In order to differentially estimate GSH and GSSG levels, it is necessary to derivatize the third groups on GSH, to prevent its oxidation to GSSG during the assay procedure. The two reagents of choice have been N-ethylmaleimide and 2-vinyl pyridine. Derivatization with N-ethyl maleimide, although rapid, involves a tedious step, namely, the removal of excess N-ethyl maleimide. This is essential as unreacted N-ethyl maleimide inhibits glutathione reductase and thus, interferes with the assay. 2-Vinyl pyridine, the other derivatization reagent that is often used, does not interfere with the assay procedure. However, its use involves careful pH adjustments in small volumes of the biological sample [4]. In view of this, we have developed the present method detailed above, which involves the deirvatization of GSH with iodoacetic acid, in presence of solid sodium bicarbonate. Iodoacetic acid has been used for a similar purpose [8], [9] for the estimation of GSH and GSSG by HPLC. However, iodoacetic acid has not been used for the derivatization of GSH, when unreacted GSSG has been estimated spectrophotometrically.

Tjhe use of iodoacetic acid for derivatization of GSH and the subsequent estimation of GSSG by the

enzymatic recycling method, offers a rapid and sensitive method for GSSG estimation in biological samples. The recovery of added GSSG from brain homogenate is almost complete (102%). Thus, the method described in this paper would prove to be particularly useful when a large number of samples need to be analyzed in a short period of time.

Acknowledgements

This work was funded by a NIMHANS sponsored research project. The authors thank Prof. Channabasavanna S M, Director, NIMHANS and Prof. B S Sridhara Rama Rao for their encouragement and support.

1.Meister A, Selective modification of glutathione metabolism
Science Page: 220: 472-477, 1983
2.Meister A & Anderson M A, Glutathione
In: Snell E E (Ed.) Ann. Rev. Biochem, Annual Reviews Inc., New York Page: Vol. 52, pp 711-760, 1983
3.Reed D J, Regulation of reductive processes by glutathione
Biochemical Pharmacology Page: 35: 7-13, 1986
4.Tietz F, Enzymatic method for the quantitative determination of nanogram amounts of total and oxidized glutathione
Analytical Biochemistry Page: 27: 502-522, 1969
5.Akerboom T P M & Sies H, Assay of glutathione, glutathione disulfide and glutathione mixed disulfide in biological samples. In: Jackoby W B (Ed.)
Methods in Enzymology, Academic Press, New York Page: Vol. 77, pp, 373-382, 1981
6.Akerboom T P M, Bilzer M & Sies H, The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver
Journal of Biological Chemistry Page: 257: 4248-4252, 1982
7.Sachetta P, Dicola D & Fedrici G, Alkaline hydrolysis of N-ethylmaleimide allows a rapid assay of glutathione disulfide in biological samples
Analytical Biochemistry Page: 154: 205-208, 1986
8.Griffith O W, Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinyl pyridine
Analytical Biochemistry Page: 106: 207-212, 1980
9.Reed D J, Babsen J R, Beatty P W, Brodie A E, Ellis W W & Potter D W, High performance liquid chromatographic analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides
Analytical Biochemistry Page: 106: 55-62, 1980
10.Tabor C H & Tabor H, An automated ion exchange assay for glutathione
Analytical Biochemistry Page: 78: 543-553, 1977
11.Maker H S, *In: Lajtha A (Ed) Handbook of Neurochemistry . Plenum Press, New York* Page: Vol. 3, pp 607-629, 1983
12.Alpert A J & Gilbert H F, Detection of oxidized and reduced glutathione with a recycling post-column reaction
Analytical Biochemistry Page: 144: 553-562, 1985
13.Newton G L, Dorian R & Fahey R C, Analysis of biological thiols: derivatization with monobromobimane and separation by reverse phase high performance liquid chromatography

Analytical Biochemistry Page: 114: 383-387, 1981