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A Comparison Between Different Methods for the Determination of Reduced and Oxidized Glutathione in Mammalian Tissues

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In this study, three rapid assay techniques for the determination of glutathione, one enzymatic, one fluorometric and one newly patented colorimetric method, were compared by measuring reduced (GSH) and oxidized (GSSG) glutathione in guinea-pig heart and liver. The HPLC technique was used as a standard, since it is considered the most reliable assay method. In heart, all methods measured the same levels of GSH (about 1 μmole/g wet tissue), whereas in liver the fluorometric assay gave GSH levels about half as high as those measured by the other methods (about 3 *vs.* 7 μmoles/g wet tissue). Conversely, the fluorometric assay grossly overestimated GSSG concentration (by 5 to 8 times) in both heart and liver. These results confirm previous doubts about the use of the fluorometric technique for GSSG determination in mammalian tissues and also raise some questions about its use for the measurement of GSH in liver. In this tissue, the GSH concentration determined by the fluorometric method was shown to be inversely correlated with the size of the sample, suggesting the presence of some quenching material.

Keywords: Glutathione assay—fluorometric, enzymatic, colorimetric, HPLC methods

INTRODUCTION

One of the most important roles played by glutathione in cell metabolism is protection of cells against the toxic effects of reactive oxygen species (ROS), radicals involved in about one hundred human diseases.[1] Moreover, the oxidative stress consequent to excessive production of ROS is held responsible for the side-effects of various drugs.[2] For instance, the cardiotoxicity of anthracyclines[3] and the hepatotoxicity of some quinone-containing substances[4] are generally ascribed to oxidative stress. Several methods have been devised to measure reduced (GSH) and oxidized (GSSG) glutathione. A commonly used procedure is the enzymatic assay, originally described by Tietze[5] in 1969. A fluorometric method was also proposed by Hissin and Hilf.[6] More recently, a simple, patented colorimetric method (GSH-400®, Bioxytech, France) has been made available. As GSH is by far the predomi-

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nant form of total glutathione (GSH + GSSG) in physiological conditions, it is particularly important to have an accurate method for the determination of GSSG, in order to ascertain the presence of oxidative stress.

During the course of our studies on the cardiac effects of simple quinones,[7–9] we evaluated three currently used methods for the rapid determination of glutathione: enzymatic,[5] fluorometric[6] and Bioxytech methods. As we observed considerable discrepancies between the results obtained with these three assay procedures, we decided to compare them in order to establish their relative merits precisely. For this purpose, we determined GSH and GSSG levels in guinea-pig liver and heart, two tissues with high and low glutathione content, respectively. The results of the three methods were also compared with those obtained by the HPLC technique,[10] which is universally considered as the standard method.

MATERIALS AND METHODS

Male guinea-pigs (300–400 g body wt) were killed by a blow to the head followed by exsanguination. Heart and liver were rapidly removed, frozen in liquid N_2, and maintained at –80°C until used (within 48 hours).

GSH-400® Patented Method

This method is based on the formation of substitution products (thioethers) between SH-containing substances and a patented reagent. In alkaline conditions, the substitution product obtained with GSH is transformed into a chromophoric thione with maximal absorbance at 400 nm.

Cardiac (about 50 mg wet wt) and hepatic tissues (about 250 mg wet wt), frozen in liquid N_2, were pulverized in a cooled ceramic percussion mortar after the addition of 6% metaphosphoric acid (40 ml/g tissue). The tissue homogenates were centrifuged at 27,000 × *g* for 20 min at 4°C to remove proteins. Aliquots (50 µl) of supernatants were then used for determination of GSH using the colorimetric assay kit supplied by Bioxytech (France). Before each assay, a standard curve was prepared with five concentrations (20–100 µM) of standard GSH dissolved in 6% metaphosphoric acid Absorbance at 400 nm was a linear function (r = 0.998) of GSH concentrations. The inter-assay coefficients of variation (CV) of the determinations (n = 6) at 20 and 100 µM were 7.24 and 6.69%, respectively.

Fluorometric Method

Determinations of GSH and GSSG were performed according to Hissin and Hilf[6], using *o*-phthaldialdehyde (OPT) as the fluorescent reagent.

GSH assay When GSH was determined in cardiac tissue, about 50 mg of heart were homogenized in 0.2 ml 25% metaphosphoric acid plus 0.75 ml of 0.1 M sodium phosphate-5 mM EDTA buffer (pH 8.0, exactly). The total homogenate was centrifuged at 105,000 × g for 30 min at 4°C, to precipitate proteins. 0.1 ml of supernatant were diluted by the addition of 0.4 ml of the phosphate-EDTA buffer. The final assay mixture (3 ml) contained 0.1 ml of the diluted supernatant, 2.8 ml of phosphate-EDTA buffer and 0.1 ml of an OPT solution (1mg OPT/ml of reagent-grade absolute methanol, prepared just prior to use). After mixing and incubating at 26°C for 15 min, the sample was transferred into a quartz cuvette. Fluorescence intensity at 420 nm was determined after excitation at 350 nm in a Perkin-Elmer LS-3 fluorescence spectrometer. GSH content was quantified using a standard curve obtained by dissolving known amounts of GSH (0.05–0.4 µM final concentrations) in 2.9 ml of the phosphate-EDTA buffer and mixing with 0.1 ml of the OPT solution (see above). Detected fluorescence was a linear function (r = 0.996) of GSH concentration up to 0.8 µM. The inter-assay CV of the determinations (n = 12) at 0.05 and 0.4 µM were 12.2 and 10.9%, respectively.

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When GSH was determined in hepatic tissue, about 250 mg of liver were homogenized in 1 ml 25% metaphosphoric acid plus 3.75 ml of the phosphate-EDTA buffer. After centrifugation at 105,000 × *g*, 0.5 ml of supernatant were diluted by the addition of 4.5 ml phosphate-EDTA buffer. The final mixture (3 ml) contained 0.1 ml of the diluted supernatant, 2.8 ml of the phosphate-EDTA buffer and 0.1 ml of the OPT solution.

GSSG assay For GSSG measurement in cardiac tissue, a 0.1-ml portion of the 105,000 × *g* supernatant, obtained as described above, was incubated for 30 min at 26°C with 0.04 ml of 40 mM N-ethylmaleimide (NEM) in order to prevent oxidation of GSH to GSSG. At the end of the incubation period, 0.36 ml of 0.1 N NaOH were added to the mixture. A 0.1-ml aliquot was added to 2.8 ml 0.1 N NaOH and 0.1 ml of the above-mentioned OPT solution. After mixing and incubating for 15 min at 26 °C, fluorescence intensity was measured at 420 nm (excitation wavelength 350 nm). GSSG content was quantified using a standard curve obtained by dissolving known amounts of GSSG (0.025–0.2 μM final concentrations) in 2.9 ml 0.1 N NaOH and mixing with 0.1 ml OPT solution. Detected fluorescence was a linear function (r = 0.992) of GSSG concentrations up to 0.4μM. The inter-assay CV of the determinations (n = 12) at 0.025 and 0.2 μM were 18.5 and 17.5%, respectively.

For GSSG determination in liver, 0 5 ml of the original 105,000 × *g* supernatant were incubated for 30 min at 26 °C with 0.2 ml of 40 mM NEM, after which 4.3 ml of 0.1 N NaOH were added to the mixture. The final mixture (3 ml) contained 0.1 ml of this diluted sample, 2.8 ml of 0.1 N NaOH and 0.1 ml of the OPT solution.

Enzymatic Method

The procedure used for enzymatic determination of GSH and GSSG was essentially that proposed by Tietze,[5] as modified by Anderson,[11] with minor modifications as described below. The method is based on the determination of a chromophoric product, 2-nitro-5-thiobenzoic acid (TNB), resulting from the reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) with GSH. In this reaction, GSH is oxidized to GSSG, which is then reconverted to GSH in the presence of glutathione reductase and NADPH. The rate of TNB formation, which is proportional to the sum of GSH and GSSG present, is followed at 412 nm.

GSH and GSSG contents were measured in tissue extracts obtained exactly as described above for the GSH assay with the GSH-400® patented method.

Measurement of total glutathione 0.1 ml of supernatant obtained from both liver and heart were 1:5 diluted by the addition of 0.4 ml 0.1 M potassium phosphate-5 mM EDTA buffer (pH 7.4). Subsequently, 0.1 ml of diluted supernatant was transferred into a 1-ml cuvette containing 0.785 ml of 0.1 M potassium phosphate—5 mM EDTA buffer (pH 7.4), 0.025 ml of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (prepared in 0.1 M phosphate buffer, pH 7.4) and 0.08 ml of 5 mM NADPH. After a 3-min equilibration period at 25°C, the reaction was started by the addition of 2U glutathione reductase (type III Sigma, from bakers yeast, diluted in 0.1 M phosphate-EDTA buffer, pH 7.4). The formation of TNB was continuously recorded at 412 nm at 25°C in a Shimadzu UV-160 recording spectrophotometer.

The total amount of glutathione in the samples was determined from a standard curve obtained by plotting known amounts of GSH (0.16, 0.32, 0.64, and 1.28 μM), incubated in the same experimental conditions, *vs.* the rate of change of absorbance at 412 nm (r = 0.999). The inter-assay CV of the determinations (n = 18) at 0.32 and 1.28 μM were 19.3 and 18.3%, respectively. GSH standards were prepared daily in 6% metaphosphoric acid and diluted in phosphate-EDTA buffer (pH 7.4).

Measurement of GSSG In order to minimize GSH oxidation, it was important to prepare the samples rapidly. Accordingly, soon after prepa-

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ration, the supernatant was treated for derivatization with 2-vinylpyridine at room temperature for 60 min. In a typical experiment, 0.15 ml of supernatant were placed in a plastic tube under a hood and 3 µl of undiluted 2-vinylpyridine (stored at −20°C) were added. 9 µl of triethanolamine were also added, the mixture was vigorously mixed, and the pH was checked—it was generally between 6 and 7. After 60 min, 0.1-ml aliquots of the samples were assayed by means of the same procedure described above for total GSH measurement.

The amount of GSSG present was quantified from a standard curve obtained by plotting known amounts of GSSG (0.08, 0.16, 0.32, and 0.64 µM) *vs.* the rate of change of absorbance at 412 nm (r = 0.998). The inter-assay CV of the determinations (n = 18) at 0.16 and 0.64 µM were 15.6 and 15.8%, respectively. GSSG standards were prepared daily in 6% metaphosphoric acid, diluted in phosphate-EDTA buffer and derivatized with 2-vinylpyridine.

Measurement of GSH GSH present in the samples was calculated as the difference between total glutathione and GSSG levels. For this calculation, the fact that one molecule of GSSG gives rise to two molecules of GSH upon reaction with glutathione reductase was taken into account.

HPLC Method

The procedure used was essentially that proposed by Fariss and Reed.[10] This method is based on the formation of S-carboxy-methyl derivatives from free thiol groups, followed by conversion of free amino groups to 2,4-dinitrophenyl (DNP) derivatives. These derivatives were separated by reversed-phase ion exchange HPLC. 50–100 mg (wet wt) of heart or liver were homogenized by mortar grinding in liquid N_2 in the presence of 1.5 ml 10% (v/v) perchloric acid (PCA) containing 1 mM bathophenanthrolinedisulfonic acid (BPDS). The acidic extract was sonicated, frozen and thawed, and finally centrifuged at 15,000 × *g* for 5 min. An aliquot (0.5 ml) of the resulting PCA extract was mixed with 50 µl of 15 mM γ-glutamyl-glutamic acid, used as an internal standard, and derivatized for HPLC analysis as described by Fariss and Reed.[10] Twenty µl-aliquots of the derivatized samples or standards were separated on a Waters µBondapak amine column (10µm, 300 × 3.9 mm I D.) in a Perkin Elmer Series 410 Liquid Chromatograph equipped with a solvent programmer and a variable wavelength detector set at 350 nm. A guard column (Guard-pak Precolumn Module Waters Association) was used in front of the analytical column. The column was flushed at the end of the day with highly purified "MilliQ" water followed by 20% H_2O : 80% methanol (v/v) to remove buffer salts. GSH and GSSG were eluted with a multi-step linear solvent gradient of acetate buffer pH 5.5 and methanol, essentially as described by Reed et al.,[12] at a flow rate of 1 ml/min. Recycling time between runs was 10 min. Before analysis, all tissue extracts and standards were filtered using a membrane filter GS 0.22 µm (Millipore). The peak identification of GSH and GSSG in the tissue sample was made on the basis of the retention times relative to the internal standard. The standard addition method was also used to determine peak identities.

Quantitation of GSH and GSSG in the samples was achieved by comparison with a standard curve obtained by derivatization and HPLC analysis of known amounts of GSH (5–40 µM) and GSSG (1–20 µM), in the presence of a fixed amount of the internal standard. The curves were linear (r = 0.999) from 1 to 40 µM GSH or GSSG The inter-assay CV for GSH determination (n = 5) at 5 and 40 µM were 2.8 and 2.75%, respectively. For GSSG determinations (n = 5) inter-assay CV at 1 and 20 µM were 3.0 and 1.9%, respectively.

Statistical Analysis

Comparisons between the results obtained with different assay methods were made by one-way

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analysis of variance (ANOVA) using the general linear model (GLM) procedure of the statistical analysis system [SAS (1988) Release 6.03. SAS Institute, Cary, NC, USA]. In the case of significant differences (α:0.05), the ANOVA was followed by the Bonferroni statistical test.

Materials

The kit of the GSH-400® method was from Bioxytech (Bonneuil/Marne Cedex, France). OPT, GSH, GSSG, EDTA, NEM, DTNB, NADPH, BPDS and glutathione-reductase were from Sigma (St. Louis, MO, USA). 2-Vinylpyridine and γ-glutamyl-glutamic acid were from Aldrich Chimica (Milan, Italy). All chemicals used for HPLC analysis were dissolved in highly purified "MilliQ" water. Methanol was of a HPLC grade.

RESULTS

Table I shows the GSH and GSSG levels determined in extracts from guinea-pig heart and liver using the fluorometric method of Hissin and Hilf,[6] the enzymatic method of Tietze[5] as modified by Anderson,[11] the GSH-400® Bioxytech method (not capable of detecting oxidized glutathione), and the HPLC method.[10] In the heart, all methods measured almost identical levels of GSH, whereas the level of GSSG determined by the fluorometric method was almost 5 times higher than those measured enzymatically and by the HPLC technique. In the liver, a good correspondence was found between the determinations of GSH by the patented kit, the enzymatic and the HPLC methods. By contrast, the fluorometric assay gave GSH levels about half as high as those measured by the other three methods. As with the results obtained from heart, in liver too the GSSG content measured by the fluorometric method was much higher than those determined by the enzymatic and HPLC techniques. As discussed below, overestimation of GSSG by the fluorometric method has already been observed by Beutler and West.[13] In an attempt to explain the underestimation of liver GSH, experiments were carried out with varying amounts of the 105,000 × *g* supernatant used for the GSH assay. The results reported in Table II show that the total amount of GSH detected by the fluorometric method did not increase proportionally with the volume of the sample used, although the calibration curve remained linear throughout the relevant concentration range. As a consequence, the GSH level, expressed as μmoles per g of wet tissue, decreased by about 40%.

DISCUSSION

Measurement of GSH and GSSG levels is essential for studies aimed at ascertaining oxidative stress produced by drugs or pathological conditions. Therefore, it is particularly important to

TABLE I GSH and GSSG contents of guinea-pig heart and liver

Assay Method	Heart		Liver	
	GSH	GSSG	GSH	GSSG
GSH-400®	1.157 ± 0.010	ND	7.80 ± 0.604	ND
Enzymatic	1.213 ± 0.102	0.046 ± 0.006	6.80 ± 0.314	0.044 ± 0.007
Fluorometric	1.178 ± 0.111	0.230 ± 0.042[a]	3.07 ± 0.353[a]	0.324 ± 0.091[a]
HPLC	1.260 ± 0.168	0.048 ± 0.001	6.51 ± 0.360	0.041 ± 0.005

Data are expressed as μmoles/g wet tissue. Experimental conditions are reported in Materials and Methods. Results are means ± SD from 10 determinations carried out in duplicate on 10 different heart or liver specimens. In the case of HPLC assay, the determinations were carried out on 5 different heart or liver specimens.
ND = not determinable [a] $p < 0.001$ *vs.* results obtained by other assay methods. Other differences were not statistically significant.

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TABLE II Effect of increasing supernatant amount on fluorometric determination of GSH in guinea-pig liver extracts

Amount of supernatant (ml)	Total GSH nmoles	μmoles GSH/g wet tissue
0.1	0.516 ± 0.024	4.08 ± 0.195
0.2	0.961 ± 0.006	3.80 ± 0.030
0.3	1.153 ± 0.042	3.04 ± 0.110
0.4	1.431 ± 0.047	2.83 ± 0.080
0.5	1.592 ± 0.009	2.51 ± 0.015

300 mg of liver were homogenized in 1 ml 25% metaphosphoric acid plus 3.75 ml 0.1M sodium phosphate-5mM EDTA (pH 8.0). Homogenate was centrifuged at 105000*g* for 30 min at 4°C and various amounts of supernatant, diluted to 5 ml with the phosphate-EDTA buffer, were assayed for GSH content, as reported in Materials and Methods. Results are means ± SD from three separate determinations.

have a rapid, easy and sensitive method. In this study, we compared three commonly used rapid methods, one enzymatic, one fluorometric and a newly patented colorimetric method (GSH-400®, Bioxytech, France), by determining reduced and oxidized glutathione in both guinea-pig heart and liver. The results of these methods were also compared with those obtained by the HPLC technique, which is considered the standard assay method.

The enzymatic method used was that originally described by Tietze[5] as modified by Anderson.[11] The fluorometric method was that proposed by Hissin and Hilf[6] in 1976. The GSH-400® (Bioxytech) method is a newly patented colorimetric procedure which is, however, limited by its inability to detect the oxidized form of glutathione. Since GSH is the predominant form of total tissue glutathione in physiological conditions (>99%), its measurement is in most cases sufficient. However, in the presence of oxidative stress, the GSH/GSSG ratio alters, so that this method is not applicable when such an alteration is suspected.

This study shows that the performances of the three rapid methods used for GSH determination differ according to the tissue in which measurements are carried out. In heart, all methods detect the same amount of GSH. By contrast, the present results raise some doubts about the reliability of the fluorometric method for GSH detection in liver. We followed in every detail the protocol suggested by Hissin and Hilf[6] for the extraction of hepatic tissue, carefully checking the pH of the media which, according to the authors, is the most critical parameter. Nevertheless, the liver GSH level, as measured by the fluorometric method, was found to be only about half as high as that measured by the other two rapid methods. These results were validated by the HPLC technique which also measured a GSH content twice as high as that determined by the fluorometric assay. The finding that the liver GSH level measured by the fluorometric method decreases as the amount of the assayed extract is increased suggests that a soluble quenching substance is responsible for the poor performance of this analytical assay. Because of its sensitivity, the fluorometric method is still widely used for the determination of glutathione in various tissues.[14,15] However, our observations suggest that the data obtained by means of the fluorometric technique must be taken with caution unless this method has preliminarily been validated with the tissue of interest.

The present results also demonstrate that markedly different levels of GSSG are measured by the fluorometric and enzymatic methods in both heart and liver. By contrast, very similar GSSG values were detected by the enzymatic and HPLC methods. In guinea-pig liver, the GSSG level detected by the fluorometric method is almost 8 times as high as that measured by either the enzymatic or the HPLC procedure, while in heart it is about 5 times higher. Considering the normal physiological conditions of the livers and hearts used for these assays, the GSSG levels measured by the fluorometric method (9 and 16% of total glutathione in liver and heart, respectively) do not appear plausible. Our results are in excellent agreement with those of Beutler and West[13] who, following the protocol of Hissin and Hilf,[6] found GSSG levels in rat liver supernatants 8 times as high as those measured by the enzymatic assay. The former authors demonstrated the pres-

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ence in rat liver extracts of an OPT-reacting material which may be separated from real GSSG by Dowex-1 chromatography. However, the need to treat samples by chromatographic techniques before the GSSG assay drastically limits the usefulness of the fluorometric assay.

In conclusion, our results confirm previous doubts[13] on the use of the fluorometric technique for GSSG detection in mammalian tissues, and also raise some questions about its general applicability for measuring GSH, since tissue-specific interferences may be encountered. Although easy to perform and reliable, the colorimetric GSH-400® method is limited by its inability to determine GSSG and may therefore be usefully applied only when negligible levels of oxidized glutathione are present. Of the three rapid methods considered, only the enzymatic assay appears to be of general applicability since it offers a reliable determination of both GSH and GSSG. Therefore, it can be considered a valid alternative to the much more time-consuming HPLC technique.

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