Methods Compared for Determining Glutathione Peroxidase Activity in Blood
Bahram Faraji, Han K. Kang,¹ and Jane L. Valentine²

We compared four methods for determination of glutathione peroxidase (EC 1.11.1.9, glutathione:hydrogen-peroxide oxidoreductase) in human blood can be measured "directly," by determining the amount of substrate remaining, or "indirectly," by measuring the production of product. In the substrate assay the amount of unconsumed reduced glutathione (GSH) remaining at specific time intervals is determined in the presence of small amounts of peroxide. In the product assay the rate of glutathione oxidation by peroxide is measured by monitoring the decrease of NADPH as oxidized glutathione (GSSG) is converted to GSH. The overall reaction mechanism for both assay types is as follows:

\[ 2 \text{GSH} + \text{H}_2\text{O} \xrightarrow{\text{GSH-Px}} \text{H}_2\text{O} + \text{GSSG} \xrightarrow{\text{GSSG-R}} 2 \text{GSH} \]

Both types of assay have been used to assess the relationships of GSH-Px activity to certain hemolytic diseases (1-5) and to the selenium status of humans (6-9), and to assess the role the enzyme may play in the biochemical adaptation of animals against oxidative damage (10, 11).

Researchers have commented on the suitability of both types of method. Emerson et al. (5) contend that assay of the product is superior in all respects, with reproducibility being excellent as compared with the 50% variability with the substrate assay. Flohe and Brand (12) also reported on the specificity and reliability of the product measurements. Except for the limited report of Emerson et al. (5), no extensive evaluation has been made by a single laboratory to compare the two types of assay or to relate the values obtained with them.

In the studies cited above, various approaches were used to make these measurements. Intercomparison of the results of the studies are hampered by slight differences in the pH, temperature, buffer conditions, and enzyme activity used (13).

To remedy this situation, we assayed blood samples from 52 healthy volunteers by both kinds of techniques. We evaluated the substrate measurement by using the methods of Groes et al. (4) and Hafenman et al. (14). The assay products were measured by the methods of Paglia and Valentine (15) and Thomson et al. (7). We sought to assess the degree of precision and reproducibility of each method, and the inter-method correlation of results, and to determine normal reference intervals for each of the four methods.

Materials and Methods

Equipment

GSH-Px activity was determined with a Model 8451A "diode array" spectrophotometer, which includes the operating system of the HP85A personal computer with a built-in cathode-ray terminal and printer/plotter (all from Hewlett-Packard, Fullerton, CA 92631). Erythrocytes and hemoglobin were determined with a "Coulter S plus IV with QC" (Coulter Electronics, Inc., Hialeah, FL 33010).

Venous blood was collected in blue-top Vacutainer Tubes containing sodium heparin as anticoagulant (Becton Dickinson and Co., Rutherford, NJ 07071).

Reagents

For the enzyme assays we used glutathione reductase (EC 1.6.4.2), Type III, purified from yeast by ammonium sulfate fractionation (150 kU/g protein); reduced glutathione (GSH); nicotinamide adenine dinucleotide phosphate, reduced form (NADPH); and t-butyl hydroperoxide (all from Sigma Chemical Co., St. Louis, MO 63178); hydrogen peroxide, 300 g/L; sodium azide; K₂Fe(CN)₆; and KCN (all from Fisher Scientific Co., Fair Lawn, NJ 07410); met phosphoric acid; Triton X-100 surfactant; Na₂EDTA; sodium bicarbonate; sodium chloride; sodium phosphate, dibasic; and sodium phosphate, monobasic (all from J. T. Baker Chemical Co., Phillipsburg, NJ 08865); tri-sodium citrate (Malnickrodt, Inc., St. Louis, MO 63147); and 5,5'-dithiobis-(2-nitrobenzoic acid) (Aldrich Chemical Co., Inc, Milwaukee, WI 53233).

Methods

GSH-Px activity determined by measuring consumed NADPH. The two methods we used here (7, 15) are essentially the same except for the type of peroxide used and the amounts of reagents relative to hemolyte. The specifics of each method are detailed for comparison purposes.

Method I. This procedure involves an initial cell wash: mix 1 mL of whole blood with 1 mL of cold (4°C) isotonic saline (NaCl, 9 g/L) and centrifuge at 1000 x g for 5 min. Repeat this procedure three times and dilute the final wash to 2 mL with isotonic saline.

¹Present address: VA Office of Environmental Epidemiology, Armed Forces Institute of Pathology, Washington, DC 20306.
²Author to whom correspondence should be addressed.
³Nonstandard abbreviations: GSH-Px, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GSSG-R, glutathione reductase; Hb, hemoglobin.

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To prepare lysate samples, add 0.4 mL of water to 0.1 mL of the cell suspension. Freeze and thaw the mixture three times in a solid CO2/ethanol mixture, prepare the sample and measure enzyme activity by the procedure of Paglia and Valentine (15). Record the conversion of NADPH to NADP+ by monitoring absorbance at 340 nm between 2 and 4 min after initiating the reaction. Subtract nonenzymatic oxidation from the hemolysate determination to obtain the true enzyme activity, reported as micromoles of NADPH per minute per 10^10 cells or per gram of hemoglobin (Hb).

Method II: Prepare hemolysate for the enzyme assay by diluting 0.5 mL of blood to 1.0 mL with isotonic saline solution. Follow the method of Thomson et al. (7) exactly, adding a final hemolysate volume of 0.3 mL to an incubation mixture of 2.7 mL. Allow this solution to equilibrate at 25°C for 10–15 min, then initiate and measure the enzymatic and nonenzymatic oxidation of GSH as described in Method I, but substitute 10 μL of 30 mmol/L tert-butyl hydroperoxide solution for 0.1 mL of 2.2 mmol/L H2O2 solution. Results are reported as micromoles of NADPH oxidized per minute per gram of Hb.

GSH-Px activity determined by measuring unconsumed GSH. The direct measurement of unconsumed GSH remaining after incubation with hemolysate (without the enzyme) was performed by two methods, those of Gross et al. (4) and Hafeman et al. (14). These two methods differ basically in the relative amount of hemolysate to reagents in the incubation mixture. The specifics of each are given briefly below.

Method III. In this procedure, wash the cells three times, each time mixing 3 mL of isotonic saline with 0.6 mL of whole blood. After the third wash, dilute the cells to 3 mL with de-ionized water. Prepare the incubation mixture and determine the enzymatic and non-enzymatic oxidation by hydrogen peroxide exactly as described by Gross et al. (4). Measure spectrophotometrically at 410 nm the formation of the yellow color from the addition of 5,5′-dithiobis-(2-nitrobenzoic acid) reagent to the supernate containing the remaining GSH. Calculate GSH-Px activity in terms of the first-order reaction rate constant (k) per gram of Hb per minute, k being defined as

\[ K = \frac{2.3(t_2 - t_1)}{\log \text{[GSH at } t_1 \text{ (1 min)]/GSH at } t_2 \text{ (3 min)}} \]

Method IV. Take 1 mL of fresh whole blood, wash it three times with 3-mL portions of buffered isotonic saline (pH 7.4), then dilute to 3 mL with distilled de-ionized water. For the incubation mixture, mix 0.1 mL of hemolysate (prepared as above) with the reagents as specified by Hafeman et al. (14). Initiate the enzymatic reaction by adding hydrogen peroxide, then determine the GSH remaining in the protein-free supernate and measure the nonenzymatic GSH oxidation (in a sample with water substituted for hemolysate). Express enzyme activity as a decrease in log GSH of 0.001 per minute per milligram of Hb after subtracting the decrease in log GSH per minute of the nonenzymatic reaction.

Subjects

Blood was sampled from 52 healthy volunteers (UCLA faculty, staff, students, and their relatives). Each subject was queried as to medication and vitamin and mineral (especially selenium) usage. Information on age and area of residence was also taken. Ages of the subjects ranged from 20 to 75 years, with a mean of 32 years (60% of the participants were younger than 35). There was no undue exposure to selenium or other substance that could affect the activity of GSH-Px. We assayed one sample of blood from each participant for GSH-Px activity by each of the four methods.

Results

Precision

Within-run evaluations. We evaluated the repeatability of each of the four methods by analyzing blood from one subject, whose erythrocyte count, hemoglobin content, and hematocrit were \(5.81 \times 10^{12}/\text{mm}^3\), 176 g/L, and 50.5%, respectively. The results are given in Table 1. The values for enzyme activity were different by each method owing to differences in activity definition and reagents used. We performed this assessment with one blood sample so we could compare enzyme activity units among the methods.

Within-run coefficients of variation (CVs) for Methods I and II were 5.6% and 5.0%, respectively. In contrast, the within-run CVs for direct measurement of the amount of GSH remaining were 20.7% and 28.1% for Methods III and IV, respectively.

Methods III and IV utilize log transformations in the calculations for expressing enzyme units. Changes in precision could result when comparing these with linear expressions. To compensate for such possible differences, data for these two methods were computed in the same units as Methods I and II, micromoles of NADPH oxidized per minute per gram of hemoglobin. The relationship of GSH to NADPH utilization in the presence of the enzyme was used for the conversion. Table 2 presents these data for the

<table>
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<th>Method and expressions of enzyme units</th>
<th>Within-run (n = 10)</th>
<th>Between-run (n = 4)</th>
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<tr>
<td>Mean CV, %</td>
<td>Mean CV, %</td>
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<tr>
<td>Method I (15) µmol of NADPH oxidized per minute per 10^10 cells</td>
<td>6.2</td>
<td>5.6</td>
</tr>
<tr>
<td>Method II (7) µmol of NADPH oxidized per minute per gram of Hb</td>
<td>10.2</td>
<td>5.0</td>
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<tr>
<td>Method III (4) K'g of Hb*</td>
<td>47.7</td>
<td>20.7</td>
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<tr>
<td>Method IV (14) (log [GSH]/min) × 0.001 × mg of Hb</td>
<td>8.9</td>
<td>28.1</td>
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*See equation in text.

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<th>Method I</th>
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\[ X = 20.49 \]
\[ \bar{X} = 10.10 \]
\[ \bar{X} = 6.34 \]
\[ \bar{X} = 7.55 \]
\[ s = \pm 1.17 \]
\[ s = \pm 0.50 \]
\[ s = \pm 4.28 \]
\[ s = \pm 2.06 \]
\[ CV = 5.7\% \]
\[ CV = 4.9\% \]
\[ CV = 67.4\% \]
\[ CV = 27.3\% \]
within-run evaluation. Precision (CV) estimates on the now-transformed data yielded values of 67.4% and 27.3% for methods III and IV, respectively. A significant change in precision was found in the transformation of values produced by Method III, with it becoming the most imprecise. No significant change resulted from data transformation in the precision estimate of Method IV.

**Between-run evaluations.** We used blood from another subject for the between-run comparisons, determining GSH-Px activity four times by each method over a period of 17 days.

The CVs for this evaluation were computed without putting all data into the same units, using the log-transformed data as suggested for Methods III and IV. The CVs for this evaluation showed Method II to have the best between-run reproducibility (Table 1). Method IV was the least reproducible of the four methods.

We made precision evaluations when data from all four methods were expressed as micromoles of NADPH oxidized per minute per gram of hemoglobin. CVs of 8.1%, 5.5%, 24.7%, and 38.1% were produced, respectively, for Methods I, II, III, and IV.

**Comparison of Methods**

The means (±SD) for erythrocyte counts, hemoglobin concentrations, and hematocrit values for 52 subjects were 4.87 (±0.47) × 10⁷/mm³, 147.4 (±14.5) g/L, and 43.2 (±3.85)%, respectively—all within normal reference intervals (16).

GSH-Px activities as determined by the four methods are presented in Table 3. With few exceptions, the 52 subjects had mean activities that agreed with those in prior publications when compared with those for which the same respective method was used (Table 3). Where differences were observed, we could deduce no explanation except those suggested by the New Zealand researchers. New Zealand residents are known to have diets low in selenium, which may be reflected in their values for GSH-Px activities. Thus the values of Thomson et al. (7) are lower, possibly because of a lower dietary intake of selenium in New Zealand than in the U.S.

Using Method I as the reference method (this being the procedure of choice of most researchers), we compared the correlations of GSH-Px activity as measured by each of the other three methods (Figure 1). Linear regression of results from Methods II, III, and IV with those of Method I yielded correlation coefficients of 0.455, 0.457, and −0.035, respectively. These coefficients were significant (p < 0.001) for each comparison, except for Method IV vs Method I, which produced a nonsignificant correlation coefficient (p > 0.05).

The technique found to be most precise, even though it is not used by most researchers, was Method II. Linear regression of the other three methods vs Method II yielded correlation coefficients of 0.455, 0.890, and 0.560 for Methods I, III, and IV, respectively, all of which were significant (p < 0.001).

We computed enzyme activity for the 52 persons in terms of micromoles of NADPH oxidized per minute per gram of hemoglobin for all four methods. Mean (and SD) values of 23.76 (7.36), 14.30 (4.00), 6.60 (1.31), and 13.60 (3.36) were obtained for Methods I, II, III, and IV, respectively. As was observed for the within-run evaluations, the enzyme activity determined by each method differed. However, the relationship between the values produced by each method remained essentially the same. Correlation coefficients of 0.560, 0.447, and −0.007 were produced for the respective methods II, III, and IV when each was compared with Method I. When Method II was used as the reference, correlation coefficients of 0.560, 0.710, and 0.510 were obtained for Methods I, III, and IV (all correlations being significant, p < 0.001).

**Discussion**

Measurement of GSH-Px activity has potential clinical utility in assessing the selenium status of normal and diseased populations. Decreased activity of the enzyme has been associated with selenium deficiency in such conditions as prolonged parenteral nutrition (21, 22, 25), Keshan's disease (26), cancer (20, 22), multiple sclerosis (17), hemolytic disease of the newborn (1), and phenylketonuria (19, 27). Supplementation with various forms of selenium, such as selenomethionine, sodium selenite, and selenium-rich yeast,
We compared the utility of the methods that are most often cited in the literature. The product-based assays proved most nearly precise, with Method II giving the smallest CV. We attribute the lack of precision for the substrate assay to the quenching step (transfer to metaphosphoric acid solution followed by centrifugation and filtration) and the lack of stability of the yellow color formed with 5,5'-dithiobis-(2-nitrobenzoic acid) and GSH. Besides these drawbacks to measuring the amount of GSH remaining, both methods (III and IV) based on this measurement technique showed variations in precision when compared with each other—Method III having much better precision than Method IV for log transformed data. When the data were converted to the same units for all methods, Method III proved to be the most imprecise.

Few attempts have been made to compare methods for assaying GSH-Px activity. Emerson et al. (5), in their comparison of the two assay types, did not present values of enzyme activity by both procedures when the direct assay proved less suitable. They did, however, comment on the difficulties with sensitivity and reproducibility of the direct procedure and on the advantage of a continuous-reading process for NADPH conversion to NADP⁺ in the coupled assay. Other researchers have also commented on the inferiority of the substrate assay (1, 12, 29).

We have found that the method most often used to determine glutathione peroxidase activity, that of Paglia and Valentine (15, Method I), does not give the highest correlations with all the other types of methods. In contrast, the method of Thomson et al. (7, Method II) seems to produce results that correlate best with those by all the other methods, and also has the best precision.

Enzyme activity units by the two assay types have not previously been published. Levander (30) noted the impossibility of citing typical values for GSH-Px activity because of the lack of standardization of measurement, making inter-laboratory comparisons of results difficult. We have found that the quantity of enzyme activity measured by the four methods differs. When the values are expressed in terms of micromoles of NADPH oxidized per minute per gram of hemoglobin, Method I was found to give activity values at least twice those produced by the other three methods. We attribute such differences to the variation in preparation of hemolysate (i.e., freezing and thawing vs water addition only) and to the variability of temperature used for incubation or equilibration. Evaluation of such effects is needed before a definite statement can be made.

Our determination of GSH-Px activity for a single blood sample by within-run analysis, our between-run evaluation, and our analysis of the enzyme activity on blood from 52 normal subjects allow comparison of previously published methods for suitability. In the present study we also evaluated the normal values for enzyme activity as determined with those 52 apparently normal subjects, defining each normal value by each of the four methods. These data should be useful for researchers interested in evaluating GSH-Px activities of nondiseased populations.

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