individuals. The extent or clinical relevance of F2 formation in unhealthy individuals is unclear.

In this study population, which included both men and women, F1.2 concentrations appear to significantly increase with subject age >44 years. Although the hockey-stick model used is only empirical and other models, e.g., nonlinear, may fit the data equally well, this result substantiates a previous study in which the concentrations of F1.2 plus F2 were shown to increase with age in healthy men >42 years old (6). Our results also suggest that the trend is not sex-specific. Because F1.2 is a biomarker of thrombin generation, the question remains as to whether increased thrombin formation associated with aging reflects subclinical vascular or hemostatic changes of concern in overtly healthy individuals.

F1.2 may be useful as a thrombotic marker, and the mean concentration reported for patients with documented deep venous thrombosis is 6.2 (SD 4.3) nmol/L (2). We measured F1.2 concentrations as great as 9.0 and 20.1 nmol/L in two presumably healthy donors, the former value being from a 17-year-old donor. Although in vitro thrombin generation associated with nonroutine venipuncture of these donors cannot be ruled out, the high F1.2 concentrations may again reflect increased in vivo thrombin generation associated with subclinical vascular or hemostatic conditions of potential concern. Possibly, a single F1.2 reference interval for all ages may be useful for identifying subclinically diseased individuals; however, until the clinical relevance of increased thrombin generation during aging is understood, age-matched F1.2 reference intervals may be important for evaluating the role of F1.2 in assessing thrombotic risk and monitoring anticoagulation therapy.

References


Automated Spectrophotometric Method for Determining Oxidized and Reduced Glutathione in Liver


An enzymatic recycling method has been applied to the measurement of total and oxidized glutathione with a centrifugal analyzer. When the reduced form of glutathione (GSH) was masked with 2-vinylpyridine to measure the oxidized glutathione (GSSG), the time to ensure full derivatization was three times longer than has been reported. The method is quick, simple, accurate, and precise (1.27% for GSH, 3.3% for GSSG, 2.15% for GSH, 5% for GSSG), and the automation allows large numbers of samples to be conveniently assayed.

Glutathione is a tripeptide widely distributed in nearly all mammalian cells. An important antioxidant, it protects cells against damage from free radicals and toxic endogenous and exogenous compounds either by reacting directly with these toxins or by facilitating the reduction of protein disulfide bridges (1). Concentrations of total glutathione therefore give an indication of the intracellular oxidative state.

One of the antioxidant properties of glutathione is mediated by the enzyme glutathione peroxidase (EC 1.11.1.9). During the detoxification of lipid and other peroxides produced by free radical attack, glutathione peroxidase converts glutathione from a reduced state (GSH) to an oxidized one (GSSG).2 The NADPH-dependent

2 Nonstandard abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; 2-VF, 2-vinylpyridine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); and NEM, N-ethylmaleimide.
dent enzyme glutathione reductase (GR, EC 1.6.4.2) converts GSSG back to GSH, and so almost all intracellular glutathione is reduced. During an oxidative stress, there will be flux of glutathione to the oxidized form, and the ratio of reduced to oxidized glutathione may then be an indication of this stress (7). GSH may also exist in another oxidized form, as mixed disulfides with protein thiols. Thiol–sulfide exchange reactions between GSSG and protein thiols are potentially important in biological samples, causing artifacts in measurement of glutathione. Thus, there is a need for a sensitive and reliable assay for both forms of glutathione.

Glutathione has been determined by several colorimetric, chromatographic, and enzymatic procedures. Sulphydryls can be determined in protein-free tissue extracts by using Ellman's reagent (2), giving a rapid but nonspecific measure of total glutathione. Fluorometric determination of GSH with o-phthalaldehyde is a sensitive method (3) but of uncertain specificity, and the use of o-phthalaldehyde at high pH (4) to determine GSSG does not give accurate results (5). High-performance liquid chromatography methods are sensitive, but laborious and very time consuming (6). Enzymatic methods based on GR (7, 8), however, are simple, sensitive, and specific. The disadvantage of the GR method of Tietze (8) is that it is laborious and time consuming for large numbers of samples. Because glutathione is unstable, any assay system should be as rapid as possible. We have therefore applied an enzymatic recycling procedure (1) to a Cobas Fara II centrifugal analyzer, and demonstrate the superior precision and accuracy of this automated method.

Materials and Methods

Reagents. GSH, GSSG, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, GR, and picric acid were obtained from Sigma Chemical Co. (Poole, Dorset, UK). 2-Vinylpyridine (2-VP) was obtained from Aldrich Chemical Co. (Gillingham, Kent, UK) and stored at −20 °C. Glutathione standard was prepared and diluted in water to provide two concentration ranges, 1–5 and 10–50 μmol/L. Standards were prepared in water containing picric acid such that the concentration of picric acid in the final reaction mixture was the same as for the diluted biological sample supernates (1.75 mmol/L for GSH, 0.35 mmol/L for GSSG). NADPH (0.3 mmol/L) was dissolved in 0.125 mol/L sodium phosphate buffer (pH 7.5) containing 6.3 mmol/L sodium EDTA. DTNB, 6.0 mmol/L, and GR solution (1 U/100 μL) were also prepared in this buffer. Chemical blanks containing picric acid only were prepared.

Automated analysis. The centrifugal analyzer pipettes 210 μL of NADPH reagent, 30 μL of DTNB reagent, and 30 μL of sample or standard into separate chambers of each of 30 separate rotor cuvettes, which are then spun to mix the contents and incubated at 30 °C for 5 min. Then 30 μL of GR is added, and the reaction monitored after 30 s with readings every 5 s for 5 min. For GSSG, for which the reaction time is slower, initial readings were delayed for 1 min, with readings then taken every 15 s for 7 min so that in both assays the reaction was monitored over the linear part of the curve. A standard plot was prepared from the standards and displayed to exclude errors in the automatization. If required, the change in absorbance with time for a particular sample was also rapidly assessed to ensure a straight line. The instrument then constructs a calibration curve by assaysing the glutathione standards; from the changes in absorbance over time for known concentrations, the concentrations of unknown solutions are calculated.

Analysis of biological samples. For GSH determination, 100-μL supernates in duplicate were added to 9.9 mL of water. For GSSG, 200-μL supernates in duplicate were added to 3.78 mL of water to which 40 μL of 2-VP was then added to mask the GSH. The vials were then left at room temperature—for 1 h initially, 3 h in later experiments.

Preparation of quality controls. Experiments were performed to test precision by repeated assay of biological quality-control samples. The liver of a 350-g male Wistar rat (Charles River, Margate, Kent, UK) that had been fed food and water ad libitum was homogenized with a Teflon pestle in 43 mmol/L picric acid, 5 mL of picric acid reagent per gram of tissue, within 4 min of death by cervical dislocation to prevent post-mortem oxidation of GSH. The homogenate was centrifuged (2500 × g at 4 °C for 15 min) and the supernate was divided into aliquots stored at −70 °C for use as a biological quality-control sample for determining interassay variation. Intraassay precision was tested by analyzing duplicates of two quality-control samples for each assay run.

Measurement of known standards. To test the ability of the assay to distinguish between oxidized and reduced glutathione, we made up solutions containing known concentrations of mixtures of GSH and GSSG, and added these to biological quality-control samples. The recovery of GSSG was initially erratic when the 2-VP was added 1 h before the assay, so we performed a further set of experiments to study the effect of adding different concentrations of 2-VP to standards containing 1.643 mmol/L GSH and 0.03 mmol/L GSSG (concentrations similar to those quoted for rat liver (1)). The incubation time with 2-VP was also prolonged from 1 to 3 h, and aliquots from the incubation mixture were assayed each hour.

Results

Validation of automated analysis. Interassay precision of the method as measured by the coefficient of variation of duplicates (9) was 1.27% for GSH and 3.3% for GSSG. Intraassay precision, calculated from the variation in the measurement of the biological quality-control sample, was 2.15% for GSH and 5% for GSSG (Table 1).

Validation of measurement of reduced and oxidized glutathione. Recovery of GSSG was high in initial experiments in which 2-VP was left for 1 h at room temperature (GSSG runs 1–4, Table 1). This was in keeping with the method of Griffith (7), who suggested
that GSH is fully derivatized after 60 min. However, we found it necessary to leave the samples for 180 min for full derivatization (Table 2). Indeed, at 60 min of incubation with 40 μL of 2-VP, the samples assayed could provide >100% error. At 120 min, there was still a potential error of 7%, but at 180 min, this potential was removed. Increased concentrations of 2-VP gave large underestimates of the concentrations of the known standards, because the GSH formed by the enzymatic recycling procedure can react with the excess 2-VP, although the rate of reaction is slow. When the GSSG assay conditions were optimized (in runs 5–8, Table 1) and the samples allowed to stand for 180 min after 40 μL of 2-VP was added, the percentage recoveries indicated the assays were far more accurate.

Accuracy, determined from measurements of standards of known concentrations added to biological quality-control samples, is demonstrated in Table 3. Accuracy is largely determined by the quality of the GSH used for the standards, and most errors occur during sample and standard preparation. Accuracy in the biological specimens, particularly of the low-GSSG pool, requires that the pools of GSH and GSSG do not become depleted, as may rapidly occur immediately post-mortem during hypoxia. The time between death and tissue homogenization in picric acid should therefore be minimized. Accuracy ranged from 96% to 101.5% for GSH and from 99% to 102.6% for GSSG, once the 2-VP was allowed to fully derivatize the samples for 3 h.

**Discussion**

We chose to automate the method of Griffith (7) with a frequently used method utilizing 2-VP to derivatize GSH. We preferred this to methods using N-ethylmaleimide (NEM) (10, 11) because, although NEM reacts with thiols and protein cystinyl residues to form adducts, it is not specific for thiols (12, 13), but also forms adducts with sulfite, thiosulfate, the ε-amino group of lysine residues, and the imidazole of histidine. Moreover, the adducts formed between NEM and glutathione are not stable in aqueous solution (12). In addition, methods involving NEM often require the later extraction of NEM by time-consuming, laborious techniques.

Standard methods as described by Griffith (7) are also laborious and time consuming. Even with use of a standard laboratory spectrophotometer with capacity for automated readings of cells in series, only four to six samples can be analyzed at any one time, whereas a Cobas Fara II centrifugal analyzer can analyze 30 samples at the same time. In the manual methods, the DTNB, NADPH, and sample are all added to the spectrophotometer cuvette and warmed to 30 °C. During this time, some reduction of DTNB occurs, even in the absence of glutathione. The GR is then added to each cuvette, and readings began after addition to the last cuvette, namely, at 60 s. This procedure requires a deftness of hand, and often results in inadequate mixing of the GR with the rest of the contents of the cuvette; fluctuations in the rates of reaction thereby occur. In the automated assay, however, standards and samples are placed in the same batch so that all are treated equally and simultaneously, thus minimizing differences within the assay.

Our automated method compares favorably with that of Hisin and Hilf (4), who quote a 91% recovery for GSH and 93–102% for GSSG. Our recoveries for GSSG were initially greater than expected, but extending the duration of the derivitization to 3 h eliminated this error.
In conclusion, using the Cobas Fara II centrifugal analyzer, we produced a method that is quick, reliable, precise, and accurate and can be applied to technology that is available in most laboratories. The rapidity of the method allows large numbers of samples to be quickly processed, preventing degeneration of glutathione in storage in tissue samples that have high y-glutamyltransferase contents, an enzyme that degrades glutathione.

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References