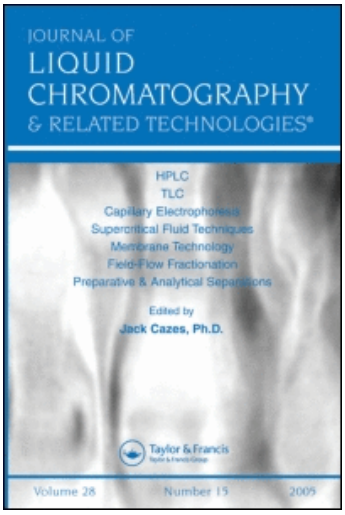

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REDUCED AND OXIDIZED GLUTATHIONE RATIO IN TUMOR CELLS: COMPARISON OF TWO MEASUREMENT METHODS USING HPLC AND ELECTROCHEMICAL DETECTION

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ABSTRACT

Two chromatographic methods for the assay of oxidized and reduced glutathione in cells have been compared in which GSH is either derivatized with OPA in an electrochemically active isoindole compound or directly measured using gold mercury amalgam electrodes specific for thiol. The first method requires a derivatization but gives efficient HPLC separation of the so-formed isoindole derivative, while in the other method, separation of the highly polar compounds requires an ion pairing eluent. Both methods allow measurement of the GSH/GSSG ratio in tumor cells.

INTRODUCTION

Tripeptide glutathione (γ-Glu-Cys-Gly, GSH) is the major free thiol in most living cells and participates in diverse biological processes such as : detoxication of xenobiotics, removal of hydroperoxides, protection against the effects of ionizing radiation, maintenance of the sulfhydryl status of proteins and of the enzyme activity by disulfide interchange (1).

Anthracyclines (Adr) are among the most important drugs in the treatment of acute leukemia and other hematological and solid malignancies, but their effectiveness may often be limited by the emergence of intrinsic or acquired drug-resistant cells. Anthracycline resistance may be associated with cross-resistance to structurally unrelated compounds, known as multidrug resistance MDR, decreased intracellular drug accumulation, increased drug degradation, alterations in the structure or activity of topoisomerase II and changes in the pattern of intracellular drug distribution (2). A characteristic feature of MDR cells is overexpression or an increased amount of a membrane glycoprotein termed P 170, but also alterations in certain enzymatic activities among which are the GSH-related enzymes.

The role of the redox cycle, an important pathway in the detoxification of reactive oxygen species, was demonstrated in cells with acquired multidrug resistance phenotype and in cells with *de novo* resistance. Studies of Batist et al., (3) showed that a 45 fold increase in glutathione S-transferase (GST) activity occurs in MCF/Adr cells and that they express a specific anionic isoenzyme of GST which presumably possess high levels of intrinsic non-selenium dependent glutathione peroxidase activity (GSH Px) (3). The Adr resistance correlated well with an increase in the GSH-PX activity, but the concentration of GSH was found either to decrease in some MDR-tumor cells (2, 4-5) or to increase in other ones (5, 6-12).

SH donors (cysteamine, N-acetyl-cysteine) or GSH depletion by diamide, diethyl maleate, buthionine sulfoximine (BSO), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or *tert*-butyl hydroperoxide (tBH) alter the glutathione redox cycle and the Adr cytotoxicity. These results demonstrate the importance of glutathione utilizing enzymes GST and GSH-Px, as well as the associated GSH redox capacity, as biochemical mechanisms contributing to Adr resistance.

In every assay, comparing sensitive tumor cells with resistant tumor cells, treated or not with drugs that alter GSH levels, GSH levels were measured but, there was little data on the GSH/GSSG ratio (5). Prior to beginning a study on MDR cells and evaluating their GSH redox state, it is worth measuring the content of GSH and GSSG in cells where GSH concentrations are weak compared to heart or liver cells. Reduced glutathione was most often assayed in cells by the enzymatic recycling method of Tietze (13), with the modification of Brehe and Burch (14), Eyer and Podhradsky (15), Griffith (16) or Akerboom and Sies (17). Other methods consisted of UV or fluorescent measurements with or without HPLC, using Sanger's reagent : 1-fluoro-2,4-dinitrobenzene (DNB) (18-20), the OPA reagent

(21-23) or the fluorescent monobromobimane reagent of Kosower et al., (24). Although GSSG measurement was possible with these methods, GSSG levels were only given by Kramer et al., (5).

We chose to test two methods of dosage of GSH and GSSG involving an HPLC separation to bring specificity, and an electrochemical detection (ED) to improve sensitivity. In the first one, we used a vitreous carbon electrode and an electrochemically active GSH derivative, in the second one a gold mercury amalgam electrode specific for some functions among which are thiols.

MATERIALS AND METHODS

Reagents

Reduced glutathione (GSH), oxidized glutathione (GSSG), orthophthalaldehyde (OPA), 5-sulfosalicylic acid (SSA), ethylenediaminetetraacetic acid (EDTA), N-ethylmaleimide (NEM), 2-vinylpyridine (2VP), sodium borohydride, dithiothreitol (DTT), mercaptoethanol (MSH), ethanethiol (ESH), heptane sulfonic acid (HSA), mercaptopropionylglycine (MPG), penicillamine (Pen) were obtained from Sigma (St Louis M.O.). Sodium acetate, disodium phosphate, orthophosphoric acid, Tris were from Prolabo (Paris, France), methanol and acetonitrile from Carlo Erba (Milano, Italy). Water was deionized.

Material

HPLC was performed using a Waters 600 E System Controller (Millipore), equipped with a U6K manual sample injector (Millipore), a Novapack (150 x 3.9 mm) CD ODS column cartridge (5 µm particle size) with an RP 18 guard cartridge (Millipore) and a BAS LC 4B amperometric detector, equipped with either a vitreous carbon or a Au/Hg working electrode. Data analysis was recorded on a Merck D2500 integrator.

HPLC eluents

Eluents were filtered (0.45 µm) and degassed with ultrasonic and by helium sparging.

Eluent 1 was MeOH and 0.1 M Na_2HPO_4 buffer (10 : 90), pH 7. Elution was performed isocratically at a flow rate of 0.5 ml/min. Peaks were detected electrochemically at + 0.75 V and 50 nA. Glutathione derivative eluted at 6.0 min, DTT-OPA at 23 min.

Eluent 2 was 0.1 M NaH_2PO_4, 3 mM HSA, pH 3.0. Elution was performed isocratically at a flow rate of 0.5 ml/min. Peaks were detected at + 0.15 V and 50 nA. Cysteine eluted at 3.5 min, glutathione at 6.4 min, DTT at 12.0 min.

GSH standard

Standard solutions of GSH and GSSG were prepared at a concentration of 10^{-2} M in 0.5% SSA (20 mM) containing 1 mM EDTA. The GSH solution as initially prepared shows a GSH/GSSG ratio of approximately 200. Aliquots were stored at -20°C and diluted immediately before using at a concentration of 10^{-5} M in 0.5% SSA. Over time, the GSH/GSSG ratio in the diluted solutions slowly decreased.

Fully reduced GSH was prepared by reducing commercial GSH with a large excess of NADPH and glutathione reductase (15, 17, 25).

OPA reagent and derivatization

OPA solution was prepared by dissolving 50 mg of reagent grade OPA in 0.5 ml methanol and diluting this to a final volume of 10 ml with 0.4 M potassium borate, pH 9.9. This solution may be stored for several weeks at 5°C without loss of activity.

Derivatization was performed at room temperature by mixing 10 µl of 10^{-5} M GSH (100 pmoles) with 10 µl OPA solution (370 nmole) in a 1.5 ml polypropylene tube. After 1 min, the derivatized samples were chromatographed. Alternatively, the derivatized samples were neutralized and diluted by addition of 100 µl of 100 mM sodium phosphate pH 7 and stored for later analysis.

Samples preparation

Two different cell lines were used : L1210 (murine leukemia cells) and MCF-7 (human breast tumor cells). They were maintained in RPMI-1640 medium (Gibco) containing 10% fetal calf serum (Gibco) in a 5% CO_2 atmosphere at 37°C. MCF-7 cells, maintained in monolayer culture, were harvested by trypsination.

10^6 cells were pelleted by centrifugation at 700 tr/min for 10 min, resuspended in 500 µl of PBS and centrifuged in the same conditions. Cell precipitate was resuspended in 200 µl of 0.5% SSA containing 1mM EDTA, then the mixture was freezed for 2 hours, thawed and centrifuged for 10 min at 3500 tr/min. In some experiments, 50 mM NEM was added to the SSA solution, in order to avoid a possible oxidation of GSH to GSSG during samples preparation (17, 26-27). 10 to 20 µl of the supernatant were either used directly to determine GSH or treated by NEM and DTT to determine GSSG. Values were the means ± SD of several determinations (nmole per 10^6 cells or per mg proteins). Protein concentrations were measured using bicinchoninic acid as described by Smith et al., (28) with bovine serum albumine as a standard.

Thiol elimination

10 µl of 10^{-5} M glutathione standard or of supernatant in SSA were mixed with 10 µl of 2 mM NEM and 10 µl of 0.1 M Tris pH 8.5. After 1 min at 0°C, the remaining NEM was consumed and the reaction performed as described above.

Disulfide reduction

To measure total glutathione (GSH + GSSX), 10 µl of 10^{-5} M glutathione standard or of the supernatant were mixed with 5 µl of 10^{-2} M DTT and10 µl of 0.1 M Tris pH 8.5. After 30 min at 0°C, DTT was eliminated by ethyl acetate extraction (3 x 500 µl) (15). Then, the reaction was rapidly performed as described above or acidified and stored. If samples were successively treated by NEM and DTT, only GSH contained in GSSX was measured.

RESULTS

GSH measurement by HPLC separation and amperometric detection of the GSH-OPA adduct

OPA and mercaptoethanol is a commonly used fluorescent label for primary amines (21,29); whereas OPA is not fluorescent, the trimolecular reaction of OPA with a primary amine and a thiol produces a highly fluorescent 1-alkylthio-2-alkyl-isoindole (29). Cohn and Lyle (22) reported a method for tissue glutathione measurement by reaction with excess OPA at room temperature in pH 8 buffer which suggests that the fluorescent OPA-GSH product also is an isoindole, where GSH supplies both the thiol and amine functional groups. Owing to the low concentrations of GSH used, the actual product is the tricyclic compound; the chemical identity of this adduct was confirmed by mass spectrometry (30).

HPLC separation with postderivatization (31-32) or with prederivatization (30) adds absolute specificity to this sensitive glutathione assay technique. Prederivatization is preferred since isoindole derivatives of amino groups (29) or of GSH (30) are less polar than parent compounds and chromatograph well by reverse-phase HPLC.

More recently, it has been shown that isoindoles may undergo anodic oxidation and can be detected electrochemically (33). This method was applied to aminoacid derivatives (33-34), to polyamines derivatives (35), but not yet to the GSH derivative. The present study was undertaken to determine the optimum conditions for the measurement of GSH by HPLC-ED after postderivatization with OPA.

Electrodynamic voltammogram

Using a vitreous carbone electrode, GSH has a very high potential, but the derivative formed by reaction with OPA should have a low potential similar to those of adducts obtained in the trimolecular reaction of amines, thiol and OPA.

The best sensitivity was obtained after determining the optimal working potential by plotting intensity vs applied potential (Fig. 1). The GSH derivative was detected from 0.5 V and reached a plateau between 0.75 and 0.9 V. Similar results were obtained by increasing or decreasing the potential, therefore these results were not

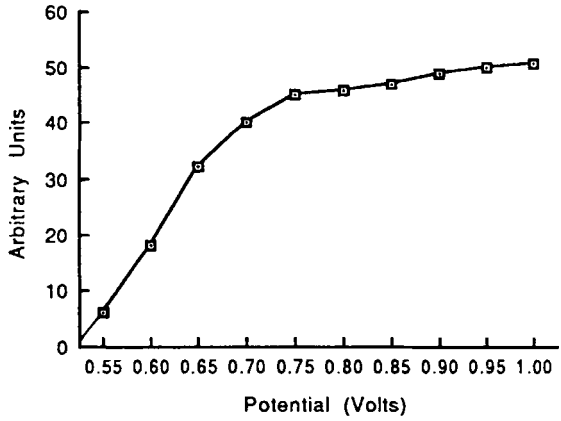

FIGURE 1. Variation of electrochemical activity of GSH-OPA derivative with vitro carbone working electrode potential.

due to passivation of electrodes. This curve was very similar to the one obtained with aminoacid-thiol-OPA adducts (33-34,36), but different from those of the polyamine-thiol-OPA adducts which showed a sharp maximum at 0.65 V (35).

The optimal potential for GSH-OPA was found to be + 0.75 V vs Ag-AgCl reference electrode. It was employed for all subsequent work with a residual current of 13 nA.

Reaction of derivatization

OPA derivatization was performed according to Neuschwander-Tetri and Roll (30), but neutralization and dilution by a phosphate buffer were omitted as the GSH contents of cell extracts were low. 0.5% sulfosalicylic acid solution and OPA reagent were used volume to volume to keep the pH at a high value. Decreasing the excess of OPA was not tested as OPA did not accumulate on the column and was not detected. Moreover, an excess was useful to entirely transform the peak corresponding to DTT in a new one corresponding to DTT-OPA.

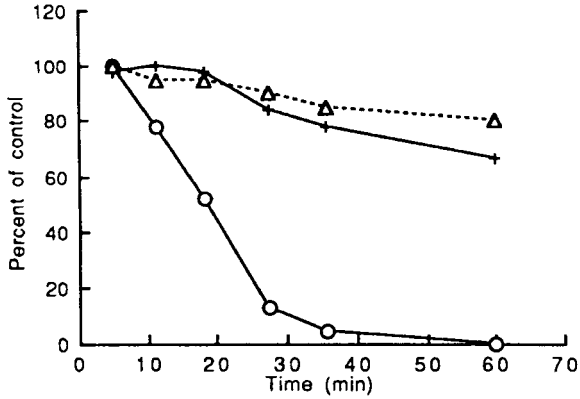

FIGURE 2. Stability of the GSH-OPA derivative under various conditions before injection onto HPLC column. The symbols represent 10^{-4} M in boric buffer (+); 10^{-5} M in boric buffer (o); 10^{-5} M in phosphate buffer (Δ).

Stability of the GSH-adduct

Breakdown of some of the OPA-amino-thiol derivatives has been reported to occur rapidly. With every compound, the adduct reached its maximum after less than 1 min, and most studies have employed reaction periods in the range of 1-2 min. Stability depended on the nature of the amine : primary amines were very stable and retained more than 90% of their original response after 4 days (29), whereas the response decreased quickly in the case of aminoacids (37), e.g. at a rate of 7%/min between 1 and 4 min for GABA-MSH-OPA adduct (34).

In the case of the GSH derivative (Fig. 2), decomposition appeared to occur rapidly in light and at pH 9.90 : with a concentration of 10^{-4} M a decrease of 50% occurred in 60 min and with a concentration of 10^{-5} M the signal disappeared in less than 30 min; usually HPLC runs were effected between 1 and 5 min after the addition of the OPA solution. But this derivative was stable in the dark and at + 4°C for at least 24 h if sodium phosphate pH 7 was added to the OPA-derivative samples as mentioned by Neuschwander-Tetri and Roll (30); if necessary, samples could be brought to pH 7 and kept in the dark at + 4°C.

HPLC conditions

A phosphate buffer pH 7 and 10% MeOH (30) were adequate to elute the GSH derivative with a retention time of about 6 min (Fig. 3 a). Changes in pH, modified both retention times and shapes of the peaks. A pH 7 mobile phase provided the best resolution and decreases in pH of the phosphate buffer or in MeOH content increased the retention times of the OPA derivatives. Ion pairing was not necessary as RT's were long enough to achieve a good resolution. A washout with MeOH was found to be useful at the end of each day to prolong the life of the column.

Linearity and limit of detection

Linearity of detector response was verified by measurement of peak currents over the range 0.1 to 100 pmoles of injected GSH derivatives. Correlation coefficients determined by regression analysis of the amount injected and the detector response were not less than 0.999.

The limit of detection at a signal to noise ratio of 2 was in the range 0.2 to 0.5 pmole (sensitivity range 2nA), though it can be improved by electronic damping when using a sensitivity range of 1 or 0.5 nA. SSA alone was used to quantify background response. This limit was far below the quantity of GSH present in 10^6 cells extracts (ca 1 to 20 nmoles) and the one of GSSG (ca 10 to 200 pmoles).

Limits of detection in the literature with fluorescent detection were 0.1 pmole for OPA prederivatization of GSH (30), 1 pmole for OPA postderivatization of GSH (32) and 5 pmoles for OPA prederivatization of primary amines (29). In the case of ED detection, the limit was 1-20 pmoles for OPA-prederivatization of aminoacids (34). It is also noteworthy that peaks given by a blank are less apparent on the electrochemical than on the fluorescent trace (33) and that quenching occurs only in fluorescence .

Other interfering products

The three thiols ESH, MSH and DTT were electroactive with glassy carbon electrodes and gave peaks respectively at 15.6, 3.6 and 5.7 min. In the presence of

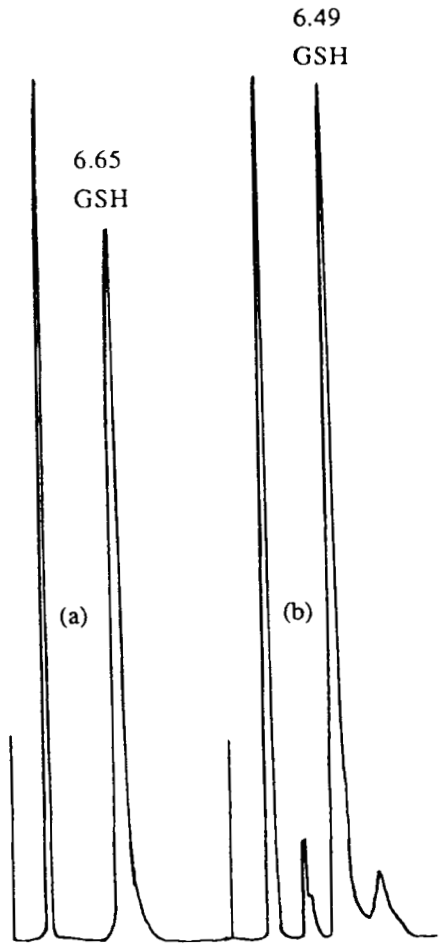

FIGURE 3. HPLC chromatograms of OPA derivatized (a) standard 100 pmoles GSH, (b) MCF-7 cells extract. Elution was performed isocratically over a Novapack C 18 column with MeOH-0.1 M phosphate buffer pH 7.0 (10:90). Flow rate 0.5 ml/min, sensitivity 50 nA, vitrous carbone electrode + 0.75V.

an excess of OPA, these peaks disappeared and late eluting peaks appeared respectively at 26.0, 4.6 and 25.2 min.

Using fluorescent detection, but no HPLC separation, Scaduto (38) reported that although aminoacids and amines did not react with OPA in the absence of an endogenous thiol, their presence in the assay of GSH caused an underestimation of the GSH concentration. If other thiols such as DTT or cysteine were also present, the GSH concentration could be overestimated. However, the method using OPA remains useful when employed in combination with a separatory technique such as HPLC and, to avoid the underestimation, an excess of OPA was used. Using DTT, reaction conditions of Xu et al., (34) and the chromatographic conditions described for GSH determination, Ala was detected at 18 min, His at 24 min and Glu at 40 min, whereas GSH had a RT of 6 min. Aminoacids or amines OPA-thiol derivatives generally required 40-60% MeOH to be eluted (34-35). When the concentration of each reagent was the one described in materials and methods, peaks did not appear, as was already noticed by Neuschwander-Tetri and Roll (30). Although the presence of DTT seemed not to hamper the measurement of GSH, it was eliminated after the reduction by AcOEt extraction as described farther.

N-(2-mercaptopropionyl)-glycine and penicillamine have been proposed as internal standards for measuring thiol compounds. They did not react with OPA in the absence of another thiol, because of the vicinity of their SH and NH_2 groups. So an external standard method was used in this case.

GSH measurement by HPLC separation and amperometric detection with an Au:Hg electrode

Liquid chromatography/electrochemistry has been used previously for the direct determination of thiols in liver samples. Glassy carbon (39) and gold electrodes have been employed at potentials between +800 and +1000 mV vs Ag/AgCl. However, lower potentials (0 to 150 mV vs Ag/AgCl) can be used with a mercury electrode or a gold mercury amalgam electrode. Due to the nature of the reaction, only compounds which complex with mercury such as thiols, halide and chelating agents give a response in this range. Operating at a lower potential, advantages are enhanced selectivity, lower detection limit and background current.

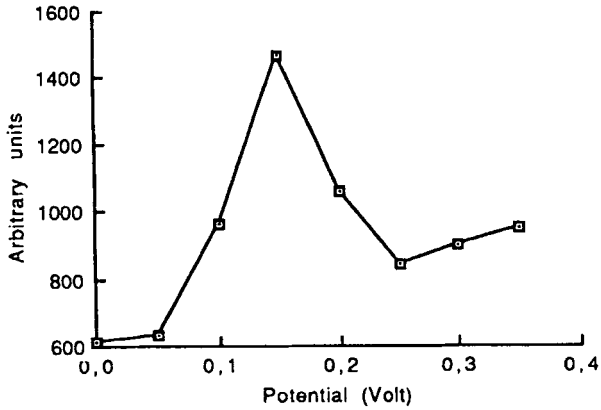

FIGURE 4. Variation of electrochemical activity of GSH with Au/Hg working electrode potential.

Electrodynamic voltammogram

The effect of applied potential on the detector's response to GSH was determined to establish the potential that produced high detector response, low background current and response stability. Response data was graphically presented in the hydrodynamic voltammogram shown in Fig. 4. The applied potential of + 0.15 V appeared optimum since response was high, reasonably stable and background current around zero. In case of decrease in response, a 10 sec cleansing voltage (- 0.2 V) can restore the response, avoiding complete polishing.

HPLC conditions

Reverse-phase ion-pair chromatography was found to be superior to cation exchange chromatography for applications requiring resolution of thiols (40). Capacity factors for the thiols were measured at different mobile phase compositions, thereby allowing the selection of an optimum mobile phase. They were measured over the pH range 2.0-3.5, at higher pH the rate of thiol oxidation increased significantly. By decreasing the pH of the eluting buffer, the retention

time of GSH could be increased, whereas that of Cys slightly increased and the DTT remained relatively constant. Cys has a very small retention time at any pH and the best separation was achieved at pH 3.0 (Fig. 5 a). At lower pH, Cys and GSH were better separated, but tailing was greater and DTT and GSH were no more separated.

To improve the separation, the effect of ion-pairing reagent (HSA) on capacity factors was studied. It has the greatest effect on selectivity, increasing retention times of Cys, GSH and Pen and decreasing those of DTT or MPG. An HSA concentration of 3 mM was used to maximize resolution between Cys, GSH, DTT and Pen and, more importantly, to move Cys away from the solvent peak (Fig. 5b). Increasing the ion-pairing agent concentration to 10 mM increased resolution for thiols, but decreased the life time of the electrodes (41).

Organic modifiers such as MeOH, CH_3CN or DMF were not used in our system since capacity factors were not very high. Fig. 6 (a) shows a mixture of standard separated in these conditions.

Linearity and sensitivity

The linearity of the detector response to reduced Cys and GSH concentration was effective over the range 0.1 to 100 pmoles. The limit of detection at a signal-to-noise ratio of 2,was 2 pmoles with a single gold-mercury electrode. Values reported in the literature with a dual gold-mercury electrode and the same signal-to-noise ratio were slightly higher : 3.5 pmoles (42), 5 pmoles (43) and 6 pmoles (41).

Other interfering products

External standards were injected every 5 to 10 samples to ensure stable detector responses. The gold-mercury electrode provided consistent response over one week before resurfacing of the electrode was necessary. The usable life span of an electrode was around 200 samples depending on the total SH/SS analyzed.

Since DTT also showed affinity for the Au/Hg electrode, its presence in large excess caused a rapid depletion of sample mercury on the electrode. The injection of several samples led to a dramatic decrease in the detector response over the period of

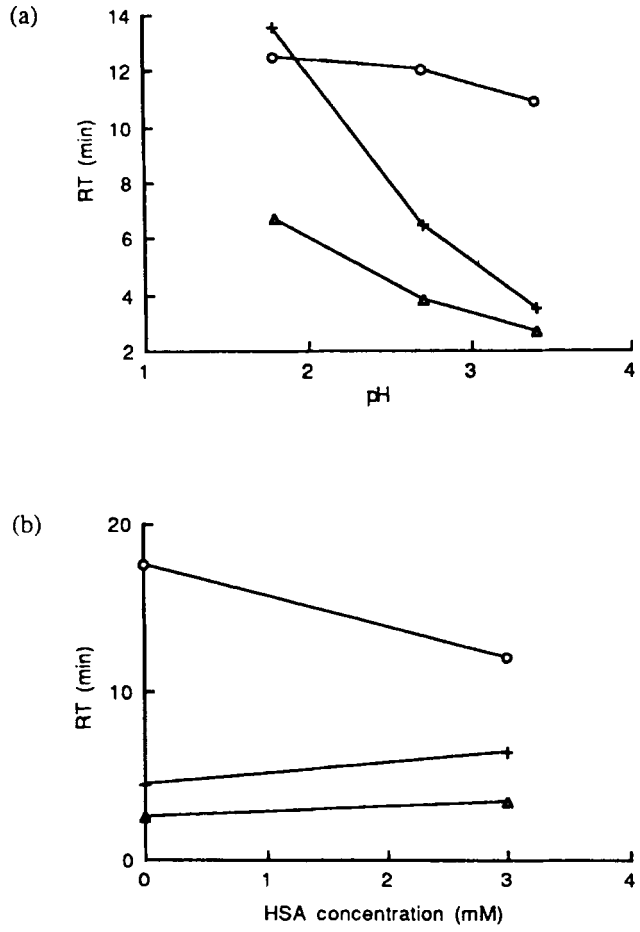

FIGURE 5. Capacity factors of GSH (+), Cys (Δ) and DTT (o) as a function of (a) mobile phase pH; (b) HSA concentration. Elution was performed isocratically over a Novapack C 18 column with 0.1 M phosphate buffer pH 3.0, HSA (a) 3mM (b) 0 to 3mM. Flow rate 0.5 ml/min, sensitivity 50 nA, Au/Hg electrode + 0.15V.

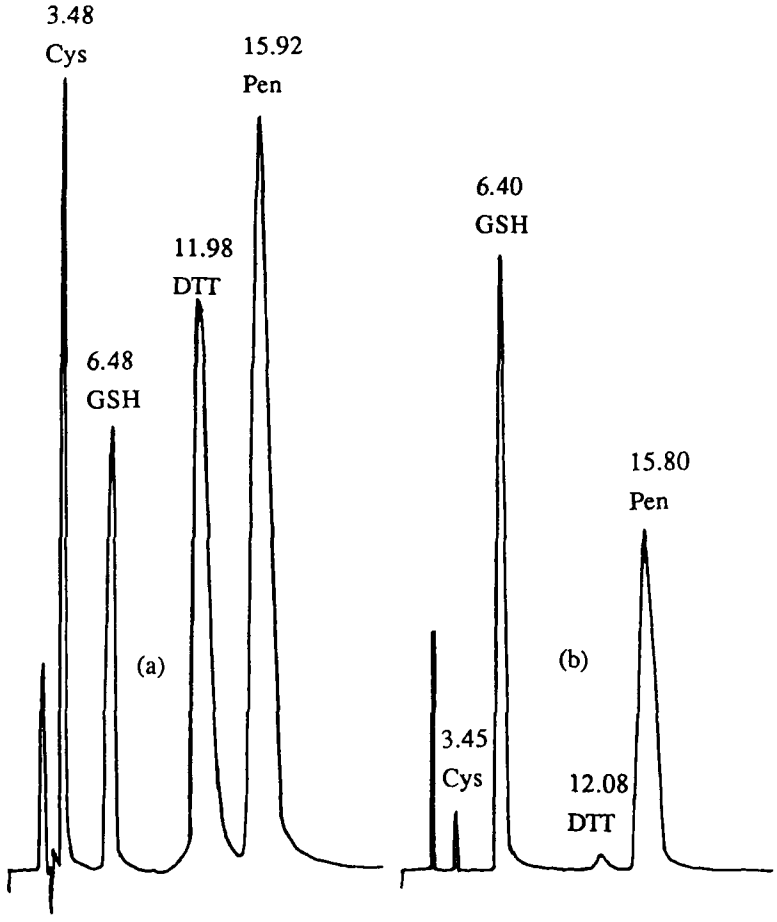

FIGURE 6. Reverse-phase ion separation of (a) standard 100 pmoles of Cys, GSH, D' and Pen; (b) MCF-7 cells extract + 50 pmoles Pen. Elution was performed isocratica over a Novapack C 18 column with 0.1 M phosphate buffer pH 3.0, 3mM HSA. Fl rate 0.5 ml/min, sensitivity 50 nA, Au/Hg electrode + 0.15V.

a few injections and a corresponding decrease in electrode life time (44). One way to avoid this problem was to eliminate DTT by ethyl acetate extraction (45).

With a similar HPLC system, γ–Glu-Cys had the same RT as GSH, whereas Cys-Gly had a higher RT (42).

Internal standard

The analytical results can be improved by the use of an internal standard added to the initial extract and used to compensate for mechanical losses during sample processing or for losses due to oxidation. The mercaptoacid N-(2-mercaptopropionyl)-glycine (36) and the mercaptoaminoacid penicillamine were tested. The last one proved to be a suitable internal standard : it was eluted with a RT value higher than that of the GSH (41) (Fig. 6 a).

Determination of GSSG in the presence of GSH

Protection or elimination of thiols

N-ethylmaleimide NEM alkylates thiol anions to form an adduct; using a pH 8.5 buffer, total alkylation was achieved in less than 1 min (15). A slowly autoxidized buffer such as Tris has been found preferable to easily autoxidized buffers e.g N-ethylmorpholine, when low thiol levels were to be analyzed.

NEM can be stored indefinitely as a concentrated solution in isopropanol (46), but NEM and its adduct were not stable for long in aqueous medium at pH values above 7, due to hydrolysis of the imide to give the corresponding maleamic acids.

The addition of NEM was successfully used to block oxidation of GSH to GSSG in tissue extracts as well as to measure oxidized glutathione in the presence of reduced glutathione (17, 26-27). Reduced glutathione was first eliminated by reaction with NEM, NEM in excess disappeared in less than 1 min in the basic medium, prior to the reduction of GSSG in GSH.

With the OPA-derivative and carbon electrodes as well as with the Au/Hg electrodes, NEM did not interfere and blocked the thiol function of GSH, completely suppressing the corresponding peak. It did not disturb these two

methods, which was not the case in the cycling method of Tietze (13) where NEM was a potent inhibitor of glutathione reductase and had to be removed.

2-vinyl-pyridine (2VP) was introduced in place of NEM, it reacts at slightly acidic pH values, does not inhibit glutathione reductase and thus excess reagent does not need to be removed (16). With our two methods, 2-VP proved to be very efficient, but NEM was preferred since 2-VP was not soluble in aqueous solutions and the reaction needs 20-60 min to take place, instead of 1 min with NEM.

Reduction of disulfides

GSH was released from GSSR by reduction with different reducing agents. Each of these were found to interfere with the electrochemical dosage of GSH. In order to choose the best one, four of them were compared.

$NaBH_4$ was found to be the less efficient even at 37°C, pH 7 and after 30 min, but excess $NaBH_4$ was destroyed by acidification (47) and no peak was detected with $NaBH_4$, in the presence of OPA or not. Dithiothreitol was previously found to be a more suitable reducing agent for disulfides than sodium borohydride (47). MSH was not used since its own peaks (MSH and MSH-OPA) interferred with the GSH-OPA peak. ESH and DTT were equivalent, but ESH was rejected because of its bad smell.

To minimize the height of the OPA-DTT peak and the passivation of the electrodes, conditions were optimized to obtain a near 100% yield with the minimum amount of DTT, as described in materials and methods. Ethyl acetate extraction was then tried and found to be satisfactory (45); three extractions were necessary to completely eliminate DTT (and NEM when present (15)) without any loss of GSH. Yield was comprised between 95 and 100% over the concentration range used.

Every assay was preceeded of a blank constituted by the solution brought to an alkaline pH in the presence of NEM, in order to verify that the GSH originally present has been completly protected by NEM and that the measured peak corresponds only to GSSG reduced in GSH.

GSH and GSSG recoveries in standard and in samples

For the determination of GSH or GSSG recovery from cell extracts, GSH or GSSG in known concentrations was added to the precipitating reagent (0.1 M SSA

+ 1 mM EDTA), in order to exclude enzymatic conversion in the cells. Samples with GSH added were then assayed for GSH and GSSX whereas samples with GSSG added were assayed for oxidized glutathione GSSX. Matched samples with either no added glutathione or only standard glutathione were also analyzed simultaneously to establish the amount of GSH or GSSX in the cells and the actual amount of GSH or GSSG added. These assays were repeated five times with the respective mean values for recovery calculations

The recoveries of GSH and GSSG standards carried through the sample preparation procedure were 100%. The calculated recoveries for externally added GSH and GSSG from a pool biological sample were 98 and 94 % respectively.

Oxidation of GSH in GSSG

Because GSH is readily oxidized, its stability was studied to find stable conditions both for standards and samples. In the stability studies on standard solutions, GSH solutions (10^{-2} M) were prepared in EDTA containing SSA solutions. These solutions were aliquoted to avoid repeated freezing and thawing. When stored at -20°C, they are reliable standard solutions but rapid transformation of GSH to GSSG was observed at higher temperature (data not shown). Similar stability studies were done with the cell extracts : GSH in the free supernatants at -80°C was stable up to two weeks, that is to say more than time required for the determination, samples to be tested by both methods were also aliquoted. On the contrary, in the cell culture medium or in SSA neutralized by the Tris buffer, GSH is more or less rapidly oxidized to GSSG.

To avoid erratic results in GSSG contents during samples preparation, we added the rapidly alkylating agent NEM to the precipitating solution to block free thiols. GSH oxidation does not change the GSH level significantly, but GSSG levels measured without NEM are 2 times higher than when NEM is present during neutralization. The accurate measurement of GSH and GSSG levels require the preparation of two SSA extracts, one with NEM to determine GSSG and one without NEM to determine total or reduced glutathione (17).

Determination of GSH and GSSG in cell extracts

Precautions were taken during sample preparation to prevent oxidation and degradation when measuring GSH and GSSG, but, as it is the case with all other

methods, ascertaining the exact *in vivo* oxidized glutathione content of tissues is achieved with difficulties.The accurate measurement of sulfur-containing compounds in biological samples relies, in part, on preparation, i.e the rapid termination of metabolic processes in cells and the prevention of thiol oxidation and thiol-disulfide interchange during the assay procedure.

To satisfy these criteria, cells were promptly treated with SSA (20 mM or 0.5%) containing a metal chelator, EDTA, to prevent oxidation of the compounds (48). Loss of thiol due to enzymatic degradation by γ-glutamyltranspeptidase or reduction of GSSG by endogenous NADPH via glutathione reductase was avoided by inactivation in acid, the method used implied protein precipitation before disulfide reduction.

Each analysis protocol included as a reagent blank, a sample treated identically to the unknown sample but without the cells. A second blank, the unknown control was prepared by reacting the thiols present in the cell extract with NEM prior to analysis, these samples served as a check on the assignment of thiols in unknown samples and prevented against the GSH/GSSG transformation.

Typical chromatograms obtained from cell extracts are shown in the following figures. A representative chromatogram demonstrating the presence of glutathione with a carbon electrode is shown in Fig. 3 (b), small peaks due to the culture medium may appear beside the one for GSH, but became insignifiant if cells were washed with a phosphate buffer before addition of SSA. A representative chromatogram demonstrating the presence of GSH and Cys with a Au/Hg electrode is shown in Fig. 6 (b). They were the only thiols detected and the cysteine peak was often masked by the solvent front, penicillamine as an internal standard was added to the cell extracts.

Reduced and oxidized glutathione were measured in two cell lines : L1210 and MCF-7; none of them was resistant. Our values estimated for one million, by the two methods are presented in the following table . Each value represents the mean ± SE (3 groups of 4).

Reduced and oxidized glutathione concentrations were equivalent for both methods. When cells were precipitated with EDTA containing SSA solutions, GSSG in MCF7 cells varied widely around 1200 pmles/10^6 cells. Addition of NEM reduced GSSG contents to 221 pmoles. When NEM was not included, but added to the acid supernatant immediately after SSA precipitation, GSSG contents were two times higher (500 pmoles).In the best case, GSH/GSSG ratio is 40. Glutathione

TABLE 1

Determination of GSH and GSSG in various cell extracts.

Cells	µg prot./10^6 cells	OPA -C electrode		Au/Hg electrode	
		pmolesGSH	pmolesGSSG	pmolesGSH	pmolesGSSG
L1210	94.68±12.62	1495±140	74± 9	1669±195	85 ± 6
MCF7	147.46±19.36	6883±511	213±16	8911±616	221±34

TABLE 2

GSH and GSSG values in cells (literature data)

Cells	Ref	mg proteins/10^6 cells	nmoles GSH or GSSG	
L1210	(9)		18.6 ± 2.6	GSH/mgproteins
MCF7	(5)		9.5 ±0.3	GSH/10^6 cells
MCF7	(5)		39.0 ±3.9	GSH/mg proteins
			3.1 ±0.05	GSSG/mg proteins
MCF7	(6)	366	16 ± 5	GSH/10^6 cells

concentrations were always lower with the OPA method than with the more direct one using Au/Hg electrode. The reason may be due to difficulties forming the intramolecular isoindole in solutions containing different amino compounds. Reduced glutathione concentrations previously published in literature were determined by independent methods.

Values were scattered and expressed in nmoles/10^6 cells or in nmole/mg protein. GSSG in most cases was not determined.

DISCUSSION

Two types of electrodes were evaluated for the LCED determination of glutathione (reduced and oxidized). Conditions were found which produced satisfactory performances with both systems.

The *OPA method* implies a derivatization reaction with a yield that appeared to be relatively constant. Chromatographic separation was very efficient, OPA derivatives were well retained and resolved on reversed-phase columns. The vitreous carbon electrode was very stable and the limit of sensitivity low.

When used with DTT, the reduction was effective and may be used with the derivatization reaction. There was a peak corresponding to DTT-OPA with a fairly late eluting retention time, but DTT could be eliminated by ethyl acetate extraction.

A limitation of the present method is that both cysteine and cystinylglycine (21,32) react poorly with OPA and thus cannot be measured; conversely, GSH and γ-Glu-Cys spontaneously react with OPA (22,32,49). A further limitation, is that neither MPG nor penicillamine can be used as internal standards.

Potential interferring peaks must contain a free thiol and an amino group. Amino acids in the presence of OPA and thiol did not interfere with the dosage as the retention times of AA-DTT-OPA were much higher than that of GSH-OPA.

The method using the*Au/Hg electrode* was found to be preferable as it involves easy and quick sample preparation, with no use of the derivatization reaction. This method allowed the measurement of Cys together with GSH in most cases, but sometimes the former was masked under the solvent peak. The low potential provides additional detector sensitivity, with insensitivity toward electrochemically inactive substances present in biological samples. However, the separation was less efficient than in the preceeding case, it was very sensitive to the pH value, and ion-pairing separation was necessary to obtain symetric peaks. Au/Hg electrodes were less stable and less sensitive than vitreous carbon.

The use of internal standards in quantitative analytical procedures is generally considered to be superior to direct calibration because it provides an inherent correction factor. Both MPG and penicillamine proved to be suitable internal standards, penicillamine being the most useful.

In the presence of DTT, the reduction was effective but, DTT contributed to a rapid passivation of the electrodes. The removal of excess DTT by ethyl acetate extraction was required before analysis and allowed correct measurement. A quicker and safer method would be to use dual Au/Hg electrodes in series (50), but this needs the use of two amperometric detectors.

The first method with OPA derivatization allowed much more selective and sensitive measurement of glutathione than was possible with direct measurement. It was used to confirm values or to afford a better sensitivity.

In the case of cells, values were equivalent for both methods, as well for GSH as for GSSG. The lack of procedure to simultaneously determine reduced and oxidized forms of redox pairs, has hampered progress in studying the redox status of cells. The application of these HPLC-ED methods may be particularly relevant to the study of the biochemical mechanisms of GSH variation during emergence of resistance, and for the determination of the redox status of cells.

REFERENCES

1. I. M. Arias W. B. Jakoby, Glutathione : Metabolism and Function, Krok Foundation Series, Raven Press, New-York, 1976, 6: pp 1-382.

2. J. Lutzky, M. B. Astor, R. N. Taub, M. A. Baker, K. Bhalla, J. E. Gervasoni Jr., M. Rosado, V. Stewart, S. Krishna, A. A. Hindenburg, Cancer Res., 49: 4120-4125 (1989)

3. G. Batist, A. Tulpule, B. K. Sinha, A. G. Katki, C. E. Myers, K. H. Cowan, J. Biol. Chem., 261: 15544-15549 (1986)

4. R. D. Olson, J. S. MacDonald, C. J. Van Boxtel, R. C. Boerth, R. D. Harbison, A. E. Slonim, R. W. Freeman, J.A. Dates, J. Pharmacol. Exp. Therap., 215: 450-454 (1980)

5. R. A. Kramer, J. Zakher, G.Kim, Science, 241: 694-697 (1988)

6. E. G. Mimnaugh, L. Dusre, J. Atwell, C.E.Myers, Cancer Res., 49: 8-15 (1989)

7. K. Suzukake, B.J. Petro and D. T. Vistica, Biochem. Pharmacol., 31: 121-124 (1982)

8. T. C. Hamilton, M. A. Winker, K. G. Louie, G. Batist, B. C. Behrens, T. Tsuruo, K. R. Grozinger, W. M. Mckoy, R. C. Young, R. F. Osols, Biochem. Pharmacol., 34: 2583-2586 (1985)

9. V. M. Richon, N. Schulte, A. Eastman, Cancer Res., 47: 2056-2061 (1987)

10. K. Mekhail-Ishak, N. Hudson, M. S. Tsao, G. Batist, Cancer Res., 49, 4866-4869 (1989)

11. M. N. Benchekroun, P. Catroux, D. Montaudon, J. Robert, Free Rad. Res. Comm., 11: 137-144 (1990)

12. B. K. Sinha, E. G. Mimnaugh, Free. Rad. Biol. Med., 8: 567-581 (1990)

13. F. Tietze, Anal. Biochem., 27: 502-522 (1969)

14. J. E. Brehe, H. B. Burch, Anal. Biochem.,74, 189-197 (1976)

15. P. Eyer, D. Podhradsky, Anal. Biochem., 153: 57-66 (1986)

16. O. W. Griffith, Anal. Biochem., 106: 207-212 (1980)

17. T. P. M. Akerboom, H. Sies, Methods in Enzymology, W. B. Jakoby, ed., Academic Press, Orlando, FL, 1981, 77, pp 373-382

18. G. L. Ellman, Arch. Biochem. Biophys., 82: 70-77 (1959)

19. J. Reeve, J. Kuhlenkamp, N. Kaplowitz, J. Chromatogr., 194: 424-428 (1980)

20. M. Fariss, D. J. Reed, Methods in Enzymology, A. Meister, ed., Academic Press, Orlando, FL, 1987, 143, pp 101-109

21. M. Roth, Anal. Chem., 43: 880-882 (1971)

22. V. H. Cohn, J. Lyle, Anal. Biochem., 14: 434-44 (1966)

23. P. J. Hissin, R. Hilf, Anal. Biochem., 74: 214-226 (1976)

24. E. M. Kosower, B. Pazhenchevsky, E.Hershkowitz, J. Amer. Chem. Soc., 100: 6516-6518 (1978)

25. M.A. Baker, G.J. Cerniglia, A. Zaman, Anal. Biochem., 190: 360-365 (1990)

26. A.J. Alpert, H.F. Gilbert, Anal. Biochem., 144: 553-562 (1985)

27. D.Gallemann, P.Eyer, Anal. Biochem., 191: 347-353 (1990)

28. P. Smith, R. Krohn, G. Hermanson, A. Mallia, F. Gartner, M. Provemzano, E. Fujimoto, N. Goehe, B. Olson, D. Klemk, Anal. Biochem., 150: 76-85 (1985)

29. S. S. Simons Jr., D. F. Johnson, Anal. Biochem., 90: 705-725 (1978)

30. B. A. Neuschwander-Tetri, F. J. Roll, Anal. Biochem., 179: 236-241 (1989)

31. Z. Friedman, H. W. Smith, J. Chromatogr., 182: 414-418 (1980)

32. J. Mårtensson, J. Chromatogr., 420: 152-157 (1987)

33. M. H. Joseph, J. Davies, J. Chromatogr., 277: 125-136 (1983)

34. X. Xu, A. L'Helgoualc'h, E. Morier-Teissier, R. Rips, J. Liq. Chromatogr., 9: 2253-2267 (1986)

35. E. Morier-Teissier, K. Drieu, R. Rips, J. Liq. Chromatogr., 11: 1627-1650 (1988)

36. S. M. Lasley, R. D. Greenland, I. A. Michaelson, Life Sci., 35: 1921-1930 (1984)

37. J. D. H. Cooper, G. Ogden, J. McIntosh, D. C. Turnell, Anal. Biochem., 142: 98-102 (1984)

38. R. C. Scaduto, Anal. Biochem., 174: 265-270 (1988)

39. I. Mefford, R. N. Adams, Life Sci., 23: 1167-1173 (1978)

40. L. A. Allison, J. Keddington, R. E. Shoup, J. Liq. Chromatogr., 6: 1785-1798 (1983)

41. G. T. Yamashita, D. L. Rabenstein, J. Chromatogr., 491: 341-354 (1989)

42. A. F. Stein, R. L. Dills, C. D. Klaasen, J. Chromatogr., 381: 259-270 (1986)

43. J. P. Richie, C. A. Lang, Anal. Chem., 163: 9-15 (1987)

44. S. M. Lunte, P. T. Kissinger, J. Liq. Chromatogr., 8: 691-706 (1985)

45. J. Butler, S. P. Spielberg, J. D. Schulman, Anal. Biochem., 75: 674-675 (1976)

46. P. C. Jocelyn, Methods in Enzymology, A. Meister, ed., Academic Press, Orlando, Fl, 1987, 143: pp 44-67

47. W. Baeyens, G. Van den Weken, P. de Moerloose, Chromatographia, 23: 717-721 (1987)

48. A. Meister, M. E. Anderson, Annu. Rev. Biochem., 52: 711-760 (1983)

49. P. G. Richman, A. Meister, J. Biol. Chem., 250: 1422-1426 (1975)

50. L. A. Allison, R. E. Shoup, Anal. Chem., 55: 8-12 (1983)

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