Cell Biology and Toxicology. 1994; **10**: 415–421.
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A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol

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Accepted 27 July 1994

Keywords: accuracy, glutathione measurement, miniaturization, reliability

Abstract

The microtiter plate technique reported by Baker and colleagues for the glutathione reductase–DTNB recycling assay of total glutathione (GSx) and glutathione disulfide (GSSG) has been modified according to Anderson's recommendations, in order to improve the reliability and accuracy of this miniaturized method for the measurement of glutathione status in cultured/isolated cells. Dilute HCl (10 mmol/L) has been used to lyse cells, before protein removal by centrifugation in the presence of 1.3% sulfosalicylic acid. The final DTNB, GSSG-reductase and NADPH concentrations in the reaction mixture have been increased to 0.7 mmol/L, 1.2 IU/ml and 0.24 mmol/L, respectively. The procedure specificity has been tested by spiking and dilution assays, showing that about 90% of the expected GSx amounts could actually be recovered, while no changes of GSSG concentrations were caused in the cells. Accuracy has been assessed by analysis of within-series precision as well as of intra- and interassay reproducibility, showing coefficient variation of <10%. Glutathione changes measured either in control rat hepatocytes or in primary cultures treated with paracetamol or menadione were in good agreement with well-known literature data. These data suggest that the experimental conditions reported in this paper are suitable for the analysis of total glutathione and glutathione disulfide concentrations in cultured/isolated cells.

Abbreviations: BSO, DL-butionine-(*S*,*R*)-sulfoximine; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GSSG, glutathione disulfide; GSx, total glutathione; SSA, 5-sulfosalicylic acid

Introduction

Glutathione, L-γ-glutamyl-L-cysteinylglycine (GSx), is the most important nonprotein thiol present in mammalian cells. This tripeptide is involved in many diverse biological processes such as protection against reactive electrophiles and peroxides, maintenance of the sulfhydryl status of proteins, modulation of enzyme activity by disulfide interchange and transport

processes (Meister, 1989). Measurement of GSx and its oxidation state may thus provide information about cellular responses to xenobiotics as well as about mechanisms of toxicity.

Among the numerous procedures (chemical, enzymatic, chromatographic) developed to analyse GSx concentrations in biological samples, the GSSG-reductase–DTNB recycling assay is generally considered to be specific, sensitive, rapid and reliable (Anderson, 1985). This technique has recently been adapted to microtiter plate conditions by Baker et al. (1990). Even though this miniaturized procedure seemed very attractive for the measurement of glutathione status in many samples, such as in studies of xenobiotic toxic effects in cultured/isolated cells, highly variable data have been obtained in our laboratory with rat hepatocytes. The Baker et al. (1990) miniaturized procedure has thus been modified according to Anderson's (1985) recommendations. The performance of this new miniaturized protocol has been studied in the experiments described.

Materials and methods

Chemicals

Concentrated HCl (37%) was obtained from Merck (Nogent-sur-Marne, France); phosphate-buffered saline (PBS), fetal calf serum (FCS) and Williams' E medium (WEM) from Gibco (Cergy-Pontoise, France); 5-sulfosalicylic acid (SSA) from Janssen Chimica (Noisy-le-Grand, France); 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) from Fluka (Mulhouse, France). GSH, GSSG and NADPH were from Boehringer (Mannheim, Germany); DL-butionine-(*S*,*R*)-sulfoximine (BSO), GSSG-reductase, menadione and paracetamol from Sigma (Saint Quentin Fallavier, France); and 2-vinylpyridine from Aldrich (Steinheim, Germany).

Isolation and culture of hepatocytes

Hepatocytes were isolated from male Sprague–Dawley albino rats (Iffa-Credo, Lyon, France) weighing 180–220 g, as described by Williams et al. (1982). Hepatocytes were seeded in 24-well plates at a density of 0.18×10^6 cells/well, in 0.3 ml of WEM supplemented with 10% FCS. Three hours later, medium was discarded and replaced by 0.3 ml of WEM alone or supplemented with BSO (2 mmol/L) menadione (50 µmol/L) or paracetamol (5 mmol/L).

Cell sample preparation

Medium was removed, acidified with 3 µl of 1 mol/L HCl and stored at –80°C until measurement of extracellular GSSG. Cells were rinsed with 300 µl of PBS, then lysed by freezing and thawing in 150 µl of 10 mmol/L HCl. Cell homogenates were then shaken before taking off 10 µl for analysis of the total protein content and transferring 120 µl to micro-centrifuge tubes, on ice. Proteins were precipitated by adding 30 µl of 6.5% (w/v) SSA. After 10 min, tubes were centrifuged for 15 min at 2000*g* and 4°C. Supernatants were stored at –80°C until assay.

Enzymatic recycling assay for glutathione

Standards (0.5–80 nmol/ml as GSH equivalent) were prepared daily by diluting in 10 mmol/L HCl, containing 1.3% of SSA, a GSH stock solution (800 mmol/L), stored at –80°C for no longer than 8 days. Stock buffer (NaH_2PO_4, 143 mmol/L; EDTA, 6.3 mmol/L; pH 7.4) was prepared in quantity and stored at 4°C. DTNB (10 mmol/L) was prepared weekly in stock buffer. NADPH (2 mmol/L) and GSSG-reductase (8.5 IU/ml) were prepared daily in stock buffer. Daily reagent was prepared by adding 10 ml of DTNB, 17 ml of NADPH and

73 ml of stock buffer. Standards, samples and daily reagent were kept on ice until being transferred to a microtiter plate. The reaction mixture in a 96-well plate was prepared by pipetting 20 μl/well of blanks, standards or samples, followed by 20 μl of stock buffer to neutralize pH. Daily reagent (200 μl) was then added to each well and the plate was kept 5 min at room temperature. The enzymatic reaction was started by quickly adding 40 μl/well of GSSG-reductase. The plate was immediately placed in a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA, USA) and the enzymatic reaction was followed kinetically for 2 min, at a wavelength of 415 nm and with regular mixing of the plate. The final concentrations of reagents were 0.73 mmol/L DTNB, 0.24 mmol/L NADPH, 0.09% SSA and 1.2 IU/ml GSSG-reductase.

Oxidized glutathione (GSSG)

GSSG was measured after GSH derivatization by 2-vinylpyridine. 2-Vinylpyridine (5 μl) was added to 100 μl of cell supernatant in a microtiter plate. The plate was then shaken for 1 h at room temperature. Measurement of GSSG was performed as described above for total GSH, except that 40 μl of blanks, standards, or samples were used, instead of 20 μl of samples (blanks or standards) plus 20 μl of stock buffer. Standards (0.5–8.0 nmol/ml as GSH equivalents) were prepared by diluting a GSSG stock solution (400 mmol/L) in 10 mmol/L HCl containing 1.3% of SSA.

Spiking/dilution and reproducibility assays

Hepatocyte cultures were incubated for up to 20 h in WEM, with or without 2 mmol/L BSO in order to obtain cell monolayers with different intracellular GSx concentrations. In the course of spiking assays, cell homogenates were prepared as described above in 150 μl of 10 mmol/L HCl alone (controls) or supplemented with 10 or 20 μmol GSH/ml (spiked samples), before protein removal by centrifugation in 1.3% SSA. In the case of dilution experiments, control cell homogenates were variously diluted in 10 mmol/L HCl, before protein removal. For analysis of technique reproducibility, supernatants from control hepatocytes were pooled and then fractionated in 20 μl aliquots. All the samples were immediately frozen at –80°C until assay.

Protein measurement

Total protein content was determined by the bicinchoninic acid protein assay kit from Pierce (Rockford, IL, USA).

Statistical analysis

Data comparisons were performed by one-way ANOVA, where appropriate. Differences were considered significant at $p \leq 0.01$.

Results

The typical standard curve obtained under the experimental conditions described is shown in Figure 1, glutathione concentrations being given as GSH equivalents per well. The curve equation was $\Delta_m DO/min = A + Bx$, where $A = 4.52 \pm 2.30$ and $B = 0.458 \pm 0.021$ (mean ± SEM of 17 experiments), with correlation coefficients always ≥0.995. Spiking of hepatocyte monolayers with 1500 or 3000 pmol GSH/culture before cell lysis and protein precipitation allowed the recovery of more than 90% of the expected total glutathione amounts (Table 1). At the same time, no significant increase in the GSSG concentration was found (Table 2). Serial dilution of hepatocyte homogenates before deproteinization allowed about 90% of the expected total glutathione

amounts to be recovered compared to the concentration measured in undiluted samples (Table 3).

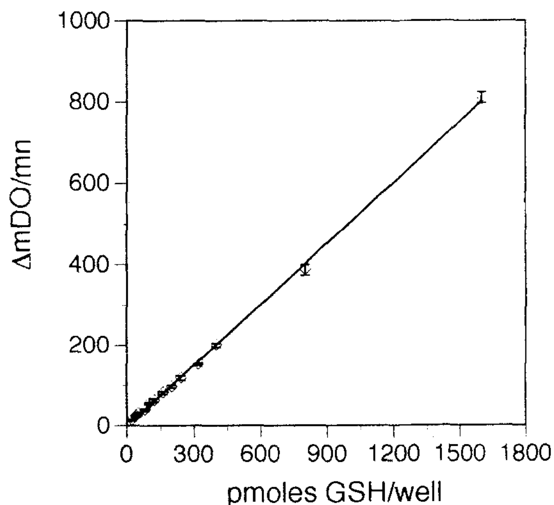

Figure 1. Standard curve for the microtiter plate measurement of GSx concentrations, by the GSSG-reductase–DTNB recycling protocol reported in this paper.

Table 1. GSx recovery percentages in rat hepatocyte cultures spiked with 1500 or 3000 pmol GSH

	Spiked amount (pmol/culture)	Recovery (%)
GSH	1500	94.0 ± 2.67 (10)
GSH	3000	92.9 ± 2.60 (10)

Hepatocytes were cultured in 24-well plates and incubated for up to 20 h in WEM with or without 2 mmol/L BSO, before adding GSH in 150 μl of 10 mmol/L HCl. GSx concentrations in unspiked hepatocytes were between 3300 and 8500 pmol/culture, as GSH equivalents. Values are means ± SEM of (*n*) assays

Repeated measurement of GSx concentrations in the same sample series showed that reproducibility of this analytical procedure was very good, as shown in Figure 2. Mean GSx concentrations obtained at days 1, 4, 5, 6, 14 and 20 corresponded to 36.07 ± 0.84, 37.96 ± 0.63, 34.78 ± 1.84, 41.29 ± 0.68, 36.12 ± 1.05 and 39.06 ± 1.92 nmol/ml, respectively, the differences among these values not being statistically significant. The within-series coefficients of variation were between 2.3% and 6.7%, while those for intraassay reproducibility were between 2.8% and 9.1%. The interassay reproducibility was characterized by a coefficient of variation of 6.3%. These experiments also showed that cell supernatants were stable for at least 20 days at –80°C.

Table 2. GSSG levels (as GSH equivalents) measured in rat hepatocyte cultures spiked with 1500 or 3000 pmol GSH

Spiked amount (pmol/well)	GSSG (pmol/culture)
0	166.0 ± 5.16 (8)
1500	180.4 ± 14.13 (8)
3000	186.7 ± 15.07 (8)

Hepatocytes were cultured in 24-well plates and incubated for up to 3 h in WEM, before adding GSH in 150 μl of 10 mmol/L HCl. Values are means ± SEM of (*n*) assays

Table 3. GSx recovery percentages following dilution of rat hepatocyte homogenates

Dilution factor	Recovery (%)
3:4	88.5 ± 2.46 (16)
1:2	84.0 ± 2.00 (24)
1:4	88.5 ± 2.52 (24)
1:8	87.3 ± 4.32 (24)
1:16	92.2 ± 5.87 (23)

Hepatocytes were cultured in 24-well plates and incubated for up to 20 h in WEM, then lysed in 150 μl of 10 mmol/L HCl and diluted in 10 mmol/L HCl, before protein removal in 1.3% SSA. GSx concentrations in undiluted homogenates were between 5500 and 9600 pmol/culture, as GSH equivalents. Values are means ± SEM of (*n*) assays

The analysis of the effects of 5 mmol/L paracetamol showed a 50% decrease of intracellular GSx concentration only, without any change of the GSSG level (Figure 3a). In

contrast, a 30-min exposure to 50 µmol/L menadione caused a 67% decrease of the intracellular GSx concentration and a clear-cut increase of intracellular as well as extracellular GSSG concentration, the GSSG in the extracellular medium accounting for about 85% of the intracellular GSx decrease, as GSH equivalents (Figure 3b).

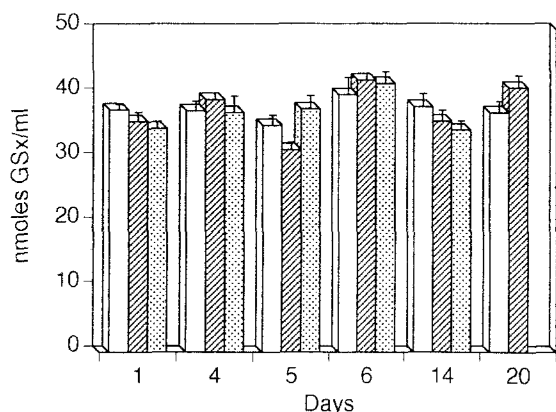

Figure 2. Reproducibility of the microtiter plate technique for the measurement of GSx concentrations by the GSSG-reductase–DTNB recycling assay protocol described in this paper. Three series of six identical samples were analyzed for several days, at three different times of the day. Open bars, series 1; hatched bars, series 2; stippled bars, series 3. Each bar corresponds to the mean ± SEM of 6 samples.

Discussion

The microtiter plate procedure described in this paper differed from that reported by Baker et al. (1990) in the final concentrations of DTNB, NADPH and GSSG-reductase (4.7-, 1.2- and 1.2-fold higher, respectively) and in the use of dilute HCl to lyse cells. However, it should be noticed that, in spite of these differences, the calibration curve parameters reported here were very close to those formerly published. Indeed, the lowest glutathione amount that could be reliably measured was 20 pmol GSH/reaction well (about 70 pmol/ml of reaction mixture), producing a 50% increase in the rate of optical density change over background. Concerning the slope of the standard curve, it should be stressed that, even though the value reported here is about 20% lower, it corresponds to the mean of 17 experiments, some of them giving slopes equivalent to that mentioned by Baker et al. (1990).

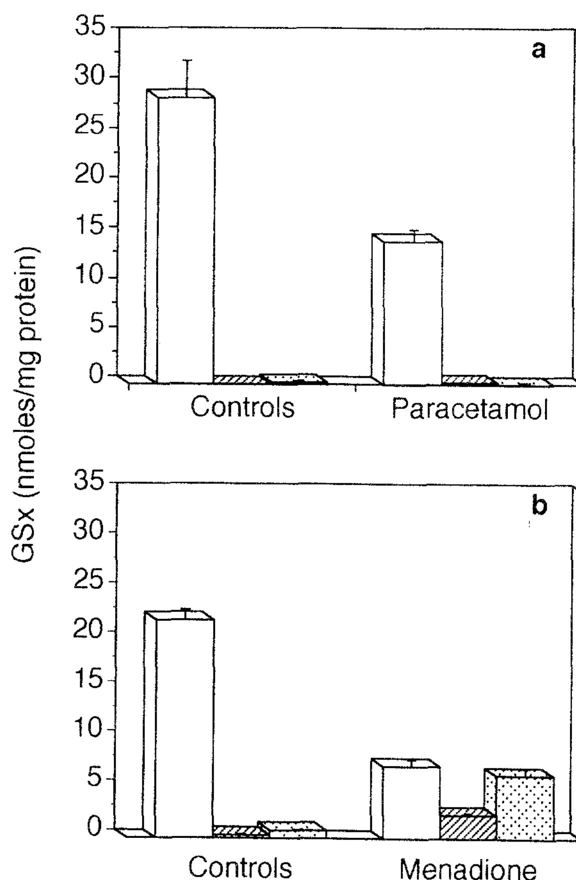

Figure 3. Effects of 5 mmol/L paracetamol (a) or 50 µmol/L menadione (b) on the glutathione status of rat hepatocyte cultures; after incubation for 2 and 0.5 h, respectively. Open bars, intracellular GSx; hatched bars, intracellular GSSG; stippled bars, extracellular GSSG.

To better define performances of this new microtiter procedure, experiments were done to study its specificity and accuracy. Spiking and dilution assays have shown that about 90% of the expected GSx could be correctly recovered over a range of about 40–1300 pmol GSH/reaction well. Moreover, addition to the

hepatocyte monolayers of GSH amounts 18- to 36-fold higher than basal GSSG levels did not produce any significant increase in measured glutathione disulfide. These data suggest that, under the experimental conditions reported here, glutathione losses consequent on formation of mixed disulfides or on transpeptidation and hydrolysis reactions were very limited and that intracellular GSH was effectively protected from spontaneous oxidation processes.

The overall precision of this microtiter plate protocol has been evaluated by analysis of the within-series as well as interassay and intraassay coefficients of variation (CV). The results of these experiments were quite satisfactory as CVs lower than 10% were always observed, in contrast to values as high as 50% obtained in our laboratory in the course of early within-series precision assays with the Baker et al. technique (data not shown). On the basis of results reported in this paper it cannot be ascertained whether such improvement in precision was due to the use of dilute HCl to lyse cells or to the increase in DTNB, NADPH and GSSG-reductase final concentrations. These reagent concentrations were clearly higher than those regarded as optimum by Eyer and Podhradsky (1986), on the basis of their analytical study of the reaction kinetic parameters. However, it should be pointed out that concentrations close to those described in this paper have largely been used in the literature (Anderson, 1985; Roberts and Francetic, 1993; Tietze, 1969). The aim of the experiments reported here was to miniaturize an already existing analytical protocol and to test its accuracy, instead of the optimization of the experimental conditions for a microtiter plate assay. In this context, the results presented here clearly show that the microtiter plate adaptation of the experimental conditions recommended by Anderson (1985) is quite a reliable procedure for measurement of glutathione as well as its oxidation status in cultured/isolated cells.

In order to test the applicability of this miniaturized protocol to measurement of xenobiotic effects on cell glutathione status, GSx and GSSG concentrations were determined in control as well as in paracetamol- or menadione-treated rat hepatocyte cultures. The GSSG concentrations in control hepatocytes were between 1.5% and 3.3% of the total GSx content. Even though this percentage was somewhat higher than 0.5%, as generally reported in the literature (Anderson, 1985), it should be noted that the very low GSSG concentrations found in control cells generally corresponded to the lower limit of the standard curve (20 pmol/reaction well) or slightly below, thus diminishing the accuracy of these measurements. However, the effects of 5 mmol/L paracetamol or 50 μmol/L menadione on the glutathione status were in good agreement with the literature data on the toxicity mechanism of these two compounds. Indeed, it is well known that paracetamol is oxidized to form *N*-acetyl-*p*-benzoquinoneimine, a reactive intermediate readily conjugated to reduced glutathione (Timbrell, 1991). According to results reported by Tee et al. (1987) and Wilson et al. (1991), a 50% decrease of intracellular GSx concentration was observed after incubation for 2 h in the presence of 5 mmol/L paracetamol, without any increase in intra- and extracellular GSSG. As regards menadione, this quinone undergoes a reduction process and is transformed into a semiquinone radical, reacting with oxygen to form reactive oxygen species (ROS) and to produce an oxidative stress (Monks et al., 1992). Metabolism of ROS by glutathione peroxidase depletes intracellular glutathione, with production of GSSG (Monks et al., 1992). According to the results obtained by Di Monte et al. (1984), exposure of rat hepatocytes to menadione was followed by a rapid decrease of intracellular GSx and a sharp increase in glutathione disulfide. The highest GSSG concentrations were found in the extracellular medium, probably as a consequence of an active transport process to protect cells from GSSG toxic effects (Meister and Anderson, 1983).

In conclusion, the various results reported in this paper suggest that this microtiter plate procedure for the glutathione reductase–DTNB recycling assay of glutathione is a reliable protocol for measurement of GSx concentrations as well as its oxidation status in cultured/isolated cells.

References

Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. In: Meister A, ed. Methods in enzymology. vol. 113. Orlando, FL: Academic Press; 1985: 548–55.

Baker MA, Cerniglia GJ, Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal Biochem. 1990;190: 360–5.

Di Monte D, Ross D, Bellomo G, Eklöw L, Orrenius S. Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. Arch Biochem Biophys. 1984;235:334–42.

Eyer P, Podhradsky D. Evaluation of the micromethod for determination of glutathione using enzymatic cycling and Ellman's reagent. Anal Biochem. 1986;153:57–66.

Meister A, Anderson ME. Glutathione. Annu Rev Biochem. 1983;52:711–60.

Meister A. Metabolism and function of glutathione. In: Dolphin D, Poulson R, Avramovic O, eds. Coenzymes and cofactors. vol. III: Glutathione: chemical, biochemical and medical aspects, part A. New York: Wiley; 1989:367–474.

Monks TJ, Hanzlik RP, Cohen GM, Ross D, Graham DG. Contemporary issues in toxicology: quinone chemistry and toxicity. Toxicol Appl Pharmacol. 1992;112:2–16.

Roberts JC, Francetic DJ. The importance of sample preparation and storage in glutathione analysis. Anal Biochem. 1993;211: 183–7.

Tee LBG, Davis DS, Seddon CE, Boobis AR. Species differences in the hepatotoxicity of paracetamol are due to differences in the rate of conversion to its cytotoxic metabolite. Biochem Pharmacol. 1987;36:1041–52.

Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem. 1969;27:502–22.

Timbrell JA. Principles of biochemical toxicology. London: Taylor & Francis; 1991.

Williams GM, Laspia MF, Dunkel VC. Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and noncarcinogens. Mutat Res. 1982;97: 359–70.

Wilson RA, Hart J, Hall T. The concentration and temporal relationships of acetaminophen-induced changes in intracellular and extracellular total glutathione in freshly isolated hepatocytes from untreated and 3-methylcholanthrene pretreated Sprague–Dawley and Fischer rats. Pharmacol Toxicol. 1991;69:205–12.

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