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Colorimetric Assay for
Glutathione
Product No. GT 10

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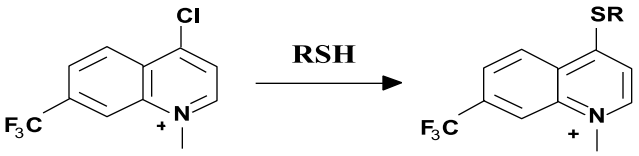
INTRODUCTION

The Analyte

Glutathione (gamma-glutamylcysteinylglycine or GSH) is a naturally occuring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of most aerobic cells.[1] GSH plays a critical role as a coenzyme with a variety of enzymes including, glutathione peroxidase, glutathione S-transferase, and thiol transferase. GSH also plays major roles in drug metabolism, calcium metabolism, the γ-glutamyl cycle, blood platelet, and membrane functions. In addition, GSH is crucial to a variety of life processes, including the detoxification of xenobiotics, maintenance of the -SH level of proteins, thiol-disulfide exchange, removal of hydroperoxides and free radicals, and amino acid transport across membranes. Physiological values of intracellular GSH generally range from 1 to 10 mM. Although many methods have been described for the assay of GSH, the reliable ones are labor intensive and not easy to use.[2]

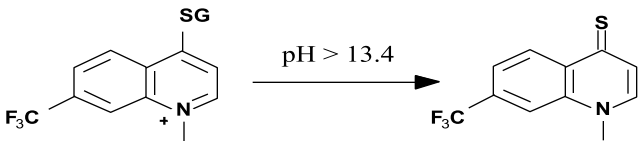
Principles of the Procedure

This method is based on a chemical reaction which proceeds in two steps. The first step leads to the formation of substitution products (thioethers) between a patented reagent, R1 (4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate), and all mercaptans (RSH) which are present in the sample:



Reagent R1 **Thioethers**

The second step is a β-elimination reaction which takes place under alkaline conditions. This reaction is mediated by reagent R2 (30% NaOH) which specifically transforms the substitution product (thioether) obtained with GSH into a chromophoric thione which has a maximal absorbance wavelength at 400 nm:



GSH-thioether **Thione**

This method makes it possible to specifically assay glutathione with only one sampling and one colorimetric measurement. A modification of this method can be used to assay other mercaptans. This is based on the measurement of substitution products, thioethers, which absorb light at 356 nm in the absence of reagent R2.

Because of its simplicity, this method is especially well adapted to the assay of glutathione in large series of biological samples. The main advantage of the method is the specificity for glutathione and it does not require an enzyme as a reagent.

REAGENTS

Materials Provided (for 100 tests)

These three solutions are ready for use.

- Reagent (R1) — Solution of chromogenic reagent in HCl. 1 x 5.5 mL
- 30 % NaOH (R2) — 1 x 20 mL
- Buffer (Solution 3) — Potassium phosphate, containing diethylenetriamine pentaacetic acid (DTPA) and lubrol. 1 x 100 mL

Materials Required But Not Provided

- Spectrophotometer capable of light absorption measurements at 356 and 400 nm, from 0-2 absorbance units.
- Disposable plastic cuvettes, 1 mL with 1 cm optical length.
- Adjustable pipettes with disposable tips.
- Disposable glass test tubes and vortex.
- Water bath kept within 22-28°C temperature range.
- Reduced glutathione (GSH), purity >98%.
- Metaphosphoric acid (MPA), purity 33-37%.

Warnings and Precautions

- For in vitro use only.
- Do not smoke, eat, or drink in areas where reagents and samples are manipulated.
- Wear disposable gloves when handling reagents and samples.
- Mouth pipetting is not recommended.
- Avoid skin and eye contact with the reagents R1 and R2.
- Reagent R2 contains 30% sodium hydroxide, which can cause severe burns.
- In case of accidental exposure of skin, eyes, or mucous membranes thoroughly wash the exposed area with water for 15 minutes.

Reagent Storage and Handling

Reagent bottles should always be kept tightly closed and stored at 0-4°C. For each experiment, take out the required amount of buffer and reagents. Transfer should be made by using clean pipette tips to avoid contamination. Immediately close the remaining buffer and reagent bottles, and store them at 0-4°C. Do not leave reagent bottles open on the bench, at room temperature, or exposed to light. Under the above conditions, all reagents are stable until the indicated expiration date.

PROCEDURE

Assay for Standard Curve

Before each new series of assays, prepare a standard curve with at least five distinct concentrations of GSH. These five concentrations should cover the range 20-100 µmol/L in the reaction medium (spectrophotometric cuvette). Prepare a MPA working solution by dissolving 5 grams MPA in 100 mL water. Prepare a 0.5 mmol/L GSH working standard solution by dissolving GSH into MPA working solution.

Add 50 µL of R1
⇓
Vortex
⇓
Add 50 µL of R2

⇓

Vortex

⇓

Incubate at 25 ± 3°C for 10 minutes in the dark

⇓

Read absorbance at 400 nm

Table 1: GSH Standard Curve

[GSH] µmol/L	0 (Blank)	**20**	**40**	**60**	**80**	**100**
Buffer S3 (µL)	**900**	**860**	**820**	**780**	**740**	**700**
GSH 0.5 mmol/L (µL)	**0**	**40**	**80**	**120**	**160**	**200**

A least-squares linear regression should demonstrate that the absorbance at 400 nm (A) is a linear function of GSH concentration. The apparent molar extinction coefficient, ε, of the measured product is equal to the slope of the corresponding straight line. An example of a standard curve obtained at 400 nm is shown in Figure 1.

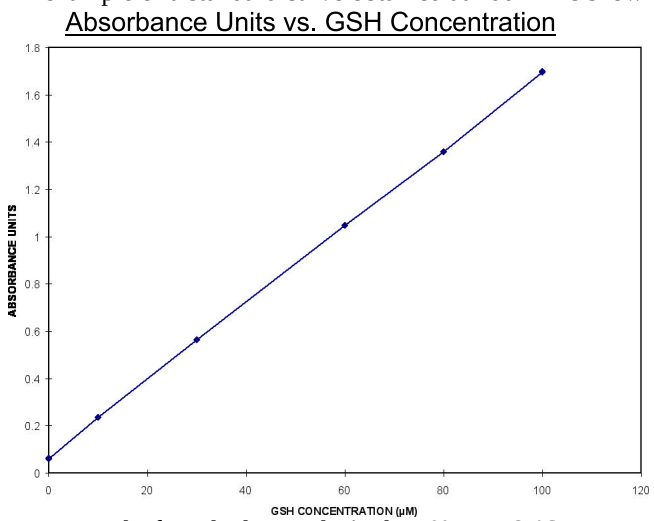


Figure 1: Example of standard curve obtained at 400 nm at 25°C.

Assay for Samples

Before each series of measurements:

- Adjust the spectrophotometer absorbance to zero at 400 nm, with buffer only (solution 3).
- Perform three independent measurements of blank absorbance (A_0) at 400 nm. The mean value of A_0 will be subtracted from the absorbance values obtained with sample (A). The blanks should be measured once only after 10 minutes of incubation.

For each measurement, the reaction mixture is prepared as follows:

1. Take an initial volume (Vi) sample (20-300 µL).
2. Bring to 900 µL final volume with buffer (Volume of solution 3 = 900 µL - V_i).
3. Add 50 µL of solution R1 and thoroughly mix.
4. Add 50 µL of solution R2 and thoroughly mix.
5. Incubate at 25 ± 3°C for 10 minutes in the dark*.
6. Measure the final absorbance (A) at 400 nm.

* Samples are stable for 1 hour after 10-minute-incubation if they are kept in the dark.

Erythrocyte Lysates

1. Centrifuge a minimum of 500 µL of whole blood at 2500g at 4°C for 5 minutes.
2. Discard plasma supernatant. If not assayed immediately, store erythrocyte pellet at -70°C*.
3. Resuspend erythrocyte pellet in 4 volumes of MPA working solution, 0-4°C.
4. Thoroughly mix and centrifuge at 3000g at 4°C for 10 minutes.
5. Collect the upper clear aqueous layer and keep at 0-4°C for the assay (within 1 hour).

whole blood
⇓
centrifugation
(2500g, 4°C, 5 min)
⇓
pellet (erythrocytes)*
⇓
4 volumes MPA (working solution)
⇓
vortex
⇓
centrifugation
(3000g, 4°C, 10 min)
⇓
collect clear supernatant and place at 0-4°C for assay

* Erythrocyte pellet can be stored at -70°C for 15 days.

Liver Homogenates

1. Wash tissue in 0.9% NaCl solution.
2. Blot tissue on paper and weigh.
3. Mince tissue in ice-cold MPA working solution.
4. Homogenize minced tissue.
5. Centrifuge homogenate at 3000g, 4°C for 10 minutes.
6. Collect the upper clear aqueous layer* and keep at 0-4°C for the assay (within 1 hour).

* Cloudy supernatant should be filtered through 0.2 µm filters.

Hepatocyte Lysates

1. Resuspend hepatocyte* pellet, from rats or mice, in 500 µL of ice-cold MPA working solution.
2. Homogenize cell suspension.
3. Centrifuge homogenate at 3000g, 4°C for 10 minutes.
4. Collect the upper clear aqeous layer and keep at 0-4°C for the assay (within 1 hour).

* Approximately 2.5-3.5 x 106 cells are used (5-8 mg of total protein).

Calculations

Measurement of Total Mercaptans (RSH): Assay at 356 nm

The GSH-400 method can be used for the measurement of other mercaptans (RSH), which include GSH. The assay is carried out in the absence of reagent R2 at 356 nm. The absorbance at 356 nm is a linear function of [RSH] concentration in the sample, but it is not GSH-specific. If the sample essentially contains GSH and a single other mercaptan i.e., N-acetylcysteine, these two mercaptans can be assayed by using the same single sample for two measurements. The first measurement is made at 356 nm before the addition of reagent R2 as described below, and the second measurement is made, after the addition of R2, at 400 nm.

Adjust the spectrophotometer absorbance to zero at 356 nm with buffer only (solution 3). The reaction mixture is prepared as in Section 3, with the omission of step 4. The absorbance (A) is measured at 356 nm.

A standard curve must be prepared with the corresponding mercaptan in Table 2, to calculate the concentration. Figure 2 gives two examples of standard curves obtained with glutathione and N-acetylcysteine at 356 nm.

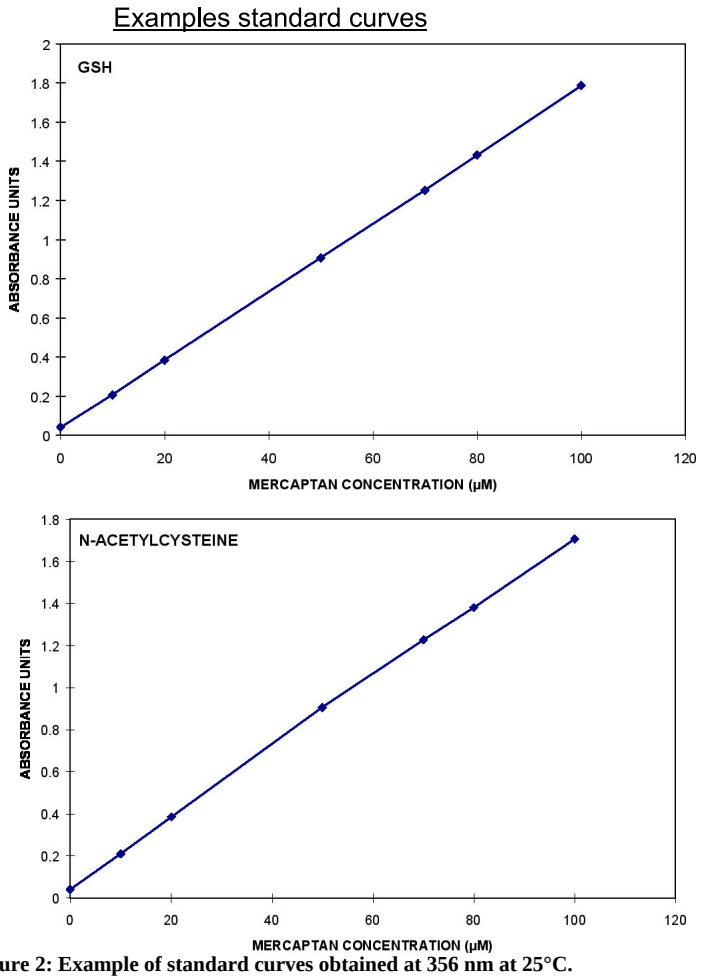


Figure 2: Example of standard curves obtained at 356 nm at 25°C.

GSH Concentration

The calculation is based on the following equation:

$$[GSH] = \{(A-A_0)/(\varepsilon_{400} \times l)\} \times D$$

where:

[GSH]	is the initial glutathione concentration in the sample, expressed as molar concentration.
A and A_0	are the absorbances measured in the presence and in the absence of sample, respectively.
ε_{400}	is the apparent molar extinction coefficient of the product measured at 400 nm.
l	is the optical path (cm).
D	is the dilution factor of the sample.

Total Mercaptan Concentration

The calculation is based on the modified version of the equation used for GSH:

$$[\text{total RSH}] = \{ (A - A_0) / (\varepsilon_{356} \times I) \} \times D$$

where :

[total RSH]	is the concentration of total mercaptan in the sample.
A and A0	are the absorbances measured in the presence and in the absence of sample, respectively.
ε_{356}	is the apparent molar extinction coefficient of the product measured at 356 nm.
I	is the optical path (cm).
D	is the dilution factor of the sample.

Note:

- Do not add reagents R1 and R2 in reverse order.
- The temperature, (25 ± 3°C), should be kept constant throughout the experiment.
- The final reaction volume (1 mL) should not vary from one measurement to another.
- The optical density at 400 nm is proportional to glutathione concentration. It is stable for 60 minutes, provided that the reaction mixture is kept in the dark.
- The sensitivity of the assay for mercaptans at 356 nm is of the same order of magnitude as that of glutathione at 400 nm as shown in Table 2.

PERFORMANCE CHARACTERISTICS

Accuracy

With a series of 30 measurements performed on the same day and under the same experimental conditions, using GSH (20-200 µM), the standard error of the mean value (SEM) was less than 2%.

Reproducibility

With the same experiments performed twice at three-day intervals, using the same samples, the new SEM calculated on the two measurement series was lower than 2%.

Sensitivity

From 30 repeated measurements performed on the control ([RSH] = 0) on the same day, under the same experimental conditions, the detection limit of the assay was 5 µmol/L in the final reaction mixture (spectrophotometric cuvette). For example, using the maximum volume of 300 µL of a sample would therefore give a detection limit for glutathione of about 17 µmol/L.

Interferences

Interferences are mainly due to the presence of proteins in samples. If the proteins are not precipitated in MPA working solution, the absorbance measured at 400 nm may not be stable.

Excess cysteine and alkylamines relative to glutathione concentration in samples gives an absorbance at 395 nm, which interferes with the glutathione assay. This interference is not usually seen in cell lysates or tissue homogenates, even if these samples contain very low levels of glutathione.

Oxidized glutathione (GSSG) is partially decomposed to GSH upon the addition of R2. Therefore, under conditions where the ratio of GSSG/GSH is equal or superior to 5%, GSSG significantly contributes to the absorbance reading at 400 nm.

Other mercaptans tested (Table 2) do not interfere significantly.

Table 2: Spectroscopic features of substitution products of various mercaptans with reagent R1 at 356 nm

Mercaptans	Apparent molar extinction coefficient 356 ($M^{-1}cm^{-1}$)	λ max (nm)
Glutathione	17400 ± 4 %	356

Mercaptopropionylglycine	17000 ± 4.8 %	353
Cysteinylglycine	15100 ± 7.5 %	350
L-Cysteine	24270 ± 2.5 %	352
L-Homocysteine	18000 ± 6.5 %	352
N-Acetyl-L-cysteine	16700 ± 1.7 %	358
DL-Penicillamine*	5600 ± 3.7 %	354
Dithiothreitol*	32200 ± 2.2 %	355
Mercaptosuccinic acid**	8800 ± 16 %	362
Captopril	17400 ± 0.7 %	359

*Reaction yield is not quantitative in 10 minutes when phosphate buffer pH 7.8 is used. With these mercaptans, a 0.1 M borate buffer pH 8.8 should be used instead of solution 3, in order to obtain a 100% reaction yield in 10 minutes.

**The two sulfhydryl groups of the molecule react with reagent R1, which results in approximately twice the color yield.

REFERENCES

1. Dolphin D., Poulson R. and Avramovic O., Eds. (1989), Glutathione: Chemical, Biochemical and Medical Aspects, Vols A & B, J. Wiley and Sons.
2. Anderson M.E. (1989), Enzymatic and chemical methods for the determination of Glutathione; In: Glutathione: chemical, biochemical and medical aspects, Vol. A, Dolphin D., Poulson R. and Avramovic O. Eds., John Wiley and Sons, pp.339-365.

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