Note

Assay of γ-glutamylcysteine synthetase and glutathione synthetase in erythrocytes by high-performance liquid chromatography with fluorimetric detection

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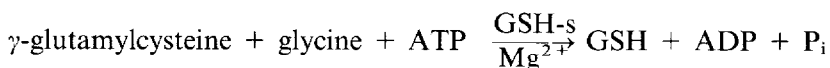*Stazione Zoologica "Anton Dohrn", Villa Comunale, Naples 80121 (Italy)*

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Glutathione (GSH) is a ubiquitous sulphydryl tripeptide with a variety of important biological functions. It serves as an intracellular reductant, as a cofactor of enzymic reactions and in the transmembrane transport of amino acids [1]. Cells synthesize GSH through the coordinated activities of the two enzymes γ-glutamylcysteine synthetase (GC-s) and GSH synthetase (GSH-s):

$$\text{L-glutamate} + \text{L-cysteine} + \text{ATP} \xrightarrow[\text{Mg}^{2+}]{\text{GC-s}} \gamma\text{-glutamylcysteine} + \text{ADP} + \text{P}_i$$

$$\gamma\text{-glutamylcysteine} + \text{glycine} + \text{ATP} \xrightarrow[\text{Mg}^{2+}]{\text{GSH-s}} \text{GSH} + \text{ADP} + \text{P}_i$$

The role of GSH as an intracellular reductant is of particular relevance to the maintenance of normal erythrocyte structure [2].

The methods presently available for the assay of the two enzymes rest principally on the quantitative determination of the inorganic phosphate (P_i) produced during the reactions [3,4], or of γ-glutamylcysteine and/or GSH either as heavy metal precipitates or as radioactive labelled compounds [5]. The spectrophotometric method described by Fouré Seeling and Meister [6] is based on a coupled enzyme procedure, which allows the formation of ADP to be followed during the reaction. This can be used on partially purified enzymes, but is unsuitable for crude preparations and hemolysates. In fact hemolysates have enzymic activities which are, according to Beutler and Gelbart [3], at the borderline of the sensitivity of this technique. Furthermore, spectrophotometric determinations of crude

preparations may be affected by interference from other ADP-forming systems.

Recently Dennda and Kula [7] described an assay of the GSH-synthesizing enzymes from microbial extracts. This employed gradient high-performance liquid chromatography (HPLC) of 5,5'-dithiobis-(2-nitrobenzoic acid) derivatized products, and reached a detection sensitivity of 200–500 pmol.

In the course of studies of the metabolism of sulfhydryl-containing compounds in erythrocytes we examined numerous samples for their GSH-synthesizing capability. We have, therefore, developed a rapid assay for the two GSH-synthesizing enzymes, which takes advantage of the high fluorescence yield of monobromobimane derivatives of thiols and of the separation techniques introduced by Newton *et al.* [8]. The method uses isocratic HPLC to quantify directly, as fluorescent derivatives, the γ-glutamylcysteine and the GSH produced by the enzymic reactions.

EXPERIMENTAL

Materials

GSH, glutathione disulphide (GSSG), L-cysteine, L-glutamic acid, DL-dithiothreitol (DTT) and carboxypeptidase A were from Sigma (St. Louis, MO, U.S.A.). The monobromobimanes (MB) were obtained with the name Thiolyte Reagent from Calbiochem (San Diego, CA, U.S.A.). All HPLC solvents were from Fluka (Buchs, Switzerland).

Preparation of hemolysates

Blood (5 ml) was collected in EDTA from six healthy subjects. Erythrocytes were obtained by centrifugation at 300 g, and washed three times with cold saline. They were hemolysed by addition of 10 volumes of cold distilled water, and the ghosts were removed by centrifugation at 12 000 g for 10 min. Haemoglobin was determined as cyanomethaemoglobin as described by Tentori and Salvati [9].

Enzyme assay

The incubation mixture for the assay of GC-s (final volume 0.3 ml) contained 0.1 M Tris–HCl (pH 8.2), 6 mM ATP, 50 mM KCl, 6 mM DTT, 20 mM MgCl$_2$, 3 mM L-cysteine and 15 mM L-glutamic acid, and was incubated at 37°C for 15 min to ensure the complete reduction of thiols. The reaction was initiated by addition of the hemolysate. For the assay of GSH-s the same incubation mixture was used, with the exception that the cysteine and the L-glutamic acid were substituted by 3 mM γ-glutamylcysteine and 30 mM glycine. During the incubation, samples of 20 μl were withdrawn from the mixture at given times for derivatization of the reaction products.

Derivatization with MB

The samples to be derivatized were added to an Eppendorf tube containing 50

μl of 50 mM N-ethylmorpholine (pH 8.4) and 10 μl of 50 mM MB in acetonitrile. The mixture was placed in the dark, at room temperature, and left to react for 15 min. The reaction was stopped by addition of 80 μl of 10% sulphosalicylic acid, and the volume was made up to 500 μl with water. After spinning down the precipitated protein in a microfuge at 12 000 g for 2 min, the supernatants were ready for HPLC analysis.

Preparation of γ-glutamylcysteine disulphide

GSSG (3 g) was dissolved in 50 ml of distilled water, and the pH was adjusted to 7.8 with NH$_4$OH. The GSSG was hydrolysed to γ-glutamylcysteine disulphide and glycine by overnight incubation at 37°C with carboxypeptidase A (100 U). The reaction mixture was paper-filtered and applied to a formate Dowex 1 (200–400 mesh) column (12 × 1.0 cm I.D.) equilibrated with 0.15 M formic glycine. The γ-glutamylcysteine disulphide was eluted with 0.3 M formic acid and dried in a rotary evaporator under reduced pressure.

The compound was checked for contamination by glycine by thin-layer chromatography on cellulose with butanol–acetic acid–water (3:1:1, v/v/v) solvent.

In the case of incomplete hydrolysis by carboxypeptidase A, any residual GSSG would co-elute from the column with 0.3 M formic acid. Its presence could be ruled out by HPLC analysis since it would appear as GSH-MB derivative.

Preparation of derivatized standards

From a freshly prepared solution of GSH or cysteine (1 mM), known amounts (10–100 nmol) were treated exactly as described above for the MB derivatization of the samples.

For the preparation of derivatized γ-glutamylcysteine standards, 10–100 nmol of disulphide were incubated for 15 min at 37°C with 10 μl of 1 M NH$_4$HCO$_3$ (pH 8) and 300 nmol of DTT to ensure complete reduction of the disulphide. For the MB derivatization a larger amount of 50 mM MB was added (20 μl) to allow for the DTT present. The subsequent steps were identical with those described for the samples and the GSH standards.

High-performance liquid chromatography

Isocratic HPLC was performed on a Beckman Model 330 fitted with a Waters U6K injector and provided with a Shimadzu RF-530 fluorescence detector. Excitation and emission wavelengths were 370 nm and 485 nm, respectively. The peak areas were integrated with a Varian 4290 integrator. The column was a Merck Superspher RP-18 cartridge (250 × 4.0 mm I.D.). The mobile phase was methanol–water (18:82) containing 0.25% acetic acid adjusted to pH 3.8 with NaOH, at a flow-rate of 1.0 ml/min.

Enzyme activity

One unit (U) of enzyme activity is defined as the amount that catalyses formation of 1 μmol of product per min at 37°C.

RESULTS AND DISCUSSION

The HPLC separation of the MB-labelled cysteine, γ-glutamylcysteine and GSH from each other and from other components of the enzymic reaction mixture is shown in Fig. 1. The two chromatograms were obtained during the assay of GC-s (A) and GSH-s (B).

To assess the sensitivity of the fluorimetric detection, a calibration curve of γ-glutamylcysteine and GSH-MB derivatives was established in the range 2–250 pmol. The linearity for the two compounds was satisfactory for levels as low as *ca.* 4 pmol.

For the assay of the ezymic activity, samples of 20 μl were withdrawn from the incubation mixture 10, 15, 20, 25 and 30 min after the addition of the enzyme. They were treated with MB, and the derivatized reaction products were determined by HPLC. In order to determine the recovery of the reaction products
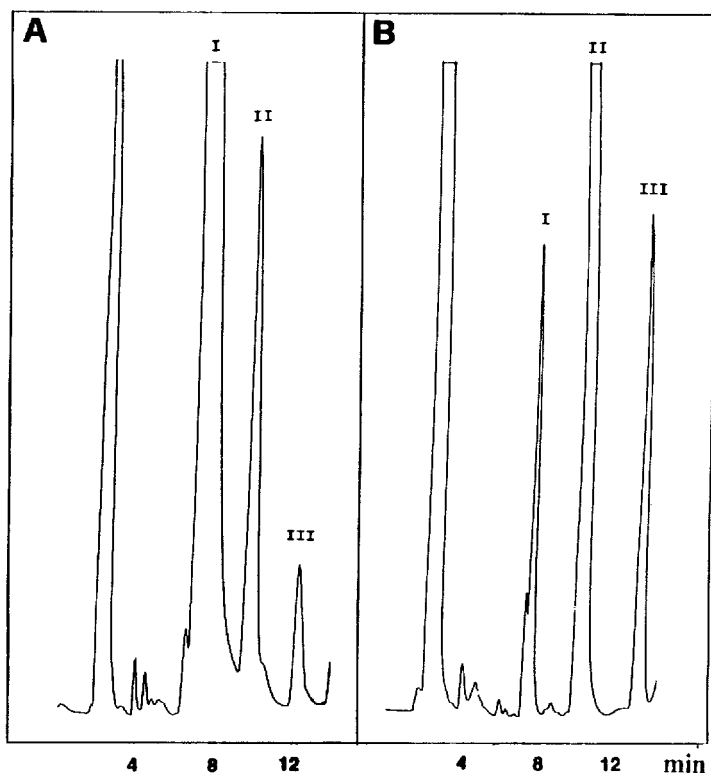

Fig. 1. Chromatographic profiles obtained during determination of (A) GC-s and (B) GSH-s. Peaks: I = cysteine; II = γ-glutamylcysteine; III = GSH. In (A) peak I represents the precursor cysteine in the incubation mixture, peak II the γ-glutamylcysteine, which increases with incubation time, and peak III the endogenous GSH from the hemolysate. In (B) the GSH peak increases with time, and I and II are the endogenous cysteine from the hemolysate and the precursor γ-glutamylcysteine, respectively.
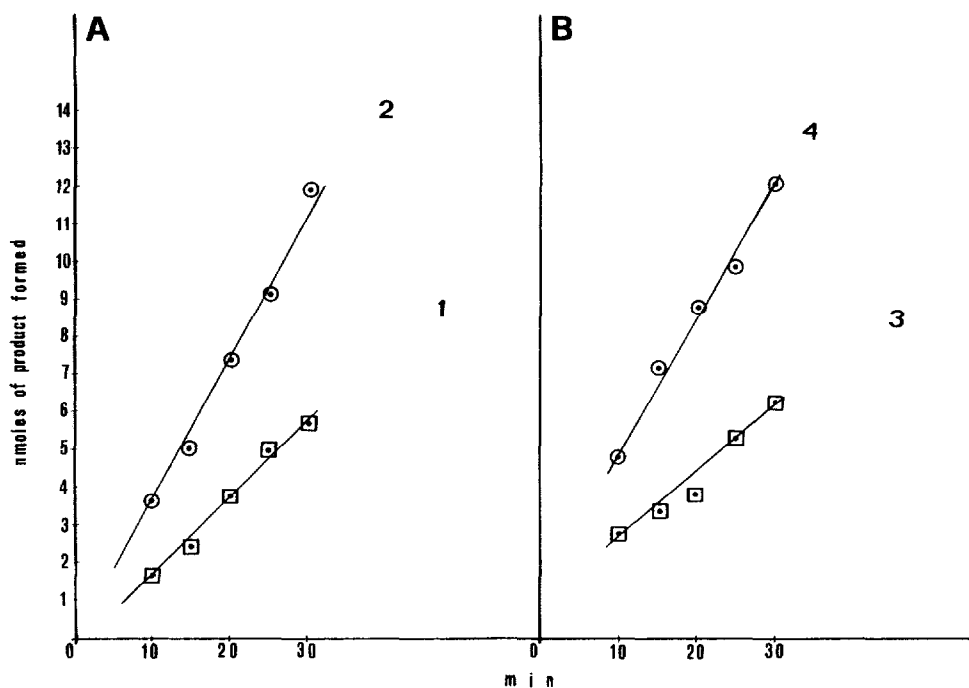
Fig. 2. Enzymic activity of (A) GC-s and (B) GSH-s in hemolysate samples containing 0.15 (1), 0.30 (2), 0.35 (3) and 0.70 (4) mg Hb.

throughout the derivatization procedure, the experiments were performed with known amounts (15 and 30 nmol) of γ-glutamylcysteine or GSH added to the reaction mixture. Complete recovery was obtained.

The derivatized samples, if not analysed immediately, could be stored frozen for several months without significant decrease in the peak areas. As shown in Fig. 2 the rate of product formation was constant up to 30 min and decreased thereafter. The addition of an ATP-regenerating system (5 mM phosphocreatine and 1 U of phosphocreatine kinase) to the reaction mixture, extended this time to at least 60 min. Reproducible and linear activities were obtained for both enzymes with reaction mixtures containing form 0.1 to 8 mU.

We have examined the possible interference of hydrolysing enzymes, such as γ-glutamyltranspeptidase (γGT) or γ-glutamylcyclotransferase (γCT) which could, if present in the sample, destroy part of the reaction products.

It has been reported [4] that the γCT activity is 0.082 U/g Hb (corresponding to $ca.$ 10% of the GC-s activity). To check for the presence of this hydrolysing enzyme, control incubations were run under our experimental conditions. Known amounts of γ-glutamylcysteine were added to the reaction mixture containing the hemolysate but in absence of the precursors. Determinations performed at 5-min intervals during 30 min did not show any decrease in γ-glutamylcysteine.

TABLE I

COMPARISON OF ENZYMIC ACTIVITIES, OBTAINED BY DIFFERENT METHODS, IN HU-
MAN ERYTHROCYTES

	GC-s (U/g Hb)	GSH-s (U/g Hb)
Minnich et al. [5]	0.43 ±0.04	0.19 ±0.03
Blume et al. [12]	0.216±0.003	0.172±0.0031
Lestas and White [4]	0.72 ±0.07	–
Beutler and Gelbart [3]	1.05 ±0.18	0.34 ±0.05
Present study (n = 6)	1.32 ±0.26	0.54 ±0.07

Although it has been reported that the membrane-bound γ GT activity is not present in erythrocytes [10], this point remains controversial. Indeed, recent studies [11] showed the presence of moderate γGT activity in erythrocytes of healthy adults, and an increase of this activity in pathological conditions. To avoid any possible contamination by γGT, the erythrocyte membranes were removed by centrifugation from the hemolysates used in the present study. Further, control runs were performed in presence of known amounts of GSH exactly as described above for the γCT.

We expressed the enzymic activities of GC-s and GSH-s as U per g of haemoglobin (Hb). This is in keeping with previously reported data obtained from human erythrocytes by other methods [3,5,12] and facilitates comparison of the results. For both enzymes the values obtained in the present study were somewhat higher than those of earlier studies (see Table I). The explanation may be that other authors used techniques requiring several purification steps for the isolation of reaction products which could lead to their losses.

It has been suggested that when working with whole hemolysates in the presence of DTT, endogenous GSH may contribute to the feedback inhibition of the GC-s activity [4]. However the concentration of GSH from the hemolysate in the reaction mixture is of order of 0.1 mM, which is far below the concentrations considered important in the GSH inhibition studies [4,13].

CONCLUSIONS

The available enzymic assays based on the determination of reaction products, although very sensitive, involve a series of manipulations, such as purification of products and scintillation counting, which renders them cumbersome and time-consuming. The choice of MB derivatization for fluorimetric determination coupled with HPLC analysis offers, in our opinion, the following advantages: (1) a very high sensitivity, and (2) separation and quantitative determination of the reaction products in a single step. It could, therefore, be useful whenever the need

arises for rapid screening of erythrocytes GC-s and GSH-s activities in various pathological conditions.

REFERENCES

1 A. Meister and M. E. Anderson, *Ann. Rev. Biochem.*, 52 (1983) 711.
2 N. S. Kosower and E. M. Kosower, *Int. Rev. Cytol.*, 54 (1978) 109.
3 E. Beutler and T. Gelbart, *Clin. Chim. Acta*, 158 (1986) 115.
4 A. N. Lestas and J. M. White, *Ann. Clin. Biochem.*, 20 (1983) 241.
5 V. Minnich, M. B. Smith, M. J. Brauner and P. W. Majerus, *J. Clin. Invest.*, 50 (1971) 507.
6 G. Fouré Seeling and A. Meister, *Methods Enzymol.*, 113 (1985) 379.
7 G. Dennda and M. R. Kula, *Biotechnol. Appl. Biochem.*, 8 (1986) 459.
8 G. L. Newton, R. Dorian and R. C. Fahey, *Anal. Biochem.*, 114 (1981) 383.
9 L. Tentori and A. M. Salvati, *Methods Enzymol.*, 76 (1981) 707.
10 S. K. Srivastava, Y. C. Awasthi, S. P. Miller, A. Yoshida and E. Beutler, *Blood*, 47 (1976) 645.
11 D. Sundarsingh Daniel, B. S. Ramakrishna and K. A. Balasubramanian, *Clin. Chim. Acta*, 162 (1987) 319.
12 K. G. Blume, N. V. Paniker and E. Beutler, *Clin. Chim. Acta*, 45 (1973) 281.
13 P. Richman and A. Meister, *J. Biol. Chem.*, 250 (1975) 1422.