# Assay of γ-glutamylcysteine synthetase activity in *Plasmodium berghei* by liquid chromatography with electrochemical detection

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## Abstract

This work describes a high-performance liquid chromatography (HPLC) method to determine γ-glutamylcysteine (γ-GC), the intermediate product of glutathione biosynthesis. Separation relies on isocratic reversed-phase chromatography using a Symmetry C18 HPLC column, particle size 5 μm, $4.6 \times 250$ mm i.d. The mobile phase is methanol–dibasic sodium phosphate (pH 6.6; 2.8 mM) (10:90, v/v) at the flow-rate of 0.5 ml/min and detection is operated electrochemically ($+200$ and $+550$ mV) with a pre-column derivatisation reaction using *ortho*-phthalaldehyde (OPA) as reagent. Under these conditions the calibration range of γ-GC was 0.3–10 μg/ml; the limit of quantification was 0.3 μg/ml; accuracy, expressed as %Bias, was $< 10$ and precision (%CV) was $< 6$. The proposed HPLC assay was used to quantitate the γ-glutamylcysteine produced by the γ-glutamylcysteine synthetase of the rodent malaria parasite *Plasmodium berghei* in an in vitro enzymatic assay. © 2001 Elsevier Science B.V. All rights reserved.

## 1. Introduction

Glutathione (GSH) is the most abundant intracellular non-protein thiol. It has a fundamental role in cellular metabolism and in protecting cells from oxidative damage. GSH synthesis is catalyzed by the consecutive actions of two ATP-dependent enzymes: γ-glutamylcysteine synthetase (γ-GCS) producing γ-glutamylcysteine (γ-GC) and GSH-synthetase leading to the formation of GSH tripeptide.

Various endogenous and exogenous compounds influence γ-GCS activity, which is feedback-regulated by GSH itself (reviewed in [1]).

γ-GCS has been purified from a variety of sources. Recent studies have shown the presence of this enzymatic activity in *Plasmodium falciparum* [2], the agent of the most severe form of human malaria. Interestingly, *Plasmodium falciparum*, which infects the host's erythrocytes, es-

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sentially produces all the GSH present in infected cells, while de novo synthesis of GSH by the host cells is impaired [2]. In addition, GSH-mediated degradation is one route through which malaria parasites react to the toxic effect of heme generated by host hemoglobin degradation [3,4].

The key role of GSH in important processes during the intra-erythrocytic life of the malaria parasite greatly increases interest in studying the *Plasmodium* enzymes involved in its biosynthesis, in view of their possible use as targets for the design of new effective drugs.

The widely used methods for the determination of γ-GC synthetase activity have been based on the estimation of either the GC synthesized [5] or the inorganic phosphate ($P_i$) released during the enzymatic reaction [6,7]. Foure et al. [8] described a spectrophotometric method where γ-GCS activity was determined from the rate of formation of ADP, generated in the enzymatic reaction, measuring it by linking to the lactate dehydrogenase–pyruvate–kinase system. However, this approach can be applied only when partially purified enzymes were available.

More recently, HPLC methods have also been reported which use pre-column derivatisation coupled to spectrofluorimetric detection for quantitative determination of γ-GC [9–17]. In the present study a chromatographic method, which couples HPLC with electrochemical detection after pre-column OPA derivatisation, has been developed and validated. The method guarantees high reliability when small sample volumes are used. It was successfully used to quantitate γ-GC pro-

duced in an in vitro assay using, as enzyme source, cell extracts prepared from the rodent malaria parasite *Plasmodium berghei*.

## 2. Experimental

### 2.1. Chemicals and reagents

L-Glutamic acid, L-cysteine, L-buthionine-[*S*,*R*]-sulfoximine (BSO), DL-dithiothreitol (DTT) and γ-glutamylcysteine (γ-GC) as trifluoroacetate salt were supplied by Sigma (Sigma chemical, St. Louis, MO, USA). Adenosin-5′-triphosphate (ATP) and complete™ protease inhibitor cocktail-tablets were purchased from Boehringer Mannheim. *O*-Phthalaldehyde (OPA) was obtained from Pickering laboratories (Pickering laboratories, Suite S Mountain view, CA, USA), 2-mercaptoethanol was obtained from Merck (Whitehouse Station, NY, USA). Methanol was HPLC grade (Lab-Scan Analytical Science, Dublin, Ireland). Water was bidistilled and all other chemicals were of analytical reagent grade.

### 2.2. Chromatographic apparatus and conditions

The chromatographic apparatus consisted of a Labflow 3000 pump, a Rheodyne model 7125 injection valve with a 20-μl sample loop (Rheodyne, Berkeley, CA, USA) and a Coulochem model 5100A electrochemical detector (ESA, Bedford, MA, USA) equipped with an analytical cell (Model 5011).

The working parameters for the electrochemical detector were +200 mV for the first electrode, and +550 mV for the second. Maxima Chromatography Software (Millipore Corp.) was used for data collection and calculation. Isocratic separation was achieved at room temperature using a Symmetry C18 HPLC Column, particle size 5 μm, 4.6 × 250 mm i.d. (Waters Corporation, Milford, MA). The mobile phase was methanol–dibasic sodium phosphate (pH 6.6; 2.8 mM) (10:90, v/v) at the flow-rate of 0.5 ml/min. The mobile phase, filtered through 0.45-μm filters (Geeman, Sciences, MI, USA) before use, was continuously recirculated to the solvent reservoir and freshly
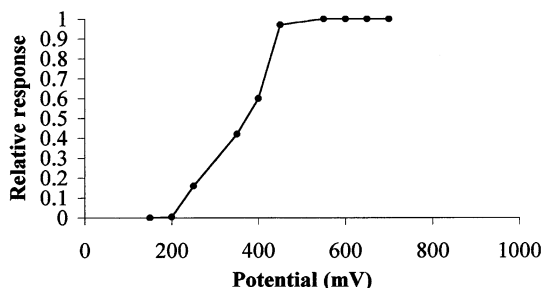


Fig. 1. Hydrodynamic voltammogram for γ-GC. The relative response is the ratio of the peak area measured at a given potential to that at plateau level.
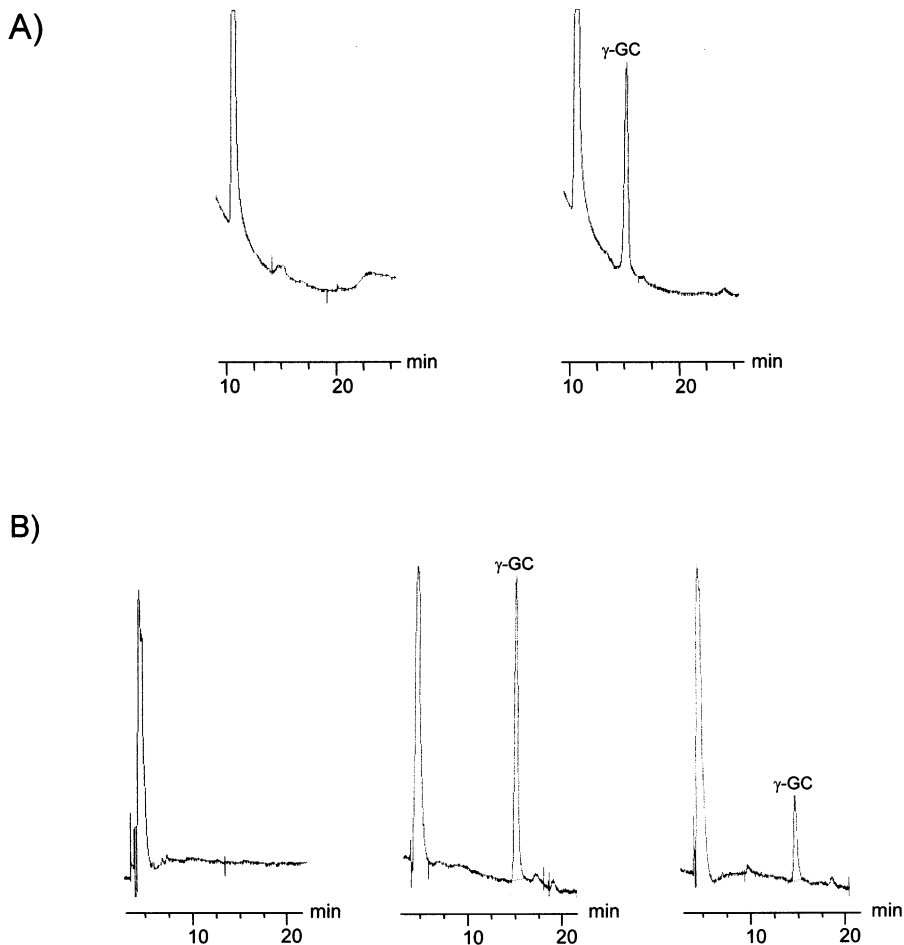
Fig. 2. HPLC separation of γ-GC produced in an in vitro assay using *Plasmodium berghei* extracts (B, central panel), or uninfected mouse hemolysates (A, right panel), as γ-GCS source. Enzymatic reaction in the absence of cysteine is shown in A and B, left panels. The inhibitory effect of BSO on *P. berghei* γ-GCS is shown in B, right panel. Column and chromatographic condition are described in Fig. 3.

prepared weekly. Cleaning of the electrode system was performed weekly with a methanol–water mixture (70:30, v/v).

### 2.3. Standard solutions

A standard stock solution of γ-GC (1 mg/ml) was prepared in 50% methanol and stored at −20°C. No significant degradation occurred over a period of 30 days. Working standard solutions were obtained by diluting the stock solution with 0.01 M HCl and treating and processing them as a sample.

### 2.4. Parasite propagation and preparation of cellular extracts

*Plasmodium berghei* infection (clone 8417HP) was performed on Swiss mice by intraperitoneal punctures. Infected erythrocytes, collected after bleeding, were passed through plasmodipur filters (Euro-Diagnostica, Arnhem, The Netherlands) to eliminate leukocytes and then washed twice with PBS. Erythrocyte lysis was obtained by adding two volumes of sterile distilled water. Saline concentration was restored, immediately after lysis,
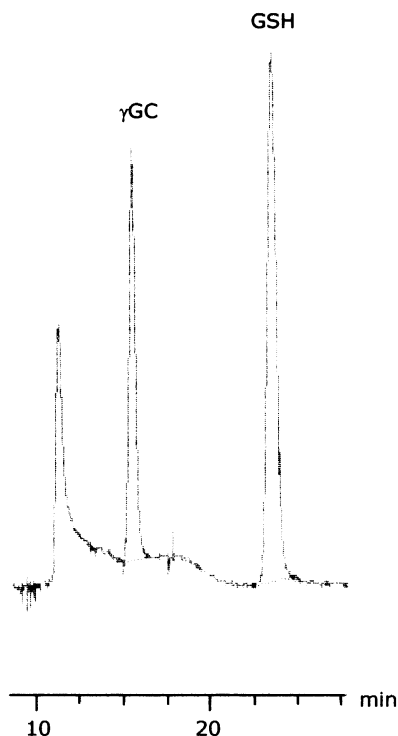
GSH

γGC

min

10          20

Fig. 3. Chromatogram corresponding to a standard solution of GSH and γ-GC OPA-adducts. Column: Symmetry C18 HPLC Column, particle size 5 μm, 4.6 × 250 mm i.d.; mobile phase: methanol–dibasic sodium phosphate (pH 6.6; 2.8 mM) (10:90, v/v); Flow-rate: 0.5 ml/min.

by adding an equal volume of $2 \times$ PBS. Free parasites, collected by centrifugation at $4000 \times g$ and washed twice with cold PBS, were resuspended in an appropriate volume of a buffer containing: 3.5 mM $MgCl_2$, 110 mM KCl, 40 mM

NaCl, 20 mM Hepes, pH 7.4 and complete antiprotease cocktail. Parasite lysis was achieved through three successive freezing (liquid nitrogen) and thawing (37°C) processes. Aliquots corresponding to $3 \times 10^7$ parasites were added to the reaction mixture. Emolysates from uninfected erythrocytes were prepared as described in Nardi et al. [18].

### 2.5. Enzyme assay

γ-GCS enzymatic assay was performed at 37°C for 30 min in 0.3 ml of: 0.1 M Tris–HCl (pH 8.2), 6 mM ATP, 50 mM KCl, 6 mM DTT, 20 mM $MgCl_2$, 1.2 mM L-cysteine and 15 mM L-glutamic acid as described in Nardi et al. [18]. The reaction was initiated by adding parasite extract. After incubation, 100 μl of a 50% sulfosalicylic acid was added to the samples and incubated for 5 min in ice. Supernatants recovered after centrifugation were subjected to derivatisation.

The reaction mixes without either L-cysteine or L-glutamic acid (substrates of the γ-GCS) were used as blank samples to determine the specificity and accuracy of the assay (see Section 3).

The possible interference of hydrolyzing enzymes, which could lower the activity values of endogenous γ-GCS, was checked by adding known amounts of γ-GC to blank samples, in the same condition of the enzyme assay. No significant decrease of γ-GC was observed.

Inhibition of γ-GCS activity was achieved by adding BSO, 10 mM final concentration, to parasite extracts prior to starting the enzymatic reaction.

Table 1
Determination of γ-glutamylcysteine precision and accuracy

| Nominal standard concentration (μg/ml) | Calculated concentration (μg/ml) mean ± S.D.[a] | Precision %CV | Accuracy Bias%[b] |
|---|---|---|---|
| 2.98 | 2.73 ± 0.02 | 0.73 | −8.33 |
| 1.49 | 1.38 ± 0.08 | 5.80 | −7.27 |
| 0.745 | 0.814 ± 0.01 | 1.23 | 9.31 |

[a] Results are the mean of three experiments.
[b] Bias% = [(measured amount − true amount)/true amount] × 100.

## 2.6. Preparation of derivatized standards and samples

The derivatisation reaction was carried out at room temperature. OPA (27 mg) was dissolved in 1 ml methanol, then 5 μl 2-mercaptoethanol and 9 ml 0.1 M sodium tetraborate pH 9.3 were added. OPA stock solution was stored at −20°C for 1 month. The OPA working solution was prepared daily by diluting 10 μl of the OPA stock reagent (OPA/βMe) with 500 μl of sodium tetraborate 0.1 M. The derivatising agent (70 μl) was left to react for 2 min with 10 μl of standard or sample solutions before 10 μl was injected onto the analytical column. The stability of γ-GC-OPA adduct after derivatisation was examined for 30 min. No significant variation of γ-GC concentration was observed.

## 3. Results and discussion

### 3.1. Validation of the method

The method reported here for the determination of γ-GC, employs HPLC and a coulometric detector with two working electrodes. The two electrodes were used in the oxidative screen mode, the optimal oxidation potentials were determined through repeated on-column injections of γ-GC stock solution. γ-GC current–voltage curves were obtained by varying the electrode potential from +200 mV to +700 mV in 50-mV steps (Fig. 1). On the basis of these results, the potential of the first electrode was set at +200 mV to remove compounds with lower oxidation potential than the analyte, while the potential of the second electrode was fixed at +550 mV because the signal response of the dipeptide–OPA adduct reached a plateau at this value. The signals generated by the second electrode were used for quantitation.

Under the conditions described above, specificity, linearity, detection and quantitation limits, accuracy, precision and robustness were determined.

The specificity of the detection assay was determined by analyzing blank samples prepared as described in Section 2. No interfering peaks were detected at the retention time of the γ-GC peak (Fig. 2). Standard solutions of glutathione, glutamic acid, cysteine and glycine derivatized with OPA/βMe did not interfere with the analytical method. A chromatogram corresponding to standard GSH and γ-GC OPA-adducts is shown in Fig. 3.

The linearity of calibration curves was calculated by linear regression in a range from 0.3 to 10 μg/ml, giving a correlation coefficient of 0.998. The equation for the calibration curve was $y = 20662x - 1894.4$. The coefficients of variation of the slope and the intercept were 0.75 and 0.19, respectively.

The limit of detection, defined as the injected amount of γ-GC giving a signal-to-noise ratio > 3, was 0.1 μg/ml while 0.3 μg/ml was established as the limit of quantitation. This value was calculated with precision (2.19) and accuracy (−3.33) and it was included in the calibration curve as the lowest concentration level.

As shown in Table 1, the accuracy and precision of the assay, over the concentration range of the standard curves, was determined by adding standard solutions of γ-GC to a blank sample (three injections for each standard).

The robustness of a method lies in its ability to remain unaffected by small changes in parameters, such as organic content percentages, pH of the mobile phase and temperature, etc.

The assay was not affected by slight variations in either pH values (range: 6.00–7.00) or electrode potentials (range: +500 to +650 mV). Slight variations in the retention times were observed using mobile phases prepared on different days and injecting the sample solution on four columns from the same manufacturer containing the same brand of packing material.

### 3.2. Applicability of the method

The described method (involving HPLC separation of γ-GC following an in vitro enzymatic assay) was applied to detect γ-GCS activity in *Plasmodium berghei*, the rodent model of malaria disease, and in uninfected host erythrocytes for comparison. Parasites were grown in laboratory

mice and parasitemia controlled through microscopic inspection. Free parasite extracts, or hemolysates from uninfected mouse erythrocytes, prepared as described in Section 2, were used as γ-GCS source. An HPLC profile of the reaction products is shown in Fig. 2. Under the assay conditions (extracts from $3 \times 10^7$ cells reacted for 30 min at 37°C) $\sim 25$ nmol of dipeptide was produced by the parasite extract, while $\sim 55$ nmol was produced by uninfected mouse hemolysate, as calculated after HPLC separation. The addition of glycine (required in the second step of GSH biosynthesis) causes a significant decrease in the γ-GC peak due to GSH formation (not shown).

As stated above, no peak at the retention time of γ-GC was observed when the enzyme reactions were performed in the absence of either precursor (Fig. 2, left panels). The specificity of the assay was also confirmed by the use of L-buthionine-[S,R]-sulfoximine (BSO), a specific inhibitor of γ-GCS [19]. Fig. 2 B, right panel, shows a significant decrease in the peak corresponding to parasite γ-GC when the specific inhibitor was added.

## 4. Conclusions

In this study a reliable method for the detection and quantitation of γ-glutamylcysteine, the intermediate product of glutathione biosynthesis, is described. The reaction with o-phthalaldehyde (OPA) in the presence of an alkanethiol to form 1,2-disubstituted isoindoles is among the most frequently employed chemistries for derivation of primary amines [20–23]. However, the isoindole products are not stable in the presence of heterobifunctional group (glutathione and γ-glutamylcysteine), and in these cases OPA is used without co-reagent [12,13]. This technique is now generally criticized for the lacking of specificity due to the interference by endogenous compounds [23]. Since the addition of 2-mercaptoethanol improves the sensitivity and the rate of OPA reaction [13,24,25], we used HPLC separation and electrochemical detection with OPA/βMe pre-column derivatisation to measure the activity of γ-glutamylcysteine synthetase (γ-GCS) in crude cellular extracts of the rodent malaria parasite Plasmod-

ium berghei. In the conditions used no other compound present in the reaction mixture interferes with the chromatographic profile of the analyte. The method was also applied to detect and quantify the production of γ-GC from uninfected mouse erythrocytes. In this case the amount of γ-GC obtained in our conditions ($\sim 55$ nmol using $3 \times 10^7$ red blood cells), agrees well with that obtained using HPLC and fluorimetric detection [18].

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