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Fluorimetric determination of monobromobimane and *o*-phthalaldehyde adducts of γ-glutamylcysteine and glutathione: application to assay of γ-glutamylcysteinyl synthetase activity and glutathione concentration in liver

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Abstract

The reversed-phase HPLC separation of fluorescent *o*-phthalaldehyde (OPA) derivatives has been applied to the assay of hepatic γ-glutamylcysteine and glutathione (GSH) levels and the enzymes producing these peptides. The method has been compared to the assay using monobromobimane (MB) as the derivatizing agent. The OPA method has the advantage of faster derivatization, the lack of need to adjust the pH, isocratic separation and selectivity for GSH and γ-glutamylcysteine. The MB method requires pH adjustment following derivatization and gradient elution chromatography. MB is also non-selective, yielding fluorescent derivatives of all biological thiols and more interfering peaks on the chromatogram. MB-based analyses are also approximately sixty times more expensive per sample. MB yields fluorescent degradation products on exposure to light. OPA adducts are stable for up to ten days when stored at −20°C. OPA detection is sensitive to 12.5 pmol in the sample, at a signal-to-noise ratio of 2.5. The two methods correlate well. Hepatic γ-glutamylcysteine synthetase in the same liver preparation was found to be 4.85 ± 0.47 nmol min^{-1} mg^{-1} protein by the OPA method and 4.42 ± 0.52 nmol min^{-1} mg^{-1} protein by the MB method. GSH concentrations were found to be 90.4 ± 6.5 nmol/mg protein for the OPA method and 92.5 ± 3.4 for the MB method.

1. Introduction

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is present in almost all cells in millimolar concentrations. It functions as an intracellular reductant and plays important roles in catalysis, metabolism and transport [1,2]. It protects cells against free radicals, reactive oxygen and other toxic compounds. GSH synthesis is intracellularly catalyzed by two enzymes, γ-glutamylcysteinyl synthetase (γ-GC synthetase; EC 6.3.2.2) and GSH synthetase (EC 6.3.2.3). The former is the rate-limiting enzyme in GSH synthesis. Various endogenous and exogenous chemicals influence γ-GC synthetase activity, while GSH itself regulates activity by a feedback mechanism [2].

Although there are numerous methods for the determination of GSH [3–5], relatively few are available for the determination of γ-glutamylcysteine (γ-GC) [6,7]. Reeve et al. [8]

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used Ellman's reagent to convert cysteine, GSH and γ-GC to the mixed disulfides of 5-thiol-2-nitrobenzoic acid, separated them by HPLC and detected them spectrophotometrically at 280 nm. This method, however, has low sensitivity and suffers from interference by various peptides and other non-thiol biological compounds absorbing at 280 nm. Newton et al. [9] have reported a sensitive and specific method for the fluorimetric determination of biological thiols, based on conversion of biological thiols to fluorescent derivatives of monobromobimane (MB) and separation of the derivatives by reversed-phase HPLC. However, MB is relatively expensive, and chromatographic peaks from MB and its hydrolysis products are troublesome [10].

In view of the limitations of the available methods, we have developed a rapid and simple HPLC method for detection of γ-GC and GSH, based on derivatization with *o*-phthalaldehyde (OPA) to form a stable fluorescent product. The reaction of free thiols with OPA in the presence of primary amines yields isoindoles. Formation of these fluorescent derivatives is the basis for detection of free amino acids by pre-column derivatization in the presence of an added thiol such as ß-mercaptoethanol [11–13]. GSH contains both primary amine and thiol moieties. It therefore reacts with OPA to yield a highly fluorescent isoindole. This reaction was first reported by Cohn and Lyle [14]. Since then, this technique has been modified for fluorimetric determination of GSH [4,15–18]. However, certain endogenous compounds can also react with OPA to produce fluorescent derivatives. Thus, interference may occur [19–21]. To solve this problem, there is a need for a sensitive separation technique, such as HPLC [22].

γ-GC shows a similarity in structure with GSH, lacking only a glycyl residue. We considered, therefore, that γ-GC should react with OPA to yield a fluorescent derivative in the same manner as GSH does. We now report the pre-column derivatization, separation and quantitation of γ-GC–and GSH–OPA adducts, and compare the method with the MB derivatization method. The methods have also been compared for the determination of cytosolic GSH concentration and γ-GC synthetase activity in rat liver.

2. Experimental

2.1. Chemicals

GSH, L-cysteine, L-glutamic acid, γ-GC, N-ethylmorpholine, OPA and the kit for protein determination (Lowry method) were purchased from Sigma (St. Louis, MO, USA). MB (Thiolyte reagent) was obtained from Calbiochem (San Diego, CA, USA). The OPA solution was prepared daily by mixing an aqueous solution of 343 mg of sodium tetraborate decahydrate (pH 9.5) with a solution of 54 mg OPA in 1 ml of HPLC-grade methanol.

2.2. Equipment

HPLC was performed using a 110B solvent delivery module liquid chromatograph equipped with a programmable 421A controller, Beckman 157 fluorescence detector (Beckman, San Ramon, CA, USA) and a Varian 4290 integrator. The excitation range of the detector was 303–395 nm and the detection range was 420–470 nm.

2.3. Chromatographic procedure

Separation was achieved using a Beckman Ultrasphere ODS (250 × 4.6 mm I.D., 5 μm particle size) reversed-phase stainless-steel column maintained at room temperature. The analytical column was protected by a Supelguard (5 × 4.6 mm I.D.), pellicular reversed-phase cartridge pre-column (Supelco, Bellefonte, PA, USA).

Following OPA derivatization, elution was carried out using an isocratic program with equal volumes of 0.25% acetic acid as solvent A (pH 3.9) and methanol–0.25% acetic acid (30:70, v/v) as solvent B (pH 3.9). The flow-rate was 1.5 ml/min.

Following MB derivatization, elution was carried out using a gradient program with the same two solvents. Starting conditions were 35% B for 1 min, followed by a linear gradient to 80% B within 7 min. At 8 min, 100% B was run for 5 min, and then starting conditions were maintained for 5 min before the next injection.

2.4. Preparation of derivatized standards and samples

For MB derivatization, freshly prepared solutions of γ-GC and GSH were added to a microfuge tube containing 20 μl of 50 m*M* N-ethylmorpholine (pH 8.5) and 10 μl of 50 m*M* MB in acetonitrile. The mixtures were incubated in the dark at room temperature for 15 min. The reaction was stopped by the addition of 10 *M* HCl, and the sample was and diluted with water. The diluted sample (20 μl) was injected onto the HPLC column.

For OPA derivatization, freshly prepared solutions of γ-GC and GSH were mixed with OPA solution (see Section 2.1) at room temperature for 1 min. After dilution with water, 20 μl were injected onto the HPLC column.

2.5. Isolation of cytosolic fraction from rat liver

Male Sprague–Dawley rats (body weight 200–250 g) were obtained from the University of Arizona, Division of Animal Resources and housed two to a cage in a room maintaining 12-h light–dark cycles. They were allowed food and tap water ad libitum. To avoid differences due to diurnal variation in enzyme activities related to GSH metabolism, all rats were killed by decapitation between 9:00 and 10:00 a.m. Tissues were removed and washed two times in saline at 4°C. Each tissue was minced and homogenized in four volumes of 0.25 *M* sucrose containing 1 m*M* EDTA and 20 m*M* Tris–HCl (pH 7.4). The homogenate was centrifuged at 3000 *g* for 10 min at 4°C and then the supernatant was centrifuged at 10 000 *g* for 20 min at 4°C. Finally, the supernatant was centrifuged at 105 000 *g* for 60 min at 4°C. The supernatant (cytosol) was used to determine the GSH concentration and γ-GC synthetase activity.

2.6. Determination of cytosolic γ-GC synthetase activity

The cytosolic γ-GC synthetase activity was determined based on the formation of γ-GC in the incubation system [10]. Incubations were performed in 1.5-ml microfuge tubes containing 10 m*M* Glu, 5 m*M* cysteine, 10 m*M* ATP, 0.1 *M* Tris–HCl (pH 8.2), 0.15 *M* KCl, 2 m*M* EDTA and 20 m*M* $MgCl_2$. After preincubation for 3 min at 37°C, cytosolic protein (about 1 mg) was added to initiate the enzyme reaction. The total volume was 1.0 ml and the incubation time was 15 min. The formation of γ-GC was quantified by both MB and OPA derivatization and taken to represent enzyme activity.

2.7. Determination of cytosolic GSH concentration

Hepatic cytosol (200 μl) was placed in a microfuge tube containing 5% sulfosalicylic acid (100 μl) and centrifuged for 3 min to precipitate protein. The supernatant (100 μl) was taken and derivatized with MB and OPA as described above.

3. Results

3.1. Chromatographic separation of fluorescent derivatives

We found that γ-GC, the precursor of GSH, reacts rapidly with OPA to yield a stable fluorescence. Under the chromatographic conditions described in Section 2, both the MB and OPA derivatives of γ-GC are well separated from GSH (Fig. 1). MB is itself fluorescent and reacts with all biological thiols in the samples, and not just GSH and γ-GC. For separation of MB adducts, therefore, an additional 5 min of 100% solvent B must be run in order to clear from the column MB and other unknown MB derivatives appearing after the elution of GSH (which has a retention time of about 10 min).

Unlike MB, OPA itself shows no fluorescence. At a pH higher than 9.5, OPA reacts only with γ-GC and GSH, and not with cysteine or other biological thiols. No interference peaks appear therefore (Fig. 1).

The calibration curves for both OPA and MB derivatives of γ-GC and GSH are linear up to 2500 pmol per injection. Relative fluorescences

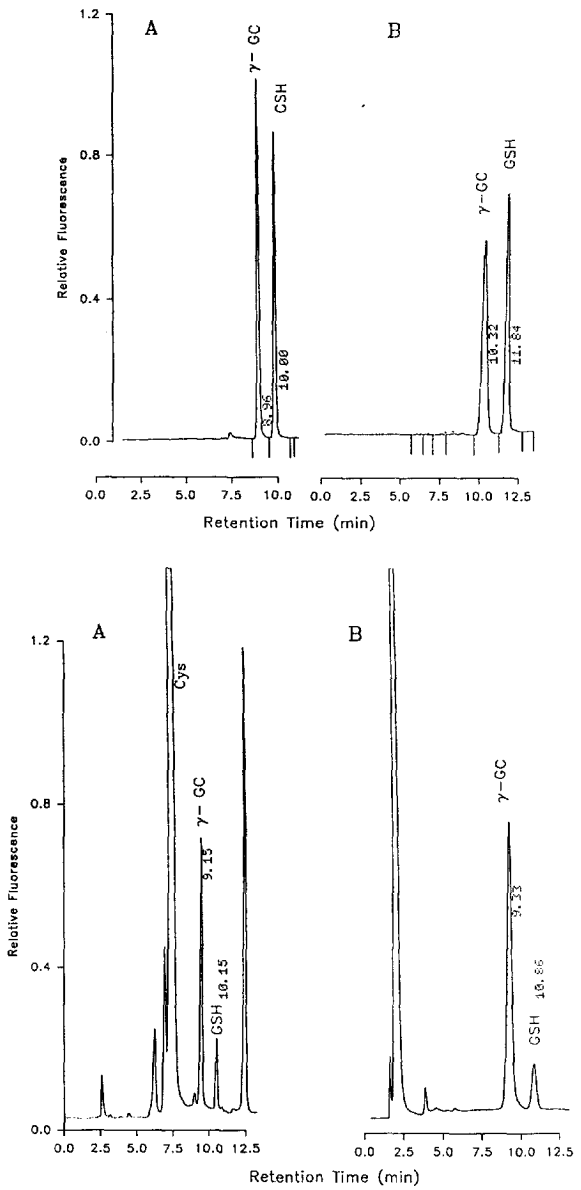

Fig. 1. Chromatograms of γ-GC and GSH derivatized with (A) MB and (B) OPA. Conditions are given in Section 2. Upper panel: chromatograms of standards; lower panel: chromatograms of samples from incubation of cytosolic protein (ca. 1 mg) at 37°C for 15 min with 10 m*M* glutamate, 5 m*M* cysteine, 10 m*M* ATP, 100 m*M* Tris–HCl (pH 8.2), 500 m*M* KCl, 0.2 m*M* EDTA and 20 m*M* $MgCl_2$.

for γ-GC and GSH for OPA were 1345 and 3184 per mol, respectively, and for MB were 2346 and 2942 pmol, respectively. Correlation coefficients were in excess of 0.995 for all four calibrations. The day-to-day variability in fluorescence yields for standards is less than 7.0%. The detection limit in the present system for OPA adducts is 12.5 pmol per injection and for MB adducts is 6.25 pmol per injection. These are the lower limits for peak recognition by the integrator.

3.2. Suitable pH for derivatization of γ-GC and GSH with OPA or MB

Derivatization of biological thiols with MB occurs under alkaline conditions (pH 8.5), and the products remain stable under acidic conditions [9]. Similarly, we find that derivatization of γ-GC with OPA is also pH-dependent, as has been shown for GSH [15]. Maximal fluorescence yield is obtained with a reaction pH between 9.5 and 12.0 (Fig. 2). The fluorescence yield decreases slowly with pH at a reaction pH between 9.5 and 6.0. As the pH drops below 6.0, the fluorescence yield decreases rapidly. At a pH lower than 4.7, almost no fluorescence is obtained from either GSH or γ-GC. Fig. 2 also shows that the pH dependency is the same for the reaction of both γ-GC and GSH with OPA.

McNeil and Beck [15] reported that GSSG

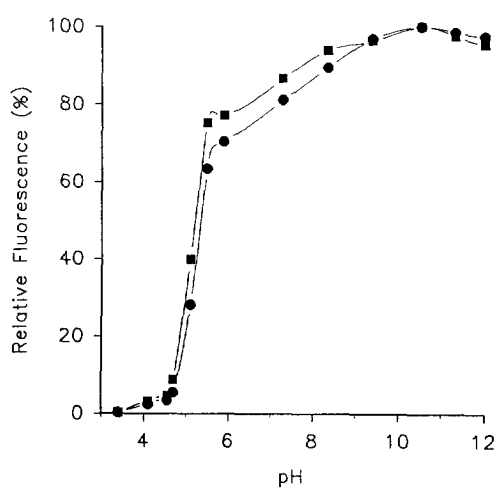

Fig. 2. Effect of pH on relative fluorescence of γ-GC–OPA (●) and GSH–OPA adducts (■). Standard γ-GC or GSH (10 μ*M*) was incubated with OPA buffered to the indicated pH. The fluorescence intensity at pH 10.5 was taken as 100%.

reacts with OPA to form a fluorescent derivative. We also found that GSSG reacted with OPA at pH 12 (data not shown) to give a fluorescent product. However, given the known cleavage of disulfides with hydroxide to give approximately 2 mol of sulfide anion [23] and the identical fluorescence spectra of both the reduced GSH–OPA adduct (formed at pH 8.5) and the oxidized GSH–OPA adduct (formed at pH 12) [18,24], it is highly probable that the two adducts are the same. Their identity is in keeping with the identity of retention time we find on HPLC chromatography (data not shown). Therefore, we chose to use a reaction pH of 9.5, the natural pH of our OPA reagent preparation, in order to reduce the interference caused by oxidized GSH. The assay, therefore, measures concentration of GSH only, and not that of GSSG that may also be present.

3.3. Stability of OPA and MB adducts of γ-GC and GSH

OPA derivatives of amino acids are light-sensitive. We compared the stability of both MB adducts and OPA adducts of γ-GC and GSH under different storage conditions. When both MB adducts and OPA adducts were stored at −20°C, fluorescence was stable for up to ten days. The reaction of OPA with γ-GC and GSH is rapid and the fluorescence yield is stable from 15 min to 24 h at both 25°C and 5°C when adducts were protected from light (data not shown). Fig. 3 shows the variation of relative fluorescence under various storage conditions for up to eight days following derivatization. In general, fluorescence increases somewhat by day 3 and then decreases slowly. The exception is that fluorescence of the GSH–OPA adduct stored at 5°C is lower at day 3 but then drifts up until day 8 (Fig. 3).

MB derivatives of γ-GC and GSH are more stable than the OPA derivatives (Fig. 4). Fluorescence is stable for three days under the storage conditions studied. After day 3, fluorescence decreases under 25°C dark storage and increases slightly under 5°C dark storage (Fig. 4).

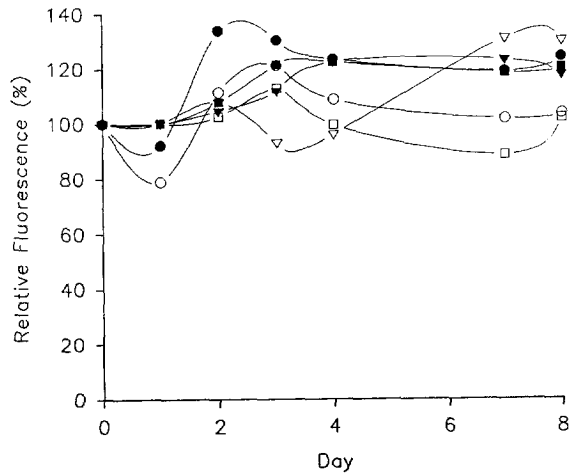

Fig. 3. Stabilities of γ-GC–OPA and GSH–OPA adducts under various storage conditions over eight days. 10 μM γ-GC (filled symbols) or GSH (open symbols) adduct was kept at 25°C in light (●, ○), 25°C in the dark (■, □) or 5°C in the dark (▼, ▽), respectively. The fluorescence intensity at 30 min was taken as 100%.

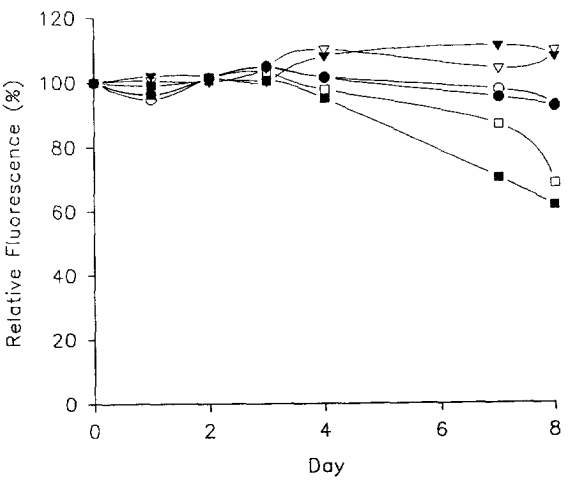

Fig. 4. Stabilities of γ-GC–MB and GSH–MB adducts under various storage conditions over eight days. 10 μM γ-GC (filled symbols) or GSH (open symbols) adduct was kept at 25°C in light (●, ○), 25°C in the dark (■, □) or 5°C in the dark (▼, ▽), respectively. The fluorescence intensity at 30 min was taken as 100%.

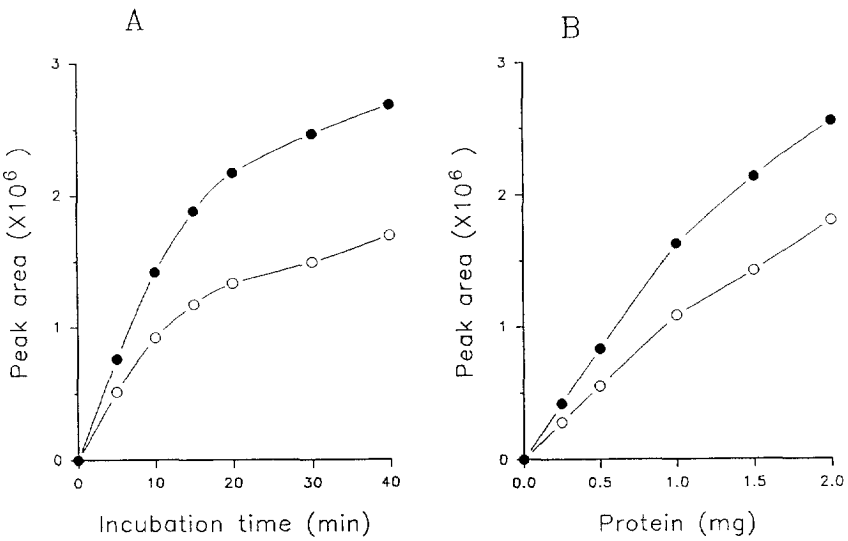

Fig. 5. Linearity of enzymatic production of γ-GC with (A) time and (B) protein. Incubation conditions were as described in Section 2, with hepatic cytosol as the enzyme source. The γ-GC–OPA adduct (○) and γ-GC–MB adduct (●) were separated and integrated by HPLC.

3.4. Determination of γ-GC activity and GSH concentration in liver cytosolic fraction

γ-GC synthetase activity was followed by fluorimetric determination of γ-GC formation. In the present incubation system, this is linear with time up to 20 min and with protein content up to 1.0 mg for both MB and OPA derivatization (Fig. 5).

Table 1 shows γ-GC synthetase activity and GSH concentration in rat liver cytosol, measured by both the OPA and MB derivatization procedures. Excellent agreement was found between the two methods for both determinations.

4. Discussion

Fluorescence detection of GSH and related thiols is both more selective and more sensitive than UV detection [25]. MB is a specific pre-column fluorescent reagent for thiols. Fluorescent derivatives of MB have been shown to be well separated by HPLC procedures (Fig. 1) [9].

Table 1
Comparison of γ-GC synthetase activity and GSH concentration in the liver cytosolic fraction of rat by OPA and MB derivatization methods

Derivatization method	γ-GC synthetase activity (nmol min^{-1} mg^{-1} protein)	GSH content (nmol/mg protein)
OPA	4.85 ± 0.47	90.40 ± 6.50
MB	4.42 ± 0.52	92.49 ± 3.35

Data are means ± S.D. for five determinations per point.

Inconveniences of the MB method include the fact that photodegradation products of MB are fluorescent and can interfere with thiol determination and that MB is not selective for GSH. Other thiols can interfere, and typically unknown peaks are observed on the chromatogram. Incubations are longer (15 versus < 1 min for OPA), and the reaction is pH-dependent, the pH needing to be dropped below 4 following derivatization [25]. The run-to-run time for MB is somewhat longer (Table 2). MB is also a relatively expensive reagent, with a current cost of $295 for 250 mg as against $36 for 1 g of OPA.

OPA is another reagent that has been used for the HPLC determination of GSH. Under alkaline conditions, GSH reacts with OPA and to give a fluorescent product [9,14,15,17,26]. Although OPA has also been postulated to react with γ-GC [22], it has not hitherto been applied to the determination of γ-GC or the assay of γ-GC synthetase. This synthetase catalyzes the rate-limiting step in GSH biosynthesis. We have now demonstrated that γ-GC forms a fluorescent adduct with OPA. The mechanism of reaction is probably the same as that of GSH with OPA, namely, that both the thiol and amine functional groups in γ-GC react with OPA to yield a fluorescent isoindole with the structure shown in Fig. 6. Both the GSH and γ-GC adducts with OPA are stable for a considerable period under a variety of storage conditions (Fig. 3). This stability permits the OPA derivatization method to be used for automated sampling analysis of GSH, γ-GC, and the enzymes for which these aminothiols are products.

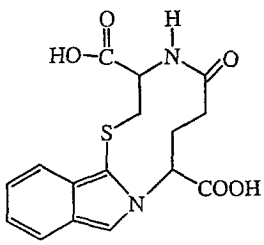

Fig. 6. Possible structure of the γ-GC–OPA adduct.

The relative advantages and disadvantages of the OPA and MB methods are compared in Table 2.

Table 2
HPLC analysis of γ-GC and GSH: comparison of OPA and MB derivatization methods

Parameter	MB method	OPA method
Limit of sensitivity (pmol) (signal-to-noise ratio = 2.5)	6.25	12.50
Variability of repeated runs on same standard (%)	1.0–6.5	3.1–7.0
Reproducibility of same sample derivatized on different days (%)	2.5–4.3	1.4–5.6
pH of derivatization	8.5	9.5
Duration of derivatization	15 min	Immediate
Stable adducts	72 h (if acidified)	24 h, dark
Fluorescence stable at −20°C?	Yes	Yes
Chromatographic conditions	Gradient	Isocratic
Run-to-run time (min)	16	13
Detection capability	Biological thiols	GC and GSH only
Interference by GSSG?	No	Yes when pH > 12
Retention time (min)	<12	<12
Interfering peak	Yes	No
Relative reagent cost per sample (OPA as 1)	60	1

Both the OPA and MB adducts can also be determined by their UV absorption at either 340 nm for the former or 380 nm for the latter. However, the sensitivity for UV detection is markedly lower than for fluorescence detection [9].

5. Conclusions

We have developed a sensitive and reproducible chromatographic method using fluorescence detection for the rapid analysis of OPA derivatives of γ-GC and GSH. The wide linear dynamic range in detector response allows the determination of γ-GC and GSH at a sensitivity of 12.5 pmol and its application for the determination of γ-GC synthetase activity and GSH concentration in animal tissues. The OPA derivatization method compares favorably to the MB derivatization method. The sensitivity, errors of the methods, stability, advantages and disadvantages are summarized in Table 2.

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