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High performance liquid chromatographic assay for the quantitation of total glutathione in plasma

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Abstract

A simple and widely used homocysteine HPLC procedure was applied for the HPLC identification and quantitation of glutathione in plasma. The method, which utilizes SBDF as a derivatizing agent utilizes only 50 μl of sample volume. Linear quantitative response curve was generated for glutathione over a concentration range of 0.3125–62.50 μmol/l. Linear regression analysis of the standard curve exhibited correlation coefficient of 0.999. Limit of detection (LOD) and limit of quantitation (LOQ) values were 5.0 and 15 pmol, respectively. Glutathione recovery using this method was nearly complete (above 96%). Intra-assay and inter-assay precision studies reflected a high level of reliability and reproducibility of the method. The applicability of the method for the quantitation of glutathione was demonstrated successfully using human and rat plasma samples.

Keywords: Total glutathione; HPLC; Plasma; Derivatization; GSH-SBDF; Isocratic separation

1. Introduction

Glutathione; γ-L-glutamyl-L-cysteinylglycine is the most abundant sulfhydryl containing tripeptide present in animal tissue [1]. It occurs mainly in the reduced form (GSH). In normal physiological conditions, the oxidized form of glutathione (GSSG) constitutes 1% of the total glutathione pool in the cell. GSH and GSSG interconvert by the actions of glutathione peroxidase and glutathione reductase [1].

The functions of glutathione have been the subject of intensive investigation for decades. It has been shown to play a major role in the protection of most cells from the effect(s) of endogenous and exogenous harmful oxidizing agents

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by acting as a scavenger for these toxic compounds [2,3]. In addition to its role as a reducing agent, it reacts enzymatically and nonenzymatically to form conjugates with various highly reactive electrophilic free radical compounds. These compounds are usually products of biotransformation reactions of endogenous metabolites as well as exogenous xenobiotics, which include therapeutic agents, toxicants, mutagens, and carcinogens. This conjugation process is an efficient detoxification mechanism that provides a means for the rapid transport and excretion of these toxic compounds [1].

Increased interest in glutathione as primary player in many cellular functions and detoxification mechanisms has led to the development of several different methods for the measurement of glutathione. A number of methods for the quantitation of glutathione in biological samples are documented in the literature [4–10]. Some of these methods are enzymatic [4], whereas others utilize high performance liquid chromatography (HPLC) [5–7] and gas chromatography (GC) [8,9]. The enzymatic assays are laborious and complicated, rendering them not suitable for routine analysis. GC and HPLC provide high degree of specificity and sensitivity. GC, however, is expensive and not readily available in most analytical laboratories, rendering the HPLC the most acceptable and widely used chromatographic technique for the separation and quantitation of biological compounds.

To the best of our knowledge, none of the published methods for the determination of glutathione in biological samples combine all the desired features for a rapid, reliable, sensitive, and a simple assay. In this report, the validation and applicability of a method for the quantitation of total glutathione is described. This method, which we adapted from a procedure that was originally described for the determination of homocysteine in plasma [10], does not require a liquid–liquid or solid-phase extraction step, and utilizes only 50 μl of sample volume. It utilizes a stable, non-hazardous reducing agent that converts GSSG to GSH. Total GSH is derivatized by a commercially available thiol-specific fluorogenic probe. Derivatized GSH is separated isocratically from other plasma thiols in a relatively short run time.

2. Materials and methods

2.1. Materials

Glutathione (99.9%), cystamine dihydrochloride (98%), homocystine (cell culture grade), and A.C.S. grade sodium acetate were purchased from Sigma Chemical Company (St. Louis, MO). HPLC grade methanol, acetic acid, and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). Trichloroacetic acid (TCA, $>99\%$) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Ethylenediaminetetraacetic acid (EDTA, electrophoresis purity) was purchased from Bio-Rad Laboratories (Hercules, CA). Phosphate-buffered saline (PBS) pH 7.4, was purchased from Life Technologies (Grand Island, NY). Tris (2-carboxyethyl) phosphine (TCEP, $>98\%$), boric acid ($\geq 99.5\%$), and HPLC grade ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBDF, $>99\%$), were purchased from Fluka (Milwaukee, WI). Autosampler vials of 12×32 mm (clear crimp-top) with 100 μl limited volume inserts were purchased from Alltech Associates Inc. (Deerfield, IL). Luna [C18 (2)] analytical column (150×4.6 mm, 5 um) and C18 octadecyl ODS guard column (4×3 mm) were purchased from Phenomenex (Torrance, CA). Water used in this project met the specifications for Type II water, according to the guidelines of the National Committee for Clinical Laboratory Standards [11]. Water was filtered through Nanopure System (Barnstead, Dubuque, IA).

2.2. Methods

2.2.1. Plasma preparation

Rat plasma specimens were obtained from whole blood collected into heparinized tubes. Blood was collected from rats by cardiac puncture immediately after sacrifice. Blood collection protocol, was approved by the Morehouse School of Medicine Institutional Animal Care and Use Committee. Within 30 min of collection, plasma was separated by centrifugation at $3000 \times g$ for 25 min at 4 °C. Supernatants were transferred to clean appropriately labeled test tubes and stored at -80 °C until analysis.

2.2.2. Preparation of the derivatization solution

The derivatizing agent was composed of three components A, B, and C. Component A was composed of 6.25 ml of 0.125 M borate buffer containing 4 mM EDTA. The pH of component A was 9.5. Component B was 1.55 M NaOH. Component C was prepared by dissolving 0.1% (w/v) SBDF into component A. The three components (A, B, and C) were mixed in a ratio of 12.5:1:5 (v/v/v), respectively. This derivatizing solution was prepared fresh on the day of analysis.

2.2.3. Sample preparation for HPLC analysis

On the day of analysis, plasma samples were allowed to thaw down to room temperature. To 50 μl of plasma, 25 μl of phosphate-buffered saline (PBS, pH 7.4), and 25 μl of internal standard solution (5 μM cystamine dihydrochloride) were added. This achieved a final internal standard concentration of 1.25 μM in a total assay volume of 100 μl. This resulted in a final free thiol cocentration of 2.5 μM (cystamine has two thiol groups). The mixture was incubated with 10 μl of 100 mg/ml tris(2-carboxymethyl phosphine) (TCEP). The reduction reaction was allowed to proceed for 30 min at room temperature. Plasma proteins were precipitated by the addition of 100 μl of trichloroacetic acid (10%, w/v) containing 1 mM EDTA. The samples were vortexed for 20 s and centrifuged at $13000 \times g$ for 10 min at room temperature. Supernatants were transferred to autosample vials containing 200 μl of freshly prepared derivatization solution (see above). Samples were transferred to a 60 °C dry bath and derivatization reactions were allowed to proceed for 1 h. At the end of the incubation period, autosample vials containing the samples were loaded onto a Hewlett Packard 1100 series HPLC system [Hewlett Packard (currently Agilent Technologies), Palo Alto, CA] for analysis.

Because glutathione-deficient plasma is not available commercially, PBS was used as the medium to construct the standard curve. For the construction of the standard curve, tubes containing 50 μl PBS were spiked with 25 μl of 5 μM cystamine (internal standard) and increasing concentrations of standard glutathione (0.3125, 0.625, 1.25, 2.5, 6.25, 12.5, 25, and 62.5 μM). The total volume of each assay tube was 100 μl. All spiked tubes used for the construction of the standard curve were treated exactly like actual plasma samples (with unknown glutathione concentrations) throughout the analysis including the protein precipitation step. Positive and negative controls were included with every batch run.

2.2.4. Chromatographic conditions

Samples (50 μl) from the autosample vials were injected into a 150×4.6 mm Luna [C18 (2)] column protected by a 4×3 mm C18 ODS (octadecyl) guard column. The mobile phase consisted of 3% methanol and 97% of 0.1 M sodium acetate buffer, pH 5.5. Separation of the derivatized thiols was achieved under isocratic conditions at a flow rate of 0.7 ml/min and a column temperature of 35 °C. Calibration was conducted daily using PBS standard curve. Derivatized glutathione and cystamine were monitored with a Hewlett Packard 1046A Fluorescence Detector (Agilent Technologies, Palo Alto, CA) at an excitation wavelength of 385 nm and emission wavelength of 515 nm. Data analyses were conducted using HP Chemstation (Agilent Technologies, Palo Alto, CA).

3. Results and discussion

As shown in Fig. 1, derivatization of deproteinated plasma samples with the thiol-specific reagent ammonium (SBDF) under the above-described HPLC conditions results in a simple and clean chromatogram with well separated plasma thiol peaks (Fig. 1A and B). The derivatized glutathione (GSH-SBDF) peak was identified by two ways (1) spiking a blank PBS sample with glutathione, and (2) adding glutathione to a plasma sample already containing glutathione and observing an increase in the corresponding symmetrical peak. PBS sample not spiked with glutathione did not show any glutathione peak. The internal standard peak (cystamine) was identified in a similar manner. Plasma samples with no internal standard showed no derivatized cystamine (CYSM-SBDF) peak. Although this method is validated for the quantitation of glu-

tathione, it also allows for the analysis of homocysteine (Fig. 1A and B).

As shown in Table 1, the retention times for GSH-SBDF and CYSM-SBDF are 6.9 and 8.5 min, respectively. The calibration curve obtained for GSH-SBDF was constructed using linear regression analysis. Linear quantative response curve was achieved over a concentration range of 0.3125–62.5 μmol/l. Analysis of the regression line resulted in a correlation coefficient of 0.999 (Table 1). The formula of the line is also shown in Table 1. The limit of detection (LOD) and limit of quantitation (LOQ) for glutathione was determined experimentally ($n = 4$). For LOQ, blank specimens were spiked with a series of decreasing concentrations of glutathione and a constant concentration (1.25 μM) of the internal standard. LOQ was defined as the concentration corresponding to a signal to noise ratio ≥ 3. LOQ was defined as the lowest quantitated concentration that was within 10% of the target concentration. As shown in Table 1, LOD and LOQ values obtained for glutathione were 5.0 and 15 pmol, respectively. Based on literature review, these values suggest that this method is significantly more sensitive than that reported with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) as a derivatizing agent [12].

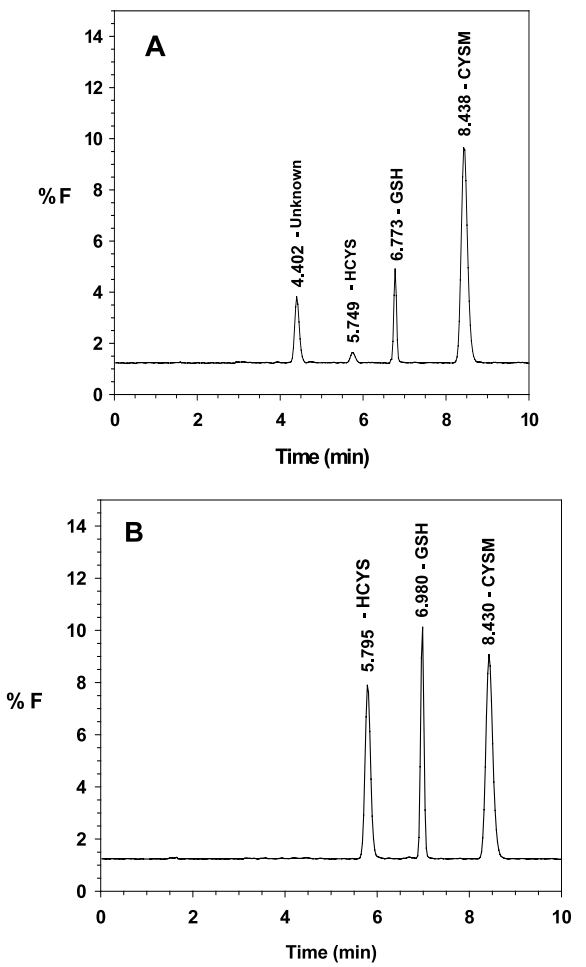

Fig. 1. HPLC Chromatogram of SBDF-derived GSH, CYSM, HCYS from (A), a PBS Standard and, (B), a Plasma Sample. PBS standard was fortified with GSH, CYSM, and HCYS to give final concentrations of 6.25, 2.5, 6.25 nmol/ml, respectively. GSH, CYSM, and HCYS concentrations in the plasma sample were 1.7, 2.5, and 1.7 nmol/ml, respectively.

Table 1
Performance parameters for the quantitation of total glutathione in plasma using HPLC

Performance parameters	Description/Value
Retention time of glutathione	6.9 min
Retention time of cystamine (I.S.)	8.5 min
Validated linearity range	0.3125–62.5 μmol/l
Equation of the line	$RR^a = 0.224 \times AR^b + 8.163 \times 10^{-4}$
Correlation coefficient (r^2)	0.999
LOD	5.0 pmol
LOQ	15 pmol

[a] RR = Response ratio; Area of analyte/Area of internal standard.

[b] AR = Amount ratio; concentration of analyte/concentration of internal standard.

Table 2 illustrates the percent mean recoveries of glutathione at three concentrations (0.3125, 6.25, and 62.5 μmol/ml). These three concentration points represent the low, middle, and high portions of the standard curve. Percent mean recoveries of GSH-SBDF at the three concentrations were virtually identical. As shown in Table 2, percent mean recoveries GSH-SBDF at the low, medium, and high concentrations were 96.3, 98.7, and 99.1%, respectively.

Intra-assay and inter-assay precision of the analytical procedure as represented by percent corre-

Table 2
Percent recovery and precision of the total glutathione SBDF assay

Glutathione concentration (umol/l)	Percent recovery (%)	Intra-assay precision		Inter-assay precision	
		Mean concentration[a] (umol/l)	%C.V.[b]	Mean concentration[a] (umol/l)	%C.V.[b]
0.3125	96.3	0.301	6.52	0.298	7.91
6.250	98.7	6.17	5.15	5.99	6.08
62.50	99.1	61.93	1.96	60.51	3.14

[a] $n = 4$; [b] Coefficients of variations (C.V.) were calculated as standard deviations expressed as percentage of mean values.

lation of variance (%C.V.) is illustrated in Table 2. Precision was determined experimentally ($n = 4$) by spiking blank PBS samples with glutathione at concentrations of 0.3125, 6.25, and 62.50 μmol/l. At the three concentrations, intra-assay precision (%C.V.) values ranged from 1.96 to 6.52% (Table 2). Inter-assay precision of the method was determined experimentally in a manner similar to that of intra-assay precision. Spiked PBS samples were analyzed on daily basis for 2 weeks. Inter-assay precision (%C.V.) ranged from 3.14 to 7.91% (Table 2). The relatively higher %C.V. values observed with the low glutathione concentrations (6.52 and 7.91 for intra-assay and inter-assay precision, respectively) are because this concentration is near the LOD where greater variation should be expected.

The applicability of this method to determine glutathione concentrations in plasma samples containing unknown amounts of glutathione was demonstrated successfully. For comparison purposes, two standard curves were constructed; one with PBS and the other with pooled plasma (by the addition method). From an ongoing study in our laboratory on the effect of oxidative stress on glutathione content and blood pressure, 15 rat plasma samples with a wide range of glutathione concentrations were assayed for total glutathione using the two standard curves (PBS and plasma). Data obtained from the two standard curves were comparable (Table 3). This suggests that with this method PBS can be used as a medium for the standard curve for total glutathione quantitation. Additionally, using 40 rat plasma samples, the applicability of this method was further tested by comparing it with an established HPLC procedure that utilizes monobromobimane as a derivatizing agent [13]. Data obtained from the two methods compared favorably with each other and the correlation (0.994) between the two methods was very good (Fig. 2).

The theory behind this method is that tris(2-carboxyethyl)phosphine (TCEP) reduces oxidized glutathione (GSSG) to the reduced form (GSH).

Table 3
Comparison of PBS and plasma standard curve for the detrmination of total glutathione concentrations in plasma

Specimen I.D.	Total glutathione concentration (μM)	
	PBS standard curve	Plasma standard curve[a]
1	0.34	0.47
2	0.69	0.81
3	1.76	2.23
4	2.26	3.01
5	3.06	3.98
6	7.94	8.89
7	9.22	10.23
8	16.31	15.56
9	29.43	31.98
10	34.12	36.01
11	48.79	44.81
12	53.60	49.98
13	59.50	56.65
14	61.19	64.03[b]
15	67.76[b]	64.87[b]

[a], Final total glutathione concentration was calculated by subtracting the total glutathione concentration (2 μM) that was originally in the pooled plasma used for the construction of the standard curve; [b], Values exceed the highest concentration used in the standard curves.

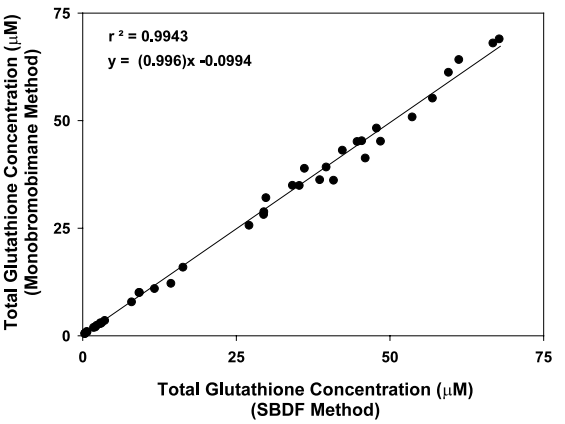

Fig. 2. Correlation between this SBDF assay and the monobromobimane assay for total plasma glutathione.

The derivatizing agent (SBDF) derivatizes the total glutathione pool (the reduced glutathione originally present in plasma plus the newly reduced glutathione by the action of TCEP). Whether reduced glutathione (rather than total glutathione) concentrations can be determined by the elimination of the reduction step is currently under intensive investigation in this laboratory.

The only drawback of the method is that it cannot be used for the determination of glutathione concentration in cardiac muscle. Preliminary data have shown that an endogenous compound in the heart muscle interferes with the internal standard (cystamine) used with this method. Work is under way in this laboratory to investigate the applicability of this method with other organs such as kidney and liver. Hitherto, preliminary data from our laboratory suggest that supernatants prepared from these tissues must be diluted for they contain significantly higher concentrations of glutathione than plasma.

In summary, this report discusses the applicability of SBDF for the measurement of total glutathione in plasma. The excellent characteristics of SPDF as a thiol-specific derivatizing agent have been described [14–16]. Unlike monobromobimane, which has been reported to show background fluorescence and to form fluorescent degradation products [17,18], SBDF exhibited no fluorescent degradation products and had no background fluorescence. More importantly, SBDF adducts showed high fluorescence.

4. Conclusion

The method presented here is simple, selective, rapid, and reliable. Because practicality and low instrument cost are central features of a routine laboratory test, this method is well within the capabilities of the average analytical laboratory. The assay shows high sensitivity, excellent precision, and nearly complete recovery of glutathione. More importantly, the method utilizes only 50 μl of sample volume rendering it suitable for pediatric samples in hospital laboratories as well as samples from small research laboratory animals. The present method has been found reliable as a routine assay in our laboratory, and about 50 plasma samples can be processed overnight.

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