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Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection

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Abstract

A HPLC method for determination of both reduced (GSH) and oxidized (GSSG) glutathione in plasma, whole blood and rat hepatocytes has been developed and evaluated. Reduced glutathione reacts with orthophthaldehyde (OPA) to form a stable, highly fluorescent tricyclic derivate at pH 8, while GSSG reacts with OPA at pH 12. At measurement of GSSG, GSH was complexed to *N*-ethylmaleimide. For the separation, reverse phase column Discovery C_{18}, 150 mm × 4 mm, 5 μm, was used. The mixture of methanol and 25 mM sodium hydrogenphosphate (15:85, v/v), pH 6.0, was used as mobile phase. The analytical performance of this method is satisfactory for both GSH and GSSG. The intra-assay coefficients of variation were 1.8 and 2.1% for whole blood, 2.0 and 1.9% for rat hepatocytes, 4.3 and 5.2% for plasma. The inter-assay coefficients of variation were 5.8 and 6.2% for whole blood, 6.6 and 7.1% for rat hepatocytes, 6.9 and 7.8% for plasma. The recoveries were as follows: 98.2% (CV 3.5%) and 101.5% (CV 4.2%) for whole blood, 99.1% (2.5%) and 102.3 (4.4%) for rat hepatocytes, 94.1% (CV 7.5%) and 103.5 (CV 8.5%) for plasma. The calibration curve was linear in the whole range tested. The limit of detection was 14.0 and 5.6 fmol, respectively. The preliminary reference ranges of reduced and oxidized glutathione in a group of blood donors are (4.69 ± 0.93) and (0.28 ± 0.12) μmol/g Hb for whole blood, (1.82 ± 0.55) and (0.154 ± 0.044) μM for plasma.

Keywords: Reduced and oxidized glutathione; Whole blood; Rat hepatocytes; Orthophthalaldehyde; *N*-Ethylmaleimide

1. Introduction

The tripeptide, glutathione (γ-glutamyl-cysteinyl-glycine) can be obtained from the diet or can be synthesized de novo in the liver [1–4]. It plays important roles in biological systems. Glutathione (GSH) can readily be oxidized to its disulfide (GSSG) and the ratio of both forms is crucial for the characterization of the oxidative stress in cells. It also plays a role in the regeneration of other antioxidants. It can regenerate ascorbic acid from dehydroascorbic acid and membrane bound α-tocopherol from the α-tocopheryl radical formed during inhibition of lipid peroxidation [5,6]. Glutathione is important in the detoxification of potentially harmful endogenous compounds and xenobiotics (e.g. α-oxoaldehydes, monoamines, polyphenoles and some drugs). It acts as cofactor for enzymes including glutathione peroxidase, and other peroxidases, dehydrochlorinase, formaldehyde dehydrogenase, glyoxylase, maleyl-acetonase isomerase, and prostaglandin endoperoxidase isomerase [7,8]. The multifunctional properties of glutathione are reflected by the growing interest in this tripeptide.

Several methods are available for the determination glutathione in biological samples. Glutathione is measured after protein precipitation by spectrophotometry, fluorometry or by HPLC. Oxidation of GSH during sample preparation represents a major problem. The blocking of thiol group with various agents such as *N*-ethylmaleimide (NEM) iodoacetic acid and 2-vinyl pyridine is often used to prevent this phenomenon [9].

The most widely used technique, enzyme recycling, measures total glutathione (GSH and GSSG) in a reaction involving NADPH, 5,5′-dithiobis-(2-nitrobenzoic acid) and glutathione reductase [10].

A variety of HPLC techniques have also been developed. HPLC with ultraviolet detection requires derivatization [11–16]. With regard to poor limit of detection it may not

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be sensitive enough for some biological samples. HPLC with fluorescence detection requires derivatization with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) [17–20], ortophthalaldehyde (OPA) [21–25], monobromobimane [26–30], dansyl chloride [31] or 5-methyl-(2-(*m*-iodoacetylaminophenyl) benzoxazole) (MIPBO) [32]. HPLC with electrochemical detection, using of either amperometric or coulometric electrodes, can measure GSH and GSSG directly. These techniques avoid typical problems associated with derivatization procedures. Coulometric detection offers superior sensitivity and selectivity to the dual-amperometric approach. However, the measurement of GSSG requires a relatively high applied oxidation potential, shortening the life of the operating electrochemical cell [33–46]. Quite recently liquid chromatography/mass spectrometry assays were developed for the determination of glutathione [47–49] or glutathione conjugates [50].

The aim of this study was to develop rapid, simple HPLC method for measurement both GSH and GSSG in different biological samples, suitable for clinical trials.

2. Experimental

2.1. Reagents and chemicals

Reduced glutathione, oxidized glutathione, *N*-ethylmaleimide, orthophthalaldehyde (OPA), sodium hydroxide, hydrochloric acid, sodium hydrogenphosphate, metaphosphoric acid, EDTA, type I collagen and trypan blue were obtained from Sigma–Aldrich, medium William's E without phenol red, fetal bovine serum, penicillin, streptomycin and glutamine from Pan Biotech GmbH, HPLC-gradient grade methanol from Merck KgaA. Collagenase crude was obtained from SEVAC (Prague, Czech Republic) and insulin (Actrapid, Novo Nordisk), glucagon (Glukagen, Novo Nordisk), prednisolone (Solu–Decortin, Merck) were from supplier mentioned in brackets. All the others chemicals were of analytical grade.

GSH and GSSG solutions were prepared daily in 1 mM hydrochloric acid and stored at 4 °C until used.

2.2. Instrumentation

Chromatographic analysis was performed with a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-10ADvp solvent delivery system, SIL-10ADvp autosampler, CTO-10ASvp column oven, RF-10Axl fluorescence detector and SCL-10Avp system controller. Data were collected digitally with CSW 32 chromatography software (DataApex, Prague, Czech Republic).

2.3. Animals

Male albino Wistar rats (BioTech, Konárovice, Czech Republic) were housed at 23 ± 1 °C, 55 ± 10% relative humidity, air exchange 12–14 times/h, and 12 h light/12 h dark cycle periods (6:00 a.m. to 6:00 p.m.). The animals had free access to standard laboratory rat chow (DOS 2B, Velaz) and tap water. All animals received care according to the guidelines set by the Institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

2.4. Hepatocyte culture

Hepatocytes were isolated from rats mentioned above with the body mass of 230–270 g by collagenase perfusion [51,52]. The viability of freshly isolated hepatocytes was more than 90% as confirmed by trypan blue exclusion. Isolated hepatocytes were suspended in William's E medium supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (10 mg/ml), insulin (0.08 IU/ml), prednisolone (0.5 μg/ml), glucagon (0.008 μg/ml) and plated in collagen-coated Petri dishes (60 mm) at a density of 2×10^6 cells/Petri dish. Hepatocytes were allowed to attach in a gassed atmosphere (5% CO_2) at 37 °C for 2 h. At the end of the incubation period, the medium and hepatocytes were collected for determination of both reduced and oxidized glutathione.

2.5. Subject and samples

Samples of peripheral venous blood with EDTA as anticoagulant were obtained from a group of healthy blood donors ($n = 70$, 35 women and 35 men in the age 27–61 years, mean age 41 years). None of the participants had a serious or chronic disease and took any medications on the day of blood sampling. A written informed consent was obtained from all participants before starting the protocol and the study was approved by the Hospital Committee on Human Research (Regional Hospital of Pardubice, Czech Republic). Plasma was separated from red blood cells by centrifugation (1700 × *g*, 15 min, 8 °C).

2.6. Sample preparation

Cold 10% metaphosphoric acid was carefully added (400 μl) to plasma, whole blood, hepatocyte samples or standards (200 μl). After incubation (4 °C, 10 min) and centrifugation (22,000 × *g*, 15 min, 4 °C) supernatants were transferred into 1.5 ml propylene tubes (50 μl for determination of GSH and 200 μl for determination of GSSG) and immediately stored at −80 °C.

2.7. Derivatization procedure

Derivatization procedure was performed by a slight modification of the method of Hissin and Hilf [53].

2.7.1. GSH assay

To 50 μl of the supernatant, 1.0 ml of 0.1% EDTA in 0.1 M sodium hydrogenphosphate, pH 8.0, was added. To 20 μl portion of this mixture, 300 μl of 0.1% EDTA in 0.1 M sodium hydrogenphosphate, and 20 μl of 0.1% OPA in methanol, was added. Well-capped tubes were incubated at 25 °C for 15 min in dark. The reaction mixture was then filtered through a 0.20 μm nylon filter (4 mm diameter, Supelco, USA) and stored at 4 °C.

2.7.2. GSSG assay

A 200 μl portion of supernatant was incubated at 25 °C with 200 μl of 40 mM NEM for 25 min in dark to interact with GSH present in the sample. To this mixture, 750 μl of 0.1 M NaOH was added. A 20 μl portion of this mixture was taken for measurement of GSSG, using the procedure outlined above for GSH assay, except than 0.1 M NaOH was employed as diluent rather than 0.1% EDTA in 0.1 M sodium hydrogenphosphate.

The stability of GSH in samples at room temperature and 4 °C, as well as glutathione-OPA adduct at 4 °C was examined.

2.7.3. Chromatography method

Chromatography of GSH and GSSG after their derivatization with OPA to form a stable, highly fluorescent tricyclic derivate [53] was accomplished using isocratic elution on Discovery C_{18}, 150 mm × 4 mm i.d., 5 μm analytical column fitted Discovery C_{18}, 20 mm × 4 mm i.d., 5 μm guard column (Supelco, USA) at 37 °C. The mobile phase consisted of 15% methanol in 25 mM sodium hydrogenphosphate (v/v), pH 6.0. The flow rate was kept constant at 0.5 ml/min. Optimum response of the fluorescent derivate was observed when the excitation and emission wavelengths were set at 350 and 420 nm, respectively. The amount of GSH and GSSG was quantified from the corresponding peak area using a CSW 32 chromatography software. The concentration of GSH and GSSG in the samples was determined from the calibration curve.

2.8. Effect of N-ethylmaleimide to prevent interference of GSH at measurement of GSSG

Effect of NEM to prevent interference of GSH at measurement of GSSG was checked using GSH standard in concentration 2 mM, well exceeding the regular levels in follow-up samples. Standard solution of GSH was processed as well as samples for the determination of GSSG (see above). The level of standard was determined.

2.9. Additional analyses

Hemoglobin in the whole blood was measured with the set HEMOGLOBIN (Lachema, Czech Republic). Briefly, in a test tube was mixed 5.00 ml of working solution (0.8 mM potassium cyanide and 0.5 mM potassium ferricyanide in 1.1 mM *N*-methyl-D-glucamine buffer, pH 8.3) with 0.02 ml of blood sample or standard. After incubation (room temperature, 10 min) the absorbance of the sample or the standard was read against working solution at 543 nm.

2.10. Statistical analysis

The data are presented as mean values ± S.D. Differences between women and men were determined by Student's *t*-test. $p < 0.05$ values were considered statistically significant.

Table 1
Precision and recovery of glutathione for whole blood, hepatocytes and plasma sample analysis

	Intra-assay (CV %)		Inter-assay (CV %)		Recovery (%)	
	GSH	GSSG	GSH	GSSG	GSH	GSSG
Whole blood	1.8	2.1	5.8	6.2	98.2	101.5
Hepatocytes	2.0	1.9	6.6	7.1	99.1	102.3
Plasma	4.3	5.2	6.9	7.8	94.1	103.5

3. Results

3.1. HPLC analysis of GSH and GSSG

HPLC chromatogram of glutathione-OPA adduct in whole blood is shown in Fig. 2. The analytical parameters of GSH and GSSG analysis have been sufficient and are shown in Table 1. The calibration curve was linear in the whole range tested: (20.0–2000.0) μM of GSH and (2.0–200.0) μM of GSSG for whole blood and rat hepatocytes, (0.2–20.0) μM of GSH and (0.02–2.0) μM of GSSG for plasma. The limit of detection (14.0 fmol for GSH and 5.6 fmol for GSSG) was estimated as the lowest GSH and GSSG concentration, which was distinguishable with 95% probability from the first point of the calibration curve.

Derivatized glutathione was separated on a reverse-phase column using an isocratic system of methanol and sodium hydrogenphosphate. Optimization of the separation was obtained after studying the effect of the concentration of sodium hydrogenphosphate (from 5.0 to 50.0 mM), the pH (from 5.0 to 7.0) and the methanol concentration. Column temperature was adjusted from 25 to 45 °C. The mobile phase conditions leading to the best separation were: 25 mM sodium hydrogenphosphate (pH 6.0) containing 15% methanol. Optimal temperature interval was from 34 to 40 °C. The criteria were the resolution, stability of the fluorescence intensity and the analysis speed. Pursuant to records we can establish that presented method is highly robustness.

3.2. The determination of GSH and GSSG in human plasma and whole blood

The preliminary reference ranges of GSH and GSSG in a group of blood donors are (4.69 ± 0.93) and (0.28 ± 0.12) μmol/g Hb for whole blood, (1.82 ± 0.55) and (0.154 ± 0.044) μM for plasma. We found no significant differences in GSH and GSSG concentration between women and men both in plasma (1.84 ± 0.62 μM versus 1.78 ± 0.49 μM, $p = 0.475$ and 0.159 ± 0.037 μM versus 0.151 ± 0.053 μM, $p = 0.399$) and whole blood (4.87 ± 0.95 μmol/g Hb versus 4.74 ± 1.18 μmol/g Hb, $p = 0.685$ and 0.31 ± 0.14 μmol/g Hb versus 0.29 ± 0.14 μmol/g Hb, $p = 0.390$). We observed both in GSH and GSSG no significant correlation between whole blood and plasma ($R = 0.2828$, $p = 0.126$, $R = 0.2524$, $p = 0.154$, respectively). We found both in GSH and GSSG no significant correlation between age and concentration ($R = 0.2154$, $p = 0.265$ and $R = 0.1958$, $p = 0.301$ for plasma;

$R = 0.3132$, $p = 0.101$ and $R = 0.2913$, $p = 0.112$ for whole blood, respectively).

3.3. The determination of GSH and GSSG in hepatocytes

We have tested the protective effect of *S*-adenosylmethionine (SAMe) on D-galactosamine (GalN)-induced damage to rat hepatocytes in primary culture. The content of reduced and oxidized glutathione in GalN-exposed cells was diminished to 1.5 and 16% of the control values after 24 h, respectively. There was more than 10-fold decrease in GSH/GSSG ratio in affected cells compared to controls.

S-Adenosylmethionine at concentration of 50 and 1000 mg/l significantly reduced lactate dehydrogenase release from cells injured by 40 mM GalN after 24 h of incubation. Content of both GSH and GSSG was significantly increased only in cells treated with the highest dose of SAMe. *S*-Adenosylmethionine at any concentration we used did not change intracellular GSH/GSSG ratio in comparison with GalN alone. Only the highest concentration of SAMe was able to increase significantly the number of cells (48%, $p < 0.001$) with polarized mitochondria. These findings indicate beneficial effect of SAMe, especially at the highest concentration, on GalN-induced toxicity to rat hepatocytes in primary culture. This action of SAMe seems to be associated with reduction of plasma membrane damage and increased synthesis of glutathione.

3.3.1. Stability of GSH in samples and stability of glutathione-OPA adduct

Stability of GSH in whole blood and plasma samples at room temperature and 4 °C as well as stability of glutathione-OPA adduct at 4 °C are shown in Table 2. The effectiveness of various protein precipitants was tested as follows. To sample and standard solution, the following precipitant reagents were added: metaphosphoric acid (10%), perchloric acid (1 M), trichloroacetic acid (10%) and sulfosalicylic acid (10%). Only metaphosphoric acid as protein precipitant led to satisfactory recoveries of GSH and GSSG. Samples treated with metaphosphoric acid were stable at 4 °C for at least 24 h (with a decline 1.1% for GSH solution, 1.4% for whole blood, 2.1% for rat hepatocytes and 1.7% for plasma). Metaphosphoric acid precipitates were stable at −80 °C for at least 3 months with slight decline.

3.4. Effect of N-ethylmaleimide to prevent interference of GSH at measurement of GSSG

Fig. 1 shows the chromatogram of a standard GSH solution (2.0 mM) and the chromatogram of identical standard, treated with NEM (40 mM) and derivatized as GSSG sample. Results indicate that this amount of NEM is entirely sufficient (recovery was approximately 100%).

4. Discussion

The research on the function of glutathione has led to the development of numerous methods for its determination. Among spectrophotometric determinations, the most considerable assay

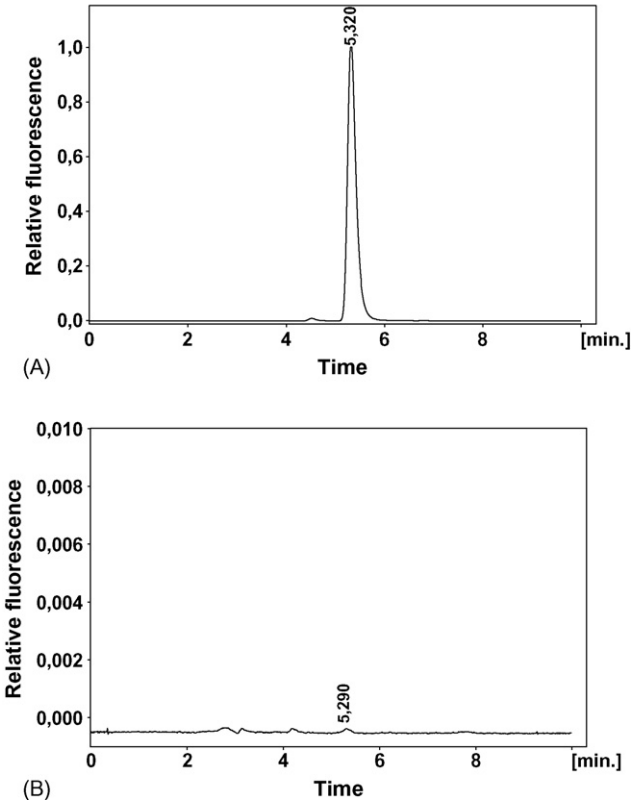

Fig. 1. (A) HPLC chromatogram of standard GSH (2.0 mM). Peak 1, glutathione-OPA adduct (GSH). (B) HPLC chromatogram of standard GSH (2.0 mM) treated with NEM (40 mM) and derivatized as GSSG sample. Peak 1, glutathione-OPA adduct. HPLC conditions: isocratic elution (mobile phase: 15% methanol in 25 mM sodium hydrogenphosphate, pH 6.0). Stationary phase was column Discovery C_{18}, 150 mm × 4 mm i.d., 5 μm (Supelco, USA) and guard column Discovery C_{18}, 20 mm × 4 mm i.d., 5 μm. The flow rate was kept constant at 0.5 ml/min, separation ran at 37 °C and glutathione-OPA adduct was monitored at excitation and emission wavelengths of 350 and 420 nm, respectively.

is based on the enzymatic recycling reaction discovered by Owens and Belcher [54] and developed by Tietze [10]. Cohn and Lyle [55] described a fluorimetric assay for GSH based on derivatization with OPA to form a highly fluorescent tricyclic derivative. Hissin and Hilf [53] modified this method for the determination of both GSH and GSSG.

Several HPLC methods with ultraviolet, fluorescence or electrochemical detection have been successfully applied to the determination of GSH and GSSG [11–46]. One of the most popular methods is HPLC with coulometric dual-channel detectors. A disadvantage of this method is the application of high oxidation potential at the measurement of GSSG [46].

The above mentioned fluorimetric method of Hissin and Hilf [53] for the determination of GSH as well as GSSG in liver tissue of rats can overestimate the amount GSSG in these samples, since more than 90% of the response arises from the reaction OPA with other reactive components [23,56,57]. Moreover, this method is not suitable for the measurement both GSH and GSSG in different biological samples, especially in human plasma. We propose a solution to this problem by using HPLC separation of the fluorescent tricyclic derivative.

Table 2
Stability of GSH in whole blood and plasma samples at room temperature and 4 °C. Stability of glutathione-OPA adduct at 4 °C. Calculated levels of GSH presented as means ± S.D.; each sample assayed in triplicate

Hours	GSH in whole blood (μM)		GSH in plasma (μM)		Glutathione-OPA adduct [GSH] (μM)	Glutathione-OPA adduct [GSSG] (μmol/L)
	4 °C	Room temperature	4 °C	Room temperature	4 °C	4 °C
0.0	842.2 ± 17.4	793.4 ± 2.2	1.52 ± 0.02	1.48 ± 0.03	983.1 ± 32.1	101.2 ± 4.8
0.25	737.6 ± 34.1	648.8 ± 22.5	1.51 ± 0.02	1.46 ± 0.02	981.1 ± 17.8	101.6 ± 3.7
0.5	702.4 ± 19.2	576.4 ± 12.8	1.48 ± 0.03	1.39 ± 0.04	973.3 ± 28.5	101.9 ± 4.2
1.0	630.2 ± 9.4	533.4 ± 12.2	1.43 ± 0.03	1.26 ± 0.05	965.4 ± 27.9	101.4 ± 2.1
2.0	539.0 ± 16.9	420.2 ± 17.6	1.36 ± 0.04	1.11 ± 0.06	972.3 ± 11.4	100.0 ± 3.0
4.0	466.8 ± 27.6	356.8 ± 6.4	1.12 ± 0.06	0.85 ± 0.05	966.4 ± 19.9	99.5 ± 5.2
8.0	413.0 ± 12.0	168.0 ± 4.2	0.93 ± 0.03	0.61 ± 0.07	968.4 ± 38.9	99.2 ± 5.6
24.0	209.0 ± 4.4	146.2 ± 7.4	0.61 ± 0.06	0.20 ± 0.05	961.5 ± 43.2	98.7 ± 2.1

Oxidation of GSH during sample preparation is the major problem at its measurement. We have found decreased concentration of GSH in whole blood by about 25% within 1 h (see Table 1). Originally, we have determined both GSH and GSSG in erythrocyte lysate. Erythrocytes were washed four-fold by isotonic solution (0.9% sodium chloride) and after their lysis by ice-cold deionized water immediately stored at −80 °C. With respect to rapid oxidation of GSH we have measured GSH in whole blood. Rossi et al. [58] suggest that trichloroacetic acid, at concentration of at least 80–100 g/l, prevents time-dependent GSH oxidation, and the use of high dose of NEM, coupled with short incubation times before acid precipitation of proteins, are the ideal conditions for the prevention of oxidation during acidification [9]. For the protein precipitation by acidification we have used 10% metaphosphoric acid. Under these conditions GSH was stable at 4 °C for at least 24 h (with a decline 1.1% for GSH solution, 1.4% for whole blood, 2.1% for rat hepatocytes and 1.7% for plasma). Metaphosphoric acid precipitates were stable at −80 °C for at least 3 months with slight decline. Hence we have immediately precipitated all the samples with cold 10% metaphosphoric acid.

Preliminary results indicated that a level of 40 mM NEM, as reported by Hissin and Hilf [53], was sufficient to inhibit the GSH-OPA reaction completely when using 2 mM of GSH (see Fig. 2). This concentration entirely exceeds the regular levels in follow-up samples.

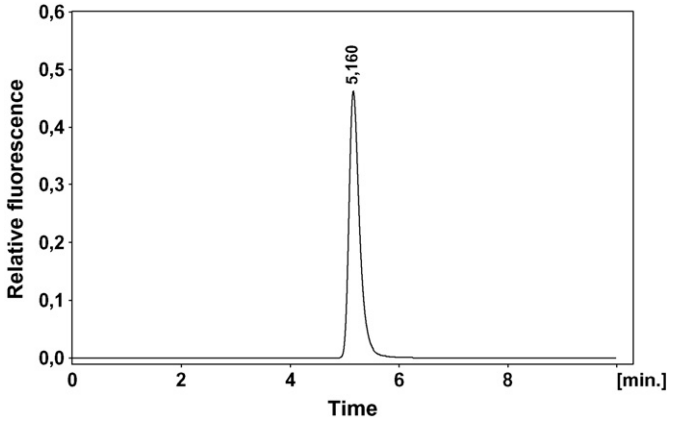

Fig. 2. HPLC chromatogram of whole blood GSH (706.76 μM, 6.01 μmol/g Hb). Peak 1, glutathione-OPA adduct (GSH). HPLC conditions: see Fig. 1.

The normal values for total, free and reduced glutathione in plasma and in whole blood differ somewhat from one laboratory to another [9]. The preliminary reference ranges of reduced and oxidized glutathione in a group of blood donors are (4.69 ± 0.93) and (0.28 ± 0.12) μmol/g Hb for whole blood, (1.82 ± 0.55) and (0.154 ± 0.044) μM for plasma. Blood glutathione amounts measured in women and men are not statistically different [9]. We found no significant differences in GSH and GSSG concentration between women and men both in plasma (1.84 ± 0.62 μM versus 1.78 ± 0.49 μM, $p = 0.475$ and 0.159 ± 0.037 μM versus 0.151 ± 0.053 μM, $p = 0.399$) and whole blood (4.87 ± 0.95 μmol/g Hb versus 4.74 ± 1.18 μmol/g Hb, $p = 0.685$ and 0.31 ± 0.14 μmol/g Hb versus 0.29 ± 0.14 μmol/g Hb, $p = 0.390$) too.

This method was used for study a protective effect of *S*-adenosylmethionine on cellular and mitochondrial membranes of rat hepatocytes against *tert*-butylhydroperoxide-induced injury in primary culture [52]. Within three years we have carried out more than 5000 analyses and have found out, that the lifetime of the analytical column is almost 3000 injects. Therefore we can establish that the presented method is inexpensive and suitable for clinical trials.

5. Conclusion

We developed a rapid, simple and very selective HPLC method with fluorescence detection for the determination of both reduced and oxidized glutathione in different biological samples. This method provides excellent sensitivity, precision, and accuracy in follow-up samples and is suitable for research whereas can be easily adapted for clinical testing purposes. By virtue of this method it is possible to determine both GSH and GSSG from filter paper spots in newborns.

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