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The Simple Isocratic HPLC–UV Method for the Simultaneous Determination of Reduced and Oxidized Glutathione in Animal Tissue

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Summary. The aim of our work was to optimize and apply simple high-performance liquid chromatography method with ultraviolet detection (HPLC–UV) for simultaneous determination of reduced (GSH) and oxidized (GSSG) glutathione in biological matrix (specifically, the rat liver tissue was used herein), since the ratio between oxidized and reduced glutathione forms (GSSG–GSH) has been recognized as an important biological marker of oxidatively depleted GSH in oxidative stress (OS)-associated diseases and poisonings. An isocratic chromatographic separation of GSH and GSSG (2.8 min and 6.3 min, respectively) was performed with the mobile phase consisted of sodium perchlorate solution (pH adjusted to 2.8) at flow rate of 1 mL min^{-1}, detection set at 215 nm, and column temperature of 40 °C. The method offers short run time, linearity in the range of 0.01–200 μM concentration for both compounds (R^2 = 1), low limits of detection and quantification (GSH: 0.18 μM and 0.56 μM, GSSG: 0.52 μM and 1.58 μM, respectively), precision, accuracy (bias < 2%), and high reproducibility.

Through suitable sample handling, an overestimation of GSSG was prevented. High recovery (>99%) was achieved. The method was successfully applied for the analysis of GSH and GSSG in liver homogenates of Wistar rats intraperitoneally exposed to cadmium (Cd) (1 mg kg^{-1} $CdCl_2$/21 days). Regardless of other Cd-mediated hepatotoxicity mechanisms, herein, we have exclusively interpreted/emphasized oxidative GSH depletion.

The presented method is acceptable for a routine analysis of GSH and GSSG in biological matrix, while the calculated ratio GSSG–GSH is considered as a valuable OS marker.

Key Words: oxidized/reduced glutathione, HPLC–UV, cadmium, hepatotoxicity

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Introduction

Within the elucidation of pathophysiological mechanisms, the interpretation of oxidative stress (OS) has attracted great attention during recent decades. Glutathione cycle is the essence of antioxidative response to OS exogenously/endogenously induced in living organisms. Considered to be a principal endogenous antioxidant [1], glutathione (GSH) reacts with metabolite intermediates forming mercapturic acid conjugates (second phase of metabolism) [2]. Additionally, a potential chelating property of GSH is taken into consideration for the detoxification of toxic metals. Glutathione is a tripeptide that consists of glycine, glutamate, and cysteine, present in micromolar (μM) concentrations in body fluids and in millimolar (mM) concentrations in tissues [3]. Under physiological conditions, reduced form of glutathione (GSH) is prevalent (~90% of total glutathione). GSH depletion occurs in accordance with OS development, due to its oxidation into disulfide form (GSSG). This is not the case within reactions of conjugation and/or chelation (when GSSG production does not occur). Thus, an increased ratio GSSG–GSH is considered as a sensitive and indicative biomarker of OS-associated diseases/poisonings [1]. Therefore, simultaneous determination of both glutathione forms is of great importance for the reliable interpretation of the GSH depletion.

Numerous methods are available for measuring GSH and GSSG in biological samples. Most of those methods are spectrophotometric, allowing determination of total and reduced glutathione, while the amount of GSSG corresponds to their difference. Several equations proposed for the calculation of total glutathione often lead to false estimation of GSSG level [1]. Previously developed techniques including enzymatic [4], fluorometric [5], and colorimetric [6] assays provided inadequate detection limits and poor specificity. Electrochemical methods avoiding derivatization have also been proposed previously [7], though loss of sensitivity of electrodes after a few hundred injections and the necessity of reconditioning are the major disadvantages of electrochemical methods. Furthermore, currently available high-performance liquid chromatography (HPLC) procedures for GSH and/or GSSG determination require a demanding sample preparation, including pre- or post-column derivatization [8]. A resulting overproduction of GSSG (GSH oxidation), which often occurs during alkalization and derivatization phase, is the major disadvantage of such sample preparation protocol [9, 10]. Although liquid chromatography–tandem mass spectrometry (LC–MS/MS) is the technique of choice for the simultaneous determination of GSH and GSSG (taking into consideration specificity and low limits of

detection and quantification), it is inaccessible to many laboratories [11–13]. Other methods, such as micellar electrokinetic capillary electrophoresis (MEKC) [14], nuclear magnetic resonance (NMR) [15], fluorescence [16], and chemiluminescence-based methods, have also been proposed [17]. The lack of total automatization and inability to determine protein-bound glutathione are the major limitations of capillary electrophoresis; NMR is not commonly available in clinical laboratories; fluorescence requires previous derivatization, while GSSG determination is not achievable by chemiluminescence methods.

Herein, we presented an isocratic HPLC–UV method enabling direct and simultaneous measurement of both glutathione forms with minimal biological sample handling avoiding derivatization. The method's applicability was tested in liver homogenates of the Wistar rats subacutely and intraperitoneally (i.p.) exposed to cadmium (Cd) and in the intact group. Cadmium may interfere with glutathione cycle through competition with essential metals at active sites of the antioxidative metalloenzymes and binding with thiol (–SH) groups of proteins, including GSH.

Materials and Methods

Experimental Protocol (Animal Study)

The experiment was conducted on male Wistar rats weighing in the range of 220–250 g randomly divided into the control group (intact group: $n = 6$) and the experimental group further subdivided into four groups ($n = 6$) according to the time of i.p. exposure to 1 mg $CdCl_2$ kg^{-1} day^{-1} for 1, 3, 10, and 21 days (Cd1, Cd3, Cd10, and Cd21 groups, respectively). The animals were sacrificed by decapitation, and liver was immediately removed and prepared for the analysis according to the proposed protocol. Experimental animals were treated according to the Guidelines for Animal Study, No. 12032014/9 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia and Montenegro). The rats were housed and adapted for the experiment under standardized housing conditions (ambient temperature of 23 ± 2 °C, relative humidity of 55 ± 3%, and a light–dark cycle of 13/11 h) and had free access to standard laboratory pellet food and tap water. All experiments were performed after a 7-day period of adaptation to laboratory conditions.

Chemicals

Glutathione reduced form (98–100% purity) and glutathione oxidized form (99% purity) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany), as well as ortho-phosphoric acid (OPA), and metaphosphoric acid (MPA). Sodium perchlorate monohydrate ($NaClO_4 \bullet H_2O$) was obtained from Acros Organics (New Jersey, USA).

Measurements

Glutathione status (GSSG–GSH ratio) was determined in the liver homogenates of Wistar rats after 1, 3, 10, and 21 days of the treatment. The presented modified HPLC–UV method was used for simultaneous determination of GSH and GSSG.

Standard solutions

Stock solutions of GSH and GSSG (1 mM) were prepared in 100 mM sodium perchlorate solution (pH was adjusted to 2.8 with 0.1% OPA) and stored at 4 °C. Working standard solutions were prepared daily via dilution in sodium perchlorate solution.

Sample collection and preparation

Tissue sample preparation required homogenization in 1 mL ice-cold saline solution followed by addition of 1 mL ice-cold MPA (5% w/v) for deproteinization. Liver homogenates were immediately centrifuged at 4 °C and 8000 rpm for 25 min. Resulting supernatant was used for chromatographic analysis or stored at −20 °C until analysis, while sediment was used for protein measurement. All tissue samples were kept on ice during the whole procedure. The results were expressed as nmol of GSH or of GSSG per mg of proteins. The Lowry method was used for protein determination [18]. The GSSG–GSH ratio was considered during interpretation of the results.

Apparatus and chromatographic conditions

HPLC analyses were performed on a Shimadzu Corporation HPLC system consisting of an LC-30AD Nexera Liquid Chromatograph Pump system, a

CTO-30A Nexera Column Oven, an SPD-M20A Prominence Diode Array Detector, a CBM-20A Prominence Communications Bus Module, an SIL-30AC Nexera Autosampler, and a DGU-20A5 Prominence Degasser, all manufactured by Shimadzu USA Manufacturing INC (Canby, OR, USA). For instrument control, data acquisition and data analyses, LabSolutions Main-system administrator, and Nexera software by Shimadzu (2008–2010) were used.

The chromatographic separation was accomplished on ZORBAX Eclipse AAA (4.6 × 150 mm, 3.5 μm) analytical column (Agilent Technologies) set at 40 °C and a flow rate of 1 mL min^{-1}. The detector was set at 215 nm. Isocratic separation was performed with the mobile phase consisting of 100 mM sodium perchlorate solution (pH was adjusted to 2.8 with 0.1% OPA). Injection volumes were 50 μL.

Identification and quantification of the GSH and GSSG peaks obtained from biological samples were ensured by comparisons with the corresponding data obtained from standard solutions.

Validation Study Protocol

Calibration curve analysis

The calibration curves were constructed by plotting peak areas against known GSH and GSSG concentrations (0.01–200 μM). In accordance with the common procedure, the low limits of detection and quantification (LLOD and LLOQ) were determined by the analysis of solutions with known concentrations of the analyte and by establishing the minimum level at which the analyte can be reliably detected (testing showed that 0.01 μM is reliably detected). Standard errors of the predicted *y*-values for each *x* in the regression (STEYX) were estimated statistically. LLOD and LLOQ were calculated based on the standard deviation of the response and the slope of defined calibration curve (3× STDEV of low concentration/slope of the calibration curve and 10× STDEV of low concentration/slope of the calibration curve, respectively).

Stability study procedure

The stability of GSH and GSSG in biological samples was tested referring to different time and temperature storage conditions, at three concentration levels in triplicate. Liver homogenates of Wistar rats were spiked with 25,

50, and 100 μM of GSH and GSSG in final concentration. The samples were subjected to short term temperature conditions for: (a) 1 h and 4 h at room temperature (the samples were stored on the HPLC autosampler); (b) 24 h at 4 °C, in the refrigerator; and (c) 24 h, at −20 °C. Stability of the samples was expressed as a percentage of decrease of GSH and GSSG peak areas related to different time (hours) and temperature (°C) storage conditions.

In order to prevent possible loss of GSH and the GSSG overestimation during sample handling, acid deproteinization was performed shortly after sample collection. Upon prompt centrifugation, acid supernatants were immediately analyzed or stored at −20 °C until analysis. Such stored supernatants gave good reproducibility for as long as 1 month, though immediately analyzed supernatants gave better chromatographic separation and peak sharpness.

Recovery study procedure

Recovery was evaluated at three concentration levels within calibration range. Taking into consideration the presence of the endogenous GSH and GSSG, liver homogenate of Wistar rat was divided in four portions; one portion was used as a biological matrix sample, and the other three were spiked with adequate portions of mixed GSH and GSSG stock solutions to obtain final concentrations of 1, 25, and 100 μM. Three different liver homogenates were fortified for recovery analyses. Upon applied experimental protocol, supernatants of spiked samples were injected for chromatographic analysis. For estimation of recovery, the peak areas of fortified samples were compared to those of neat standards.

Statistical Analysis

Data were analyzed with one-way ANOVA and Tukey's test for post-hoc multiple comparisons. PASW Statistics 18.0 software was used. Values are presented as mean ± standard deviations. Differences were considered statistically significant for $p < 0.05$.

Results and Discussion

Development and Optimization of the Method

The purpose of the study was to improve and modify previously reported HPLC methods for direct and simultaneous determination of GSH and GSSG levels in biological samples.

Analytical column selection

Multiple HPLC columns were tested in order to optimize chromatographic resolution. The highest peak resolution (>2), based on GSH and GSSG separation, was accomplished with the reversed-phase ZORBAX Eclipse AAA (4.6 × 150 mm, 3.5 μm) analytical column. Chromatographic separation was consistent throughout all performed measurements. Also, high peak resolution (>1) was accomplished with ZORBAX C8 Eclipse X-DB (4.6 × 150 mm, 5 μm) analytical column.

The column temperature was also tested for optimization. Testing showed that chromatographic resolution was the highest at 25 °C, but enhancement of the temperature to 40 °C increased peak sharpness and shortened the time of analysis.

The column needs to be daily washed with acetonitrile and then reconditioned with the mobile phase prior analysis in order to achieve consistent separation of GSH and GSSG.

Mobile phase optimization

Modifications were made in molar concentration of the mobile phase constituent. Adjustment of sodium perchlorate solution molarity to 100 mM allowed more consistent chromatographic separation of GSH and GSSG peaks in tested samples compared to 50 mM sodium perchlorate solution, even though the areas under detected peaks remained comparable. Also, due to the higher molarity, eluted peaks of GSH and GSSG were more symmetrical with less occurrence of GSH peak splitting. Chromatographic separation changed markedly due to changes in pH value of the mobile phase, resulting in the highest chromatographic resolution and consistency of retention times for GSH and GSSG peaks at pH value adjusted to 2.8. Changes in pH value above 3 or close to 2 significantly reduced chromato-

graphic resolution and separation, declined up to the point when it was not achievable. Addition of methanol and/or acetonitrile to the mobile phase severely impaired chromatographic resolution, e.g., 15% of methanol in mobile phase disrupted detection of GSH and GSSG. It was also concluded that mobile phase needs to be freshly prepared or stored overnight in the refrigerator at 4 °C. We also attempted to change the mobile phase constituent in order to use a chemical commonly used for other analyses in laboratories. The use of 100 mM potassium dihydrogen phosphate solution, with pH adjusted to 2.8, actually allowed detection of both GSH and GSSG. Though chromatographic separation was less satisfactory, the use of potassium dihydrogen phosphate solution shortened the time of analysis.

Sample preparation improvement

Sulfosalicylic (SSA) acid was tested as a protein-precipitating agent. It resulted in a large initial interfering peak overlapping GSH peak and caused the GSSG peak to disappear entirely, which lead to a conclusion that both forms of glutathione were already precipitated by the SSA. Thus, sample preparation protocol included the use of MPA as a protein-precipitating agent. The resulting chromatograms show that no interfering peaks occur due to the use of MPA. Adjusted protocol included the use of saline solution for homogenization instead of sodium perchlorate solution and EDTA, possibly leading to achievement of significantly higher recovery of GSSG compared to previous report [19].

Improvement of the method refers to high reproducibility, consistent chromatographic separation, advanced symmetry, and resolution of GSH and GSSG peaks, allowing more precise and accurate integration, while applied sample preparation protocol supports high recovery of GSSG.

Chromatograms of GSH and GSSG from standard solutions and from liver homogenates extracts

Chromatogram of the mixed standard solution of GSH and GSSG is shown in *Fig. 1a* in *Appendix B*. At used chromatographic conditions, selectivity factor ($a = k'b/\ k'a$) was higher than 3 which is within the ideal range of a (1 to 5), confirming an excellent separation of GSH and GSSG on the selected column. Optimized chromatographic conditions resulted also in short run times and sharp and symmetrical peaks of GSH and GSSG.

To demonstrate applicability of our HPLC–UV method, we measured GSH and GSSG level in liver homogenates of Wistar rats in the control group and the experimental groups *(Appendix B, Fig. 1b* and *c)*. Unidentified peaks refer to biological matrix and do not interfere with the peaks of interest.

Validation Study

Selectivity

The selectivity was demonstrated by comparing retention times and spectrums of GSH and GSSG obtained from samples (n = 10) with a corresponding set of data referring to the standard solution. The absence of interfering peaks from sample matrix and detectible amount of endogenous glutathione in both forms were demonstrated with the accomplished chromatograms. The applied sample preparation protocol along with the optimized chromatographic conditions for measurements of GSH and GSSG in biological matrix was found to be suitable for the routine analyses.

Calibration curve, LLOD, and LLOQ

Calibration curves for both compounds (GSH and GSSG) were defined for the range of 0.01–200 μM. The equations for the regression analysis were: $y = 2492.7x - 175.87$, $R^2 = 1$, for GSH and $y = 6648.8x + 743.4$, $R^2 = 1$, for GSSG (where y is the peak area and x is the concentration expressed in μM).

The lowest limits of detection (LLOD) were calculated to be 0.18 μM and 0.52 μM, while the lowest limits of quantification (LLOQ) were calculated to be 0.56 μM and 1.58 μM for GSH and GSSG, respectively.

Precision and accuracy

The coefficient of variation (C.V.) for intra-day precision test was below 2.56% for GSH and below 1.17% for GSSG. Inter-day precision test resulted with the coefficient of variation (C.V.) below 4.7% for GSH and below 2.5% for GSSG. Accuracy, calculated as a bias in the difference between expected concentration and measured concentration, was below 2%. Worthwhile analytical precision and accuracy of the method make this technique a convenient and reliable tool for glutathione determination *(Appendix A, Table I)*.

Stability

We observed that stability of biological samples does not change within 4 h when kept at room temperature, though considering the period of 24 h storage there is a difference in keeping the sample at 4 °C and at −20 °C. It was observed that there is no decrease of peak areas after 24 h storage at −20 °C, while decrease occurs after 24 h storage at 4 °C from 0.47% to 2.93% for GSH and from 1.49% to 7.6% for GSSG.

Recovery study

Achieved recoveries were high at all tested concentration levels, with an average value of 99.19% for GSH and 99.45% for GSSG. The results confirmed that sample preparation procedure does not affect GSH and GSSG analysis.

Applicability of the Modified Method

Intraperitoneal administration of Cd was chosen to mimic the inhalation of Cd through cigarette smoking, as tobacco leaves accumulate Cd from the soil. It is well known that smoking increases the risk of various cancer types, whereas Cd (in addition to various additives present in cigarettes) was recognized as the most significant contributor. It was intriguing to measure the content of GSH and GSSG in such experimental scenario because Cd depletes GSH through binding to its –SH group, as well as indirectly through interfering red-ox associated metalloenzymes when an increase of GSSG might be expected.

Interpretation of the chromatographic results for the GSH and GSSG requires expressing them in relation to the amount of proteins present in the liver tissue homogenates used for the analysis upon centrifugation. That is, chromatographically determined GSH and GSSG originate from the certain amount of the injected supernatants which were obtained after the centrifugation of the defined volume of the liver tissue homogenates. Approximately the same amounts of individual tissue sections were used to prepare the homogenates in which the proteins were measured by standard spectrophotometric Lowry method [18]. Therefore, the chromatographically acquired results were recalculated in regard to protein status.

Glutathione status (GSSG–GSH ratio) was determined in the liver of the animal subjects, intact and subacutely exposed to Cd. GSH and GSSG

were measured following 1, 3, 10, and 21 days of the treatment *(Appendix A, Table II)*.

Since elevated GSSG–GSH ratio indicates that glutathione cycling is actively involved in OS development (alluding to oxidatively depleted GSH), the presented method opens up the evaluation of OS involvement, more precisely, glutathione cycling engagement in Cd poisoning. Our results displayed a time-dependent gradual depletion of GSH in Cd-group compared with the control group *(Appendix A, Table II)*. Depletion of GSH may occur due to its ability to bind metals (toxic Cd and/or some other essential metals) or because of its oxidation into GSSG. Significant time-dependent gradual GSSG increase was achieved in the liver of the rats subacutely i.p. exposed to Cd within first 10 days, while thereafter started to decrease. Partial increase of GSSG could be explained by an increased production of free radicals (potent oxidizing agents) through Fenton reaction in which transition metals [in lower oxidation state(s)] participate together with hydrogen peroxide. In this particular case, essential metal status was changed upon exposure to Cd, because Cd competes with essential metals at active sites of antioxidative metalloenzymes. Thus, antioxidative defense was reduced and/or diminished by Cd [inhibition of antioxidative metalloenzymes, such as glutathione reductase (which catalyzes the reduction of GSSG to GSH), superoxide dismutase (which catalyzes dismutation of superoxide anion into molecular oxygen and hydrogen peroxide – the starting compound in the Fenton reaction) and catalase (which decomposes hydrogen peroxide to water and oxygen)] [20, 21].

Conclusion

The presented method for simultaneous determination of GSH and GSSG provides high reproducibility, consistent chromatographic separation under isocratic mode, short run time, advanced symmetry, and resolution of GSH and GSSG peaks (allowing more precise and accurate integration). Simplified sample preparation supports high recovery of GSH and GSSG along with the absence of interfering peaks from biological matrix. UV detection, applied instead of fluorescent, allows avoiding long-lasting derivatization procedure which entails the GSSG overestimation. Thus, the applied sample preparation protocol, along with the optimized chromatographic conditions for measurements of GSH and GSSG in biological matrix, was found to be suitable for the routine analyses.

If we acknowledge that oxidative stress pathway is initiated, then estimation of glutathione cycle involvement in certain pathophysiological scenario is fundamentally important for understanding the underlying mechanism(s) triggered by some diseases/poisonings (in this particular case, Cd).

We concluded that the GSH depletion in liver of the Wistar rats subacutely exposed to Cd, occurred either due to its binding to Cd or oxidation into GSSG by overproduced free radicals and reduced activities of the antioxidative metalloenzymes. However, we underlined that increased health risk of smoking related diseases can be associated with exposure to Cd.

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Appendix A

Table I. Accuracy and precision of GSH and GSSG measurements in spiked samples of rat liver homogenates

Parameters	GSH			GSSG		
Accuracy						
Spiked concentration (μM)	25	50	100	25	50	100
Measured concentration (μM)	25.45 ± 0.65	50.44 ± 0.47	99.58 ± 1.23	25.14 ± 0.29	49.32 ± 0.22	98.18 ± 0.37
Bias (%)	1.8	0.88	−0.42	0.56	−1.36	−1.82
Precision						
Intra-day C.V. (%)	2.56	0.94	1.24	1.17	0.45	0.38
Inter-day C.V. (%)	4.7	1.4	1.8	2.5	1.2	0.99

Measurements were done in triplicate. Liver homogenates of Wistar rats were spiked with 25, 50, and 100 ∝M of GSH and GSSG in final concentrations. Precision was expressed as relative standard deviation (%), i.e., coefficient of variation (C.V.). Intra-day precision: five times repeated measurements of each sample in a single day. Inter-day precision: each sample was measured over five consecutive days. Values were expressed as means ± standard deviation. For chromatographic conditions, see Materials and Methods section.

Table II. Concentration of glutathione in liver homogenates of Wistar rats exposed subacutely i.p. to cadmium

Calculations			GSH (nmol mg−1 prot)	GSSG (nmol mg−1 prot)	GSSG–GSH
Group	Control	0 day	32.76 ± 3.41	1.83 ± 0.09	0.056
	Cd	1 day	25.57 ± 2.57¥ **	2.12 ± 0.13	0.083
		3 days	23.49 ± 2.42¥***	3.7 ± 0.32¥***; £***	0.158
		10 days	22.21 ± 3.99¥***	4.7 ± 0.38¥***; £***; ┼***	0.212
		21 days	18.27 ± 2.01¥***; £**; ┼*	2.11 ± 0.13┼***; µ***	0.116

Untreated group of Wistar rats (control group $n = 6$) and the group i.p. exposed to 1 mg $CdCl_2$ kg^{-1} day^{-1} for 1, 3, 10, and 21 days (Cd group: $n = 24$). Liver homogenates were used as samples. For the chromatographic conditions, see section Materials and Methods. The values are expressed as means ± standard deviation. Contents of GSH and GSSG were expressed in regard to protein status in nmol mg^{-1} proteins. Tukey's test was used for multiple comparisons between the groups, and certain comparisons were assigned as follows: control and Cd — subgroups (¥); Cd1 compared to Cd3, Cd10, and Cd21 (£); Cd3 compared to Cd10 and Cd21 (┼); and Cd10 compared to Cd21 (µ). Level of significance is labeled as follows: *($p < 0.05$), **($p < 0.005$), and ***($p < 0.0001$).

Appendix B

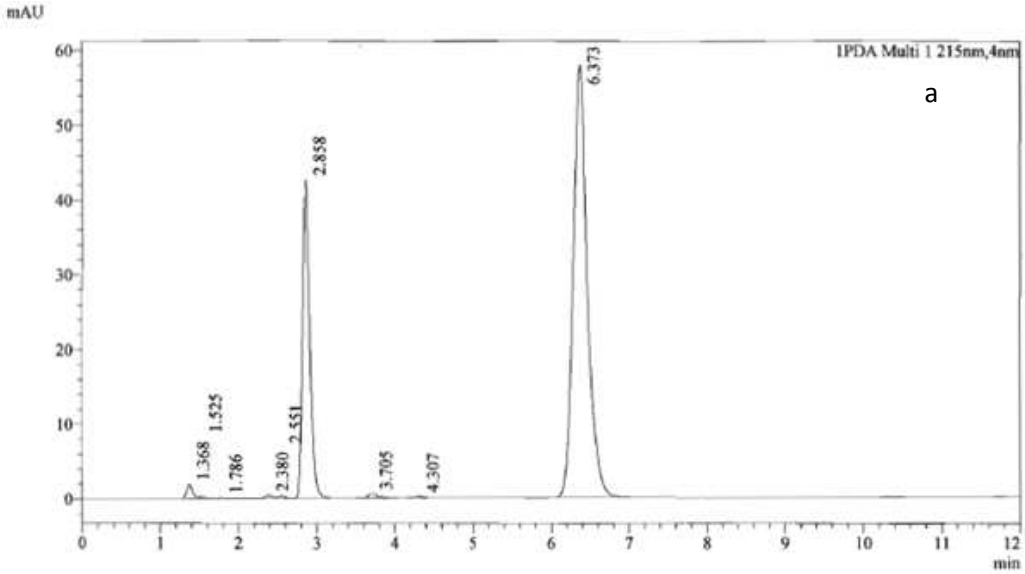

Fig. 1a. Mixed standard solution of GSH and GSSG (100 μM). The chromatogram of 100 μM mixed standard solution of reduced glutathione (GSH) and oxidized glutathione (GSSG). Chromatographic conditions: see Materials and Methods. Measurements were repeated 6 times from the same vial (n = 6). Repeatability of retention times and integrated areas of GSH and GSSG peak was presented in percentage (%) as a relative standard deviation (RSD) GSH: 2.85 min (RSD 0.19%, n = 6) and GSSG: 6.37 min (RSD 0.43%, n = 6). The obtained values for tailing factors (10%) were 1.22 and 1.13, while capacity factors (k') were calculated to be 1.09 and 3.65 for GSH and GSSG, respectively

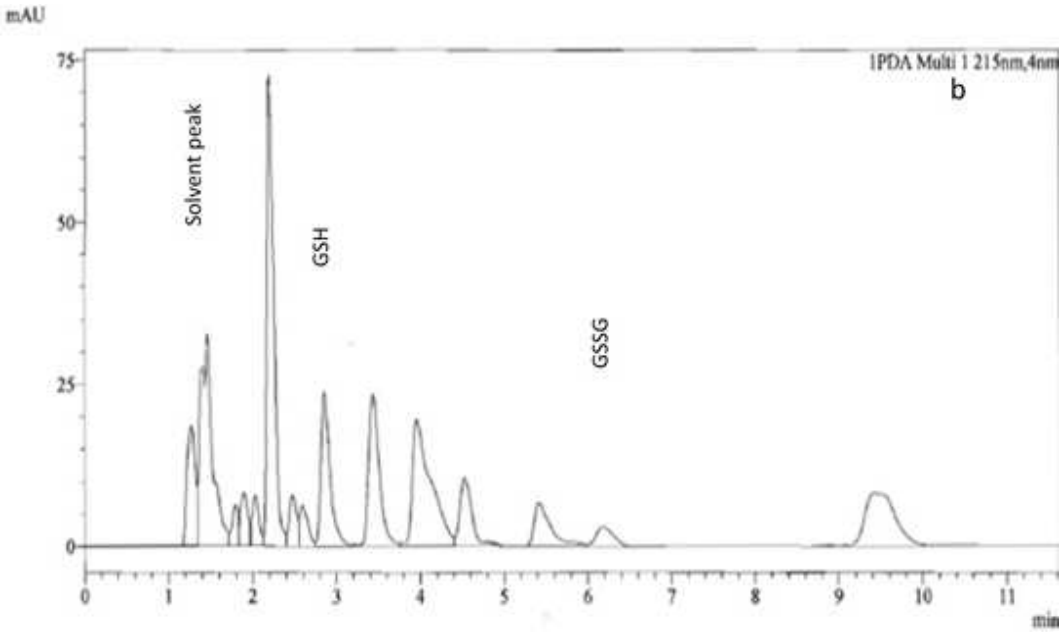

Fig. 1 b. GSH and GSSG in the liver of intact Wistar rats.
The chromatogram of the GSH and GSSG from the liver tissue homogenate of Wistar rats: control group; GSH (64.6 μM): 2.85 min, and GSSG (2.89 μM): 6.17 min; Chromatographic conditions: see *Material and Methods*. The GSH and GSSG values (expressed in μM) ought to be recalculated per mg of proteins and finally expressed as nmol (GSH and/or GSSG)/mg of proteins for the appropriate discussion of the obtained results

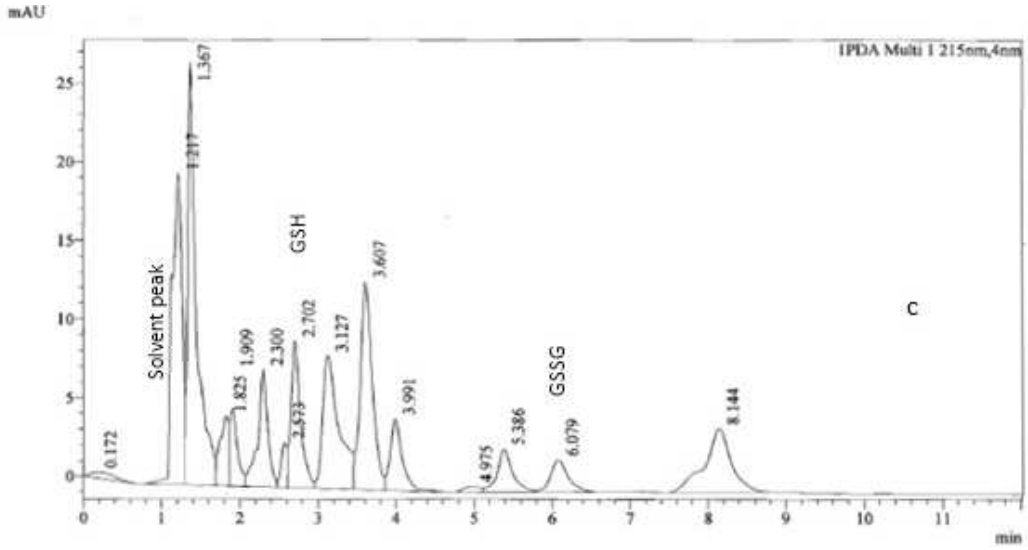

Fig. 1 c. GSH and GSSG in the liver of experimental group of Wistar rats. The chromatogram of the GSH and GSSG from the liver tissue homogenate of Wistar rats: the experimental group treated with Cd: GSH (26.24 μM): 2.70 min, and GSSG (1.24 μM): 6.07 min. Chromatographic conditions: see *Material and Methods*. The GSH and GSSG values (expressed in μM) ought to be recalculated per mg of proteins and finally expressed as nmol (GSH and/or GSSG)/mg of proteins for the appropriate discussion of the obtained results