The Application of Capillary Electrophoresis in the Determination of Glutathione in Healthy Women’s Blood

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The purpose of this work was to compare glutathione status in the blood of women taking oral contraceptives, female smokers and pregnant women using both the capillary electrophoresis (CE) and alloxan methods. The studies were performed on the whole blood prepared by deproteinization by 25% metaphosphoric acid. The reduced glutathione (GSH) was measured by alloxan and CE methods. Oxidized glutathione (GSSG) was measured by CE and the GSH/GSSG ratio was calculated. In pregnant women (Group A), women taking oral contraceptives (Group B) and female smokers (Group C), lower concentrations of GSH were observed compared with the control group (Group D) as measured by CE and alloxan methods, suggesting the presence of oxidative stress. The level of GSSG in Group C was higher than in Group D, indicating glutathione oxidation. In Groups A and B, reduced levels of GSSG were observed, which indicates that other processes besides oxidation affect glutathione status. In Groups A and C, a lower GSH/GSSG ratio was observed than in Group D, while in Group B no statistical change was observed. In conclusion, the advantage of CE is the possibility of measuring GSSG, which could allow for a more accurate interpretation of the status of GSH in the human body. Oxidation of glutathione in female smokers was indicated, while in pregnant women and women taking oral contraceptives processes other than oxidation can be associated with a decrease in glutathione levels.

Introduction

Glutathione (L-γ-glutamyl-l-cysteinylglycine) is a tripeptide of low-molecular weight containing thiol, which performs a variety of functions. Glutathione mainly acts as a nucleophilic scavenger protecting against oxidative stress and as an antioxidant in electrophilic/oxidative tissue injuries that arise from both endo- and exogenous sources (1). Additionally, glutathione participates as a mediator in many physiological processes including cellular signaling (involved in cell cycle regulation, proliferation and apoptosis), metabolism of xenobiotics, thiol disulfide exchange reactions and also as an important reservoir of cysteine (1). Given these essential roles of glutathione in cellular protection and homeostasis, glutathione availability may be important in the maintenance of health, and glutathione concentration may serve as a useful indicator of disease risk in humans (2). Changes in physiological glutathione concentration have been associated with patients suffering from Alzheimer’s disease (3), Parkinson’s disease (4), diabetes mellitus (5), atherosclerosis (6), arthritis (7) and epilepsy (8), as well as cancer (9).

It should also be noted that biological factors such as pregnancy may affect the concentration of glutathione in blood. Pregnancy is a physiological period during which high metabolic demand and elevated requirements for oxygen in tissue can be observed (10). This increased need for oxygen amplifies the rate of production of reactive oxygen species, which causes an increased risk of exposure to oxidative stress (10). Oxidative stress has previously been reported as a causative agent in pregnancy-related disorders, such as embryonic resorption, recurrent pregnancy loss, intrauterine growth restriction, pre-eclampsia and fetal death (11). To prevent damage caused by oxidative stress during physiological and pathological pregnancy, changes take place in the body’s antioxidant system (12, 13). Another factor that may affect the glutathione concentration is the use of oral contraceptives. Oral contraceptives prevent pregnancy primarily by suppressing ovulation. They generally contain two active components, estrogen and progestin, which are known to have various metabolic effects. Estradiols and progestogens alter the body’s lipid metabolism, which can cause oxidative stress (14). Furthermore, there are some reports showing that synthetic sex steroids may increase the risk of cardiovascular disease by oxidative damage (15). Moreover, concentrations of glutathione vary under the influence of environmental factors such as smoking (16). Inducers of oxidative stress have been identified in tobacco smoke, such as alkyl radicals (RO), peroxide radicals (ROO), cadmium, nickel and arsenic, which generate free-radical processes resulting in fluctuations in antioxidant levels, including glutathione (17, 18).

Glutathione is extensively present throughout the human body. In blood components, it is mostly present in the millimolar range in red blood cells, whereas in plasma it is concentrated in the micromolar range. Glutathione mainly acts in reduced form (GSH), although the production of free radicals, oxidizing agents and certain exogenous compounds may convert it into its oxidized form (GSSG) (19). The level of glutathione in blood may reflect glutathione status in other, less accessible tissues. Measurements of both GSH and GSSG in blood are considered as an index of whole-body glutathione status and a useful indicator of oxidative stress risk, which can cause various diseases.

A number of analytical methods have been developed to measure glutathione levels. The oldest method, described by Patterson and Lazarow (20), is based on the reaction of alloxan with GSH and formation of a compound characterized by an absorption maximum at 305 nm. They developed a spectrophotometric method for the estimation of GSH, which is carried out in an alkaline environment and requires sample deproteinization prior to analysis. This method is based on absorption band change being directly proportional to the amount of GSH (21). Although this method is one of the oldest, it is still used to compare the
concentration of GSH across different groups because of the low cost of analysis and its relative sensitivity. On the other hand, the disadvantages of this method are the impossibility of automation, time needed for the analysis and the inability to perform estimation in tissue.

Others methods, including the enzymatic one (22), as well as high-performance liquid chromatography (HPLC) coupled with a variety of detection types such as ultraviolet, fluorescence (23–26) and electrochemical detection (ECD) (27, 28), have also been elaborated. The analytical methods vary in their ability to assess various glutathione forms, specificity and sensitivity. Nowadays, HPLC methods are the ones most frequently applied because of their specificity and sensitivity. However, HPLC methods are disadvantaged by the long time required to complete each assay, materials consumed and high costs of instrumentation.

In recent years, some methods have been developed using capillary electrophoresis (CE) allowing for estimation of low-molecular antioxidants such as metallothionein (29), GSH and GSSG (30). CE is a technique in which analytes are separated in a narrow capillary containing a background electrolyte. In comparison with other assays, the CE method is characterized by better analytical performance (specificity in the detection of GSH and GSSG, reproducibility in migration time and peak area), simplicity of procedure, short time of analysis, low injection volume, low cost of analyses and possibility of automation (31). So far, different CE approaches with different detectors such as laser-induced fluorescence (LIF) and ECD have been reported (32, 33). CE with UV detection is also frequently used in the estimation of both forms of glutathione concentration (30, 34, 35). Furthermore, in all recent CE-UV absorbance methods, GSH and GSSG are assayed in their underivatized forms (30, 34, 35) and after following one-step sample preparation (36). Separation of compounds analyzed in a single run without any derivatization procedure represents one of the main advantages of this method, direct detection and omitting derivatization procedures prior to CE analysis may better reflect GSH oxidation.

Despite the permanent development and validation of CE methods suitable for direct measurement of GSH and GSSG (36), the estimation of glutathione status in whole blood as a routine procedure by them is still unestablished. No reference method has yet been defined for glutathione measurement in whole blood, which leads to high variability in the reference values reported in healthy subjects (36).

The aim of this study was to describe the distribution of blood GSH and GSSG in healthy adult women and verify the influence of factors such as smoking, oral contraceptives and pregnancy on glutathione status using the alloxan and CE methods.

**Materials and methods**

**Patients**

Blood samples were collected at the Department of Biomedical and Environmental Analysis, Wroclaw Medical University, and at the 2nd Department and Clinic of Gynaecology and Obstetrics, Wroclaw Medical University, Poland. The study protocol was approved by the Local Bioethics Committee of Wroclaw Medical University (KB No: 573/2005). The investigations involved 65 subjects. Personal interviews were carried out, and individuals with diagnosed diseases, as well as alcohol and drugs abusers, were excluded from the study.

The samples were divided into four groups: A, B, C and D (Table I). Group A was composed of healthy women in the third trimester of pregnancy (n = 15). Group B included female non-smokers taking oral contraceptives (over 1 year; pills containing derivatives of synthetic estrogen and progesterone) (n = 15). Group C consisted of female smokers (over 1 year; more than 5 but fewer than 20 cigarettes per day) not taking oral contraceptives (n = 15). Group D constituted non-smoking women not taking oral contraceptives (n = 20). The characteristics of each group are described in Table I.

**Sample preparation**

Blood samples were collected by venipuncture into heparin-containing tubes (ref. No.: 04.1931.001, Sarstedt, Germany). In order to obtain samples for analysis of GSH and GSSG, 1,050 μL of distilled water was added to 150 μL of whole blood, then mixed and incubated for 10 min. Next, 300 μL of 25% metaphosphoric acid (MPA) was added (ref. No. 253-433-4, Sigma-Aldrich, Germany), mixed and incubated for 10 min and centrifuged for 10 min at 3,000 × g. Plasma for cotinine and an erythrocyte pellet for hemoglobin analysis were collected according to standard procedures. Whole blood samples were centrifuged at 2,500 × g for 15 min to separate plasma from the erythrocyte pellet. The pellet was washed in an equal volume of ice-cold 0.9% NaCl. This process was repeated twice. The washed cells were lysed by the addition of ice-cold twice distilled water (1 : 1.4). All dilutions of samples were taken into account in the calculations. Plasma and erythrocyte lysate were frozen at −80°C until measurement.

**Table I**

Baseline Characteristics of 65 Healthy Women

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A (pregnant women) n = 15</th>
<th>Group B (women taking oral contraceptives) n = 15</th>
<th>Group C (female smokers) n = 15</th>
<th>Group D (control group) n = 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>26 ± 6</td>
<td>22.7 ± 0.44</td>
<td>22.5 ± 0.7</td>
<td>23.3 ± 0.5</td>
</tr>
<tr>
<td>X ± SD</td>
<td>30</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Median</td>
<td>19–37</td>
<td>22–23</td>
<td>22–23</td>
<td>23–24</td>
</tr>
<tr>
<td>Range (min–max)</td>
<td>20 ± 1.96</td>
<td>22.29 ± 1.19</td>
<td>20.58 ± 2.02</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>1.52 ± 0.54</td>
<td>2.13 ± 2.26</td>
<td>63.60 ± 18.24*</td>
<td>3.13 ± 1.26</td>
</tr>
</tbody>
</table>

*Significant (P < 0.05) when compared with the control group of women.
Determination of Glutathione in Healthy Women’s Blood

**GSH concentration measurement by the alloxan method**

The method is based on the reaction of GSH with alloxan and formation of a complex with an absorbance maximum at 305 nm (20). About 250 μL of phosphate buffer at pH 7.5 was added to 250 μL of samples (prepared by mixing 0.5 M Na₂HPO₄ and 0.5 M KH₂PO₄), 250 μL of equivalent sodium hydroxide solution (which contains 10 mL of 0.1 M alloxan solution, 10 mL of 5% NaOH and enough 0.5 M NaOH to obtain the endpoint at pH 7.5) and 250 μL of 0.1 M alloxan. Samples were incubated for 6 min and the reaction was stopped by adding 250 μL of 1 M NaOH. Control samples were prepared in a similar manner, but instead of 250 μL of alloxan, 250 μL of H₂O was added. The concentration of GSH in samples was determined by comparing the corrected optical density obtained for the sample with a standard curve, which was prepared by running a test on samples containing 7, 16, 33, 65, 98, 130 and 163 μM of GSH standard (ref. No. 200-725-4, Sigma-Aldrich) (1–20 μM) and plotting the corrected optical density at 305 nm against the GSH concentration.

**GSH and GSSG concentration measurement by CE**

CE was performed on a PA 800 Plus Pharmaceutical Analysis System equipped with an ultraviolet detector set at 200 nm. The separations were performed on a fused-silica capillary [75 μm (i.d.) × 30 cm (total length)]/10 cm (length to detector) purchased from Beckman Coulter. The instrument was set up with the anode at the inlet end of the capillary and the cathode at its outlet. The capillary was thermostated at 25 °C. GSH and GSSG measurements were conducted using the determination of GSSG and GSH in the whole blood analysis kit from Analis (ref. No.: 10-004770, Cefox™, Belgium). The calibration curves for GSH (ref. No. 200-725-4, Sigma-Aldrich) (5–40 μM) and GSSG (ref. No.: 27025-41-8, Sigma-Aldrich) (1–20 μM) were prepared by diluting the stock solutions with 5% MPA. Before each analytical run, the capillary was pressure-rinsed and filled with a borate buffer (Boric acid NaOH pH 8.2–SDS) for 0.6 min at 20 psi. The samples, prepared in advance, were injected by pressure for 8 s at 0.2 psi. The separations were performed at a constant voltage of 6 kV for 9.5 min. Data were quantified on the basis of corrected peak areas and migration times. Between each analytical run, the capillary was rinsed with 0.1 M NaOH. The results were analyzed using 32 Karat software from Beckman Coulter.

**Statistical analysis**

The data are expressed as mean (SD) values. The normality of variables was tested by the Shapiro–Wilk W-test. Differences between the groups were tested using Student’s t-test. In case of a lack of normal distribution and variance uniformity, the differences between groups were analyzed by means of a non-parametric Mann–Whitney U-test. Statistical calculations were done using the Statistica Software Package, version 9.0 (StatSoft, Poland).

**Results**

In the alloxan method, the standard curve for GSH displayed remarkable linearity ($y = 0.0048x + 0.0041$). The correlation coefficient was high ($R^2 = 0.994$), which confirms the method’s proper analytical performance. A statistically significant decrease in GSH concentration was identified in Group B (693.94 $\pm$ 394.43 μM) and Group C (692.71 $\pm$ 277.09 μM) when compared with Group D (1066.75 $\pm$ 402.57 μM), while the concentration of GSH in Group A (804 $\pm$ 301 μM) was slightly lower than in Group D (Table II). Furthermore, when converting to μM/g Hb, the differences in GSH levels between groups did not change (in Group B—3.13 $\pm$ 2.23 μM/g Hb, Group C—3.14 $\pm$ 3.14 μM/g Hb, Group D—5.32 $\pm$ 2.8 μM/g Hb), and a slight decrease was noted in Group A (3.82 $\pm$ 1.21 μM/g Hb). The GSH concentration range in all groups

**Table II**

The Comparison of GSH and GSSG Concentrations and GSH/GSSG Ratio in Female Smokers, Women Taking Contraceptives and Pregnant Women to the Control Group

<table>
<thead>
<tr>
<th></th>
<th>Group A (pregnant women)</th>
<th>Group B (women taking oral contraceptives)</th>
<th>Group C (female smokers)</th>
<th>Group D (control group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 20</td>
</tr>
<tr>
<td>Alloxan method</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GSH (μM)</td>
<td>804 $\pm$ 301</td>
<td>639.94 $\pm$ 394.43$^*$</td>
<td>692.71 $\pm$ 277.09$^*$</td>
<td>1066.75 $\pm$ 402.57$^+$</td>
</tr>
<tr>
<td>GSSG (μM)</td>
<td>325.53 $\pm$ 136.46$^+$</td>
<td>412.92 $\pm$ 186.94$^*$</td>
<td>445.11 $\pm$ 67.46$^*$</td>
<td>595.80 $\pm$ 158.63$^+$</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>1.13 $\pm$ 0.57$^+$</td>
<td>1.81 $\pm$ 0.92$^*$</td>
<td>1.44 $\pm$ 0.36$^*$</td>
<td>2.11 $\pm$ 0.54$^*$</td>
</tr>
</tbody>
</table>

$^*$Significant ($P < 0.05$) when compared with the control group of women.
groups obtained by the alloxan method was 255.011–2001.497 μM.

Using the CE method, on the basis of the electropherograms obtained from the relevant GSH and GSSG standard concentrations (Figure 1), curves were plotted (y = 0.00212590x + 0.335869 for GSH, y = 0.000872846x – 0.00339215 for GSSG) which likewise showed a remarkable linearity. The correlation coefficients in the determination of GSH and GSSG by CE were very high (R² = 0.999765 and 0.987863, respectively). Electropherograms of whole blood samples from Groups A, B, C and D are shown in Figure 2. Peaks corresponding to GSH and GSSG were eluted at ~4.85 and ~5.22 min, respectively. An unknown peak (X) eluted at 5.6 min was found in all blood samples, and peaks (Y) eluted at ~5.4 and ~6.15 min were found in blood samples of pregnant women. Concentrations of GSH and GSSG were measured simultaneously in blood samples, and peaks (Y) eluted at 5.4 min were found in blood samples of pregnant women. Concentrations of GSH and GSSG were measured simultaneously in blood samples, and the GSH/GSSG ratio was calculated. A statistically significant decrease in the GSH level was observed, as determined by CE in each group (Group A—325.53 ± 136.46 μM, Group B—412.92 ± 185.94 μM, Group C—445.11 ± 67.46 μM) when compared with the control group (595.80 ± 158.63 μM). After conversion of the units to μM/g Hb, the differences between groups remained identical (Group A—1.54 ± 1.02 μM/g Hb, Group B—2.41 ± 1.43 μM/g Hb, Group C—2.70 ± 0.90 μM/g Hb, Group D—3.63 ± 1.10 μM/g Hb). A statistically significant decrease in GSSG concentration was observed in Group B (205.58 ± 61.96 μM), as well as an increase in Group C (335.53 ± 75.79 μM) in comparison with Group D (272.73 ± 68.58 μM), whereas in Group A a slight decrease was observed in GSSG levels (259.36 ± 81.32 μM) compared with Group D. After conversion of the units to μM/g Hb, the differences between Groups B, C and D remained identical (Group B—1.16 ± 0.38 μM/g Hb, Group C—2.16 ± 0.82 μM/g Hb, Group D—1.6 ± 0.6 μM/g Hb). A statistically significant decrease in the GSSG level was observed in Group A (1.14 ± 0.49 μM/g Hb) in comparison with Group D.

A significant decrease in GSH/GSSG ratio was demonstrated in Group A (1.13 ± 0.57) and Group C (1.44 ± 0.36) when compared with Group D (2.11 ± 0.54), whereas in Group B there was no significant difference in GSH/GSSG ratio (1.81 ± 0.92) compared with Group D. The results obtained by CE showed considerable interindividual variability with concentration ranges of 188.30–889.9 μM for GSH, 101.25–476.65 μM for GSSG and 0.543–4.341 for the GSH/GSSG ratio.

Furthermore, the investigations demonstrated a statistically significant decrease in the –SH groups level in the plasma of Group C (260 ± 43 μM) compared with Group D (311 ± 61 μM), whereas in Group B (275 ± 51 μM) no difference was observed when compared with the control group.

**Discussion**

Glutathione is one of the main intracellular antioxidants, and information about its concentration is essential for proper characterization of oxidative stress in the body. Numerous measurement methods have been developed. In the 1950s, the common method for the determination of glutathione was the spectrophotometric method, based on the reaction of thiol groups with alloxan (20). Nowadays, methods for the determination of glutathione are much more advanced, such as CE. The aim of the study was to compare glutathione levels in women in pregnancy, women taking oral contraceptives and female smokers examined and measured by both the CE and alloxan methods.

In the present paper, the GSH concentrations obtained by both methods (CE and alloxan) are in the range of 150–1,500 μM, as has been reported by other authors (38). However, higher levels of GSH have been observed using the alloxan method compared with the values obtained by the CE method. The difference is probably due to the lower specificity of the spectrophotometric method elaborated by Patterson and Lazarow. The peptides cysteinylglycine and γ-glutamylcysteine present in blood can cause interference by producing substances, which may have absorption bands at or near 305 nm (21).

In the present paper, the impact of pregnancy, oral contraceptives and smoking on GSH and GSSG levels in whole blood was examined and measured by both the CE and alloxan methods.

We found a statistically significant decrease in GSH concentration as measured by the CE method in pregnant women compared with non-pregnant women, while the level of GSH measured by the alloxan method in pregnant women displays a non-significant decline. The decrease of GSH concentration in
pregnancy is concurrent with other studies (39). We observed a slight, but not statistically significant, decrease of GSSG concentration in pregnant women. However, conversion of GSSG units from μM to μM/g Hb allowed us to observe statistically significant difference in GSSG levels between pregnant and non-pregnant women. The glutathione status in pregnant women obtained by the alloxan method shows a slight decrease of GSH, which may suggest GSH oxidation. Meanwhile, we also observed a decrease of GSSG, which may indicate that the decline of GSH concentration is not related to oxidation of GSH during pregnancy. In addition, Wang and Wash (40) have reported a deficiency in glutathione peroxidase activity, an enzyme which catalyzes the oxidation of GSH to GSSG.

Only a few studies have investigated the relationship between oral contraceptives and antioxidant status among healthy women. According to some reports, oral contraceptives exert an influence on lipid metabolism, which can consequently cause oxidative stress (14). A statistically significant decrease in GSH levels, among women taking oral contraceptives as recorded using both the alloxan and CE methods, was observed in comparison with those not taking oral contraceptives, which is consistent with other authors (41, 42). This difference could suggest the oxidation of GSH; however, we observed a statistically significant decrease in GSSG levels, which may point to other processes than the conversion of GSH to GSSG, such as we observed in pregnancy. Additionally, we did not observe a statistically significant difference in –SH groups concentration and GSH/GSSG ratio between women taking and not taking oral contraceptives. As in the case of pregnant women, a decrease in the activity of glutathione peroxidase and glutathione reductase in women taking oral contraceptives was reported (43).

Another factor influencing glutathione levels in women’s blood is cigarette smoking. Tobacco smoke contains about 6,000 toxic compounds, including polycyclic aromatic carbons, nitrosamines and aldehydes and metals (44), which can cause the formation of free radicals and weaken intracellular antioxidant systems (45). In the present study, a statistically significant decrease in GSH levels was shown in female smokers compared with non-smokers, as has been reported by Michelet et al. (41). It has been shown that cigarette smoking causes a decline in GSH concentration, which consequently decreases the reductive properties of cells and contributes to the induction of oxidative stress and predisposition to tumor formation (46). Furthermore, an increase of GSSG levels and a decrease of the GSH/GSSG ratio in female smokers were observed, which suggests the oxidation of GSH into GSSG. Additionally, the process of oxidative stress was confirmed in the present work by the decrease of –SH groups concentration in the plasma of female smokers compared with non-smokers. Slightly lower levels of GSH/GSSG in the blood than those proposed by some authors were observed (36). This could be due to differences in the procedures used for preparing materials. On the other hand, the levels of GSH and GSSG presented in this study are within the range given in other publications (150–1,500 and 1–500 μM, respectively), as summarized by Rossi et al. (38).

In the present study, we can see a difference in concentrations of GSH in the same group obtained by two different methods. However, it should be noted that levels of GSH differ somewhat from one laboratory to another. This variability results from different methodologies, differences in sample processing or the selection of subjects under the influence of various factors affecting blood glutathione concentrations.

The glutathione system remains the object of extensive research, despite the ready availability of knowledge related to redox homeostasis. While the number of papers published in this research area has increased appreciably in recent times, and the development of novel techniques has led to application of contemporary methods in the measurement of GSH and congeners, standardized values of GSH and GSSG in biological samples have yet to be established.

Conclusions

Using both the CE and alloxan methods, a decrease was observed in concentrations of GSH in female smokers, women taking oral contraceptives and pregnant women compared with the control group; the values obtained by these two methods are within widely accepted ranges. It can be concluded that both methods are appropriate for evaluating GSH in blood, though they cannot be compared with each other. In addition, a particular advantage of CE is the possibility of measuring GSSG, which could allow for a more accurate interpretation of the status of GSH in the human body. In the present study, an increase of GSSG was demonstrated in the group of female smokers, which may be linked with GSH oxidation. However, a decrease of GSSG in pregnant women and women taking oral contraceptives compared with the control group was observed, which indicates a process other than oxidative stress associated with decreasing GSH concentrations.

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