Free Radical Research, Volume 38 Number 10 (October 2004), pp. 1101–1106

Taylor & Francis
healthsciences

Interference of Plasmatic Reduced Glutathione and Hemolysis on Glutathione Disulfide Levels in Human Blood

DANIELA GIUSTARINI[a,*], ISABELLA DALLE-DONNE[b], ROBERTO COLOMBO[b], ALDO MILZANI[b] and RANIERI ROSSI[a]

[a]*Department of Neuroscience, Pharmacology Section, University of Siena, via A. Moro 4, I-53100, Siena, Italy;* [b]*Department of Biology, University of Milan, via Celoria 26, I-20133, Milan, Italy*

Accepted by Professor V. Darley-Usmar

(Received 23 January 2004; In revised form 24 February 2004)

The blood reduced glutathione (GSH)/GSH disulfide (GSSG) ratio is an index of the oxidant/antioxidant balance of the whole body. Nevertheless, data indicating GSH and GSSG physiological levels are still widely divergent, especially those on GSSG, probably due to its low concentration. Standardization in methodological protocols and sample manipulation could help to minimize these discrepancies. Therefore, we have investigated how plasma reduced GSH, which is rapidly oxidized after blood withdrawal, could alter the blood GSSG measurement if the sample is not suitably processed. We have observed that an increase in plasma GSH concentration, due to red blood cell hemolysis, is responsible for a significant overestimation of blood GSSG level. Our results show that, before performing blood GSSG determination, thiols have to be rapidly blocked, to avoid possible pitfalls in GSSG measurement, in particular when hemolysis is present.

Keywords: Reduced glutathione; Oxidized glutathione; Hemolysis; Plasma; Blood

INTRODUCTION

Reduced glutathione (GSH) is the major low molecular weight thiol in mammalian cells, where it constitutes the most important antioxidant defense against radicals and reactive oxygen species. It is in balance with its oxidized form, GSH disulfide (GSSG), but, generally, intracellular GSH reductase, which utilizes NADPH as a donor of reducing equivalents, shifts this equilibrium to the reduced form.[1] An increase in oxidants unbalanced by reducing power leads to the patho-physiological status named oxidative stress.[2] The current opinion is that oxidative stress could have a key role in the development and/or progression of various pathologies, including diabetes, cancer, atherosclerosis, cardiovascular diseases, neurological disorders, acquired immunodeficiency syndrome (AIDS), to name a few.[3–10] As a consequence, the availability of markers that can provide an accurate assessment of oxidative stress is of fundamental importance.[10,11] GSH and its redox-related species have been widely investigated as possible biomarkers, by virtue of their high reactivity and their ubiquitous presence.[12,13] Moreover, since blood GSH concentrations may reflect the oxidative status in other less accessible tissues, measurement of both GSH and GSSG in blood has been considered a reliable index of whole-body GSH status and a useful indicator of disease risk in humans.[14,15] Consequently, the blood GSH/GSSG ratio is frequently investigated as an index of the overall redox status.

Nevertheless, we still lack reference values for the blood GSH/GSSG ratio, as indicated by significant divergences between different research groups.[14,15] We have measured a blood GSH concentration over two orders of magnitude higher than GSSG (1.5 mM and 3 μM, respectively) in healthy people.[14,16] Our investigations indicate that GSSG levels are much lower than generally reported, probably because some methodological errors may occur during GSSG detection, thus

*Corresponding author. Tel.: +39-577-234198. Fax: +39-577-234208. E-mail: giustarini@unisi.it

ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760400008854

leading to GSSG overestimation. Given its physiological low levels, it is likely that, under an oxidative perturbation, the raise in GSSG concentration is particularly evident, while the corresponding GSH depletion is negligible or undetectable. Therefore, to obtain some reliable information about the possible link between oxidative stress and pathological status, it is fundamental to avoid every possible methodological pitfall in blood GSSG detection.

GSSG exists in both erythrocytes and plasma at about the same concentration (i.e. 2 and 1 μM, respectively).[14,17] Since the human hematocrit value is near 50%, the blood GSSG concentration is also about 2 μM. Differently, GSH is mainly distributed in the intracellular compartment (about 3 mM), and is present in plasma at a much lower level (i.e. 2–4 μM).[14,17,18] In contrast to intracellular GSH, plasmatic GSH is rapidly oxidized to the disulfide form in a few seconds after blood withdrawal.[1] We hypothesized that this oxidation could be sufficient to significantly increase the level of blood GSSG, the plasmatic GSH concentration being equimolar with the GSSG concentration. In addition, this phenomenon could be more evident when the plasma GSH content rises as a result of hemolysis. Since erythrocyte GSH is about 3 mM,[14] even a minimal amount of hemolysis could significantly increase GSSG levels.

The aim of the present work is to investigate if oxidation of plasma GSH during sample manipulation can artifactually increase GSSG levels in whole blood leading to GSSG overestimation. Moreover, the influence of hemolysis during this measurement has been evaluated. This pitfall should explain, at least partially, the conflicting indications about the blood GSH/GSSG redox state in healthy and sick subjects found in the literature.[14,15] The guidelines to overcome this pitfall are indicated.

MATERIAL AND METHODS

Materials

HPLC-grade reagents were purchased from BDH (Poole, Dorset, England). A 4.6 × 250 mm^2 reversed-phase HPLC column Sephasil C18 was purchased from Pharmacia (Uppsala, Sweden). Monobromobimane (mBrB) was obtained from Calbiochem (La Jolla, CA, USA). All other reagents of analytical grade were from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

Sample Recruitment

Human venous blood was obtained from healthy volunteers (with consent) by venipuncture with a butterfly needle attached to a 5-ml syringe. Approximately 3 ml of blood was drawn and dropped into a tube containing K_3EDTA.

Rat blood was obtained after pentobarbital anesthesia from the abdominal aorta using K_3EDTA as an anticoagulant.

GSH Determinations in Plasma

Blood was reacted with the fluorescent thiol-labeling molecule, mBrB, as previously described[17] immediately or at specified times after withdrawal. Measurements on clear supernatant were assessed by HPLC (Bio-Tek Instruments KromaSystem 2000) with fluorescence detection (Kontron SFM 25 spectrofluorimeter). Samples were injected into a Pharmacia Sephasil column C18. Solvent A was 0.25% (v/v) acetic acid, pH 3.09, solvent B was methanol. The elution profile was as follows: 0–18 min, 20% B; 18–23 min, 23–100% B. The spectrofluorimeter was set as follows: 380 nm excitation, 480 nm emission.

GSSG Determination in Blood and RBCs

Blood, 0.3 ml, was mixed with 50 μl of 310 mM *N*-ethyl-maleimide (NEM). After 5 s, samples were deproteinized with 100 μl 50% (w/v) TCA.[14] For GSSG determination in red blood cells (RBCs), blood was washed three times with saline solution containing 5 mM glucose. The RBC pellet was resuspended in the same solution to 45% hematocrit. RBC suspension, 0.3 ml, was then reacted with 50 μl of 310 mM NEM; after 5 s, samples were deproteinized with 100 μl 50% (w/v) TCA. Deproteinized blood or RBC samples were centrifuged at 15,000*g* for 2 min. The excess of NEM was extracted from the clear supernatant with ten volumes of dichloromethane. GSSG was then measured by spectrophotometry as previously described.[14] Different amounts of hemolyzed RBCs were added to some blood samples before NEM treatment. Hemolysis was carried out by osmotic lysis of packed RBCs by addition of 1.5 volumes of water. Immediately or at different times after the hemolyzed RBC addition, blood samples were treated with NEM as above indicated and GSSG was measured by spectrophotometry.[14]

Hemoglobin Determination

Hemoglobin concentration was determined by the cyanomethemoglobin method[19] in a UVIKON 810P spectrophotometer.

Statistical Analysis

The correlation between GSSG blood levels and percentage hemolysis was calculated by applying the Spearman Test, for a non-Gaussian population.

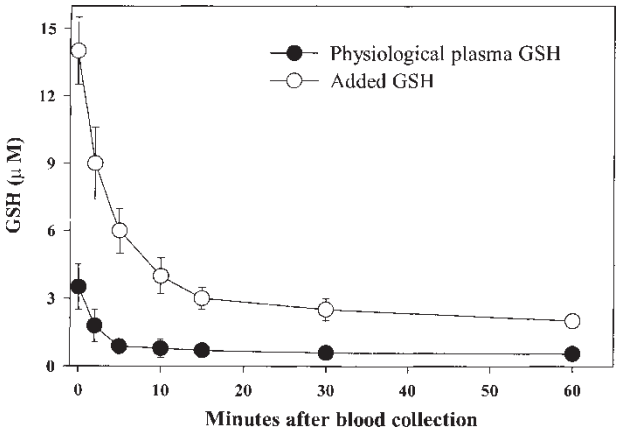

FIGURE 1 Time course of plasma reduced GSH levels. Aliquots of plasma were analysed at specified times after blood collection by means of a fluorimetric (mBrB) HPLC method. GSH levels in plasma without any extra addition (closed circles) or with 10 μM GSH addition (open circles). $n = 6$ for both experiments.

RESULTS

It is known that plasma GSH undergoes spontaneous auto-oxidation, so generating GSSG.[20,21] We quantified this process by measuring, in human plasma, levels of GSH in a 1–60 min time span after blood collection (Fig. 1, closed circles). Human blood was kept at room temperature and, at various times, plasma GSH was determined. The GSH half-life was found to be less than 5 min and about 80% of GSH disappeared 15 min after blood withdrawal. The possible contribution of intraerythrocytic GSH after slight hemolysis was mimicked by adding 10 μM GSH to blood (Fig. 1, open circles). The GSH added was oxidized as rapidly as physiological GSH. These experiments indicated that plasma GSH is highly unstable after blood withdrawal, suggesting its possible involvement in blood GSSG overestimation.

We then evaluated the possible increase in blood GSSG levels when determinations are not performed shortly after blood collection. Blood sample was maintained at room temperature and, at different times between 0.5 and 60 min, GSSG levels were measured (Fig. 2, open squares). GSSG increased with time, and after 60 min it increased by 30% of the initial value. The same experiment was repeated with rat blood (Fig. 2, open circles), and the GSSG increase was much higher than in human samples (about 190 vs. 30% higher, respectively, at 60 min). Rat plasma is characterized by a significantly higher GSH concentration than in humans. Levels of total GSH (GSH + GSSG) in rat plasma were found to be in the range of 22–27 μM, about 85% of which was in the reduced form.[22] From these data, it can be deduced that the rat plasma GSH concentration is about 20 μM, while the GSSG concentration is similar to that in human plasma. Therefore, high rat GSH levels may be responsible for the marked increase in the blood GSSG concentration we measured. To investigate further this possibility, we performed another series of experiments using RBCs instead of whole blood for both species (Fig. 2, closed squares and circles). In the absence of plasma, GSSG levels were stable in both human and rat samples (about 2 μM), even after 60 min, as expected. These data indicate that GSH undergoes auto-oxidation only in the extracellular compartment, thus increasing artifactually blood GSSG levels. Therefore, only when the blood sample is immediately processed after withdrawal can GSSG levels be measured without artificial overestimation.

GSH concentration in plasma can increase as a consequence of RBC hemolysis. Under these conditions, blood GSSG overestimation due to plasma GSH oxidation can be largely increased. Therefore, the possible contribution of hemolysis in the determination of GSSG values in human blood was evaluated. In an experiment of simulated hemolysis (Fig. 3), known amounts of hemolyzed RBCs were added to blood samples, and the levels of GSSG were measured at different times after

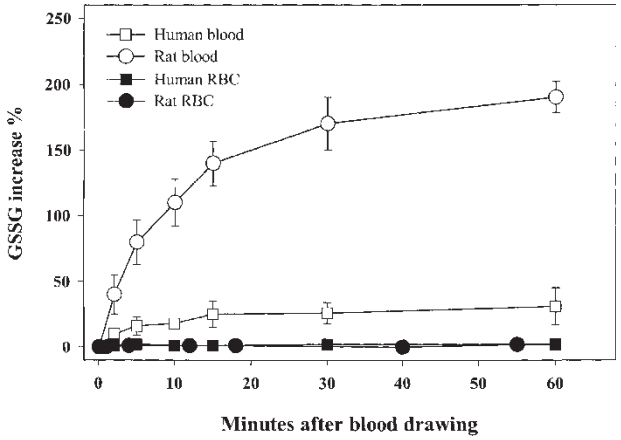

FIGURE 2 Time course of whole blood or RBC GSSG levels in human and rat. Sample aliquots were measured at specified times after blood collection by means of the spectrophotometric GSH recycling method ($n = 10$).

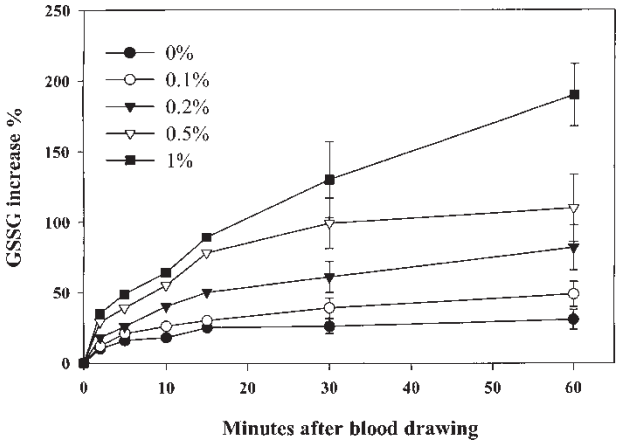

FIGURE 3 Time course of whole blood GSSG levels. Aliquots of blood were measured at specified times after collection by means of the spectrophotometric GSH recycling method. RBCs hemolysed at different final percentages were added immediately after blood collection ($n = 8$).

withdrawal. The increase in GSSG concentration was proportional to both the level of hemolysis and the time elapsed between blood withdrawal and GSSG measurement. In blood samples without hemolysate addition (Fig. 3, closed circles), a maximum increase of 20% above initial levels of GSSG was observed. In this case, the artifactual increase in GSSG levels is solely due to the oxidation of GSH physiologically present in plasma. Samples supplied with hemolyzed RBCs reached higher GSSG values due the higher GSH concentration in plasma, as a consequence of hemolysis. GSSG overestimation was negligible, even at a high level of hemolysis, only when samples were derivatized within 30 s of blood withdrawal.

Blood from subjects undergoing routine laboratory analyzes was utilized for random GSSG measurements. We chose blood samples with different levels of hemolysis to evaluate its correlation with GSSG values. Blood samples were processed about 1 h after blood collection and the GSSG values obtained were analyzed for a possible correlation with respect to % hemolysis, calculated as plasma hemoglobin content (Fig. 4). These parameters were found to be directly related ($r^2 = 0.559$, $p < 0.001$), indicating that, if GSH is not rapidly derivatized after withdrawal, the GSSG amount is related to plasma reduced GSH (that is oxidized to GSSG) rather than other factors. The higher the overestimation of GSSG the higher the % hemolysis.

DISCUSSION

The accurate evaluation of GSH and GSSG in blood samples is of pivotal importance in obtaining information on the oxidant–antioxidant balance of the body. Due to its ubiquitous distribution and high reactivity, GSH is one of the most sensitive parameters to an oxidative perturbation.[1] In pro-oxidative conditions, GSH is mainly oxidized to GSSG by either a spontaneous or enzymatic reaction (involving GSH-peroxidase). Therefore, GSH and its redox-related species have been widely investigated to study both the onset and the progression of different pathologies related to oxidative stress, like diabetes, rheumatoid arthritis, acute (adult) respiratory distress syndrome, Parkinson's disease, human immunodeficiency virus infection.[12,13,23]

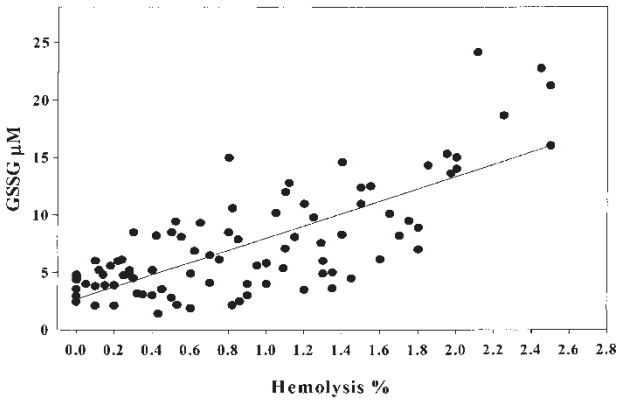

FIGURE 4 Correlation between GSSG blood levels and hemolysis percentage in healthy subjects undergoing routine laboratory tests. About 1 h after blood collection, samples were treated with NEM for spectrophotometric analysis ($r^2 = 0.559$; $p < 0.001$; $n = 85$).

While titration of blood GSH is relatively easy to perform, mostly due to its high concentration, the exact evaluation of GSSG is not free from artifacts.

In this study, we have reported that the ability of plasma to rapidly oxidize GSH to GSSG might influence blood GSSG determination leading to its overestimation. Plasma, *per se*, possesses a small amount of GSH that quickly disappears after blood collection (Fig. 1). In accordance with previous data,[22] our experiments indicate that GSH is mainly oxidized to GSSG. Therefore, if blood samples are not rapidly derivatized, this phenomenon can result in a GSSG overestimation. Due to the low concentration (2–4 μM) of plasmatic GSH, the pitfall is limited to a 20–30% increase in GSSG. Therefore, blood GSSG levels can vary from 2 to 2.5 μM when the sample is not immediately analyzed after blood collection. This phenomenon is much more evident when rat blood is used (e.g. during common laboratory experimental procedures), since it contains higher plasma GSH levels than human blood (Fig. 2). In rat blood, GSSG levels could theoretically increase up to 300–400%; in fact, since 20 μM GSH can generate 10 μM GSSG, taking into consideration the hematocrit value (about 50%), the level of GSSG in blood can vary from 2 to 7.5 μM. We have found a 190% increase in GSSG concentration 1 h after blood collection (Fig. 2), probably because the oxidative process requires more time to reach completion.

Blood GSSG overestimation can be enhanced as a consequence of hemolysis. Since erythrocyte GSH concentration is very high (about 3 mM), even a mild hemolysis can significantly increase blood GSSG levels (Fig. 3). Therefore, two different types of plasma GSH may be responsible for GSSG overestimation: (i) the physiological amount normally present and (ii) that deriving from RBC hemolysis. Both types are oxidized quickly to GSSG, following similar kinetics (Fig. 1). Blood hemolysis may have different origins. A minimal percentage (0.1–0.5%) may be due to a rapid suction of blood through the syringe needle; this could increase GSSG up to 40–70% above its real value. Some precautions should be taken to reduce this phenomenon; for example, the use of a butterfly needle for blood collection could be helpful.[24] This problem is particularly evident using rat blood, since it is more susceptible to hemolysis than human blood during withdrawal. In addition, some pathological conditions exist, besides anemias, in which hemolysis can occur. RBC susceptibility to hemolysis is increased in concomitance with hemodialysis,[25] in a pre-eclampsia complication named hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome,[26] in subjects suffering from acute post-streptococcal glomerulonephritis,[27] diabetes,[28] epilepsy[29] and hypercholesterolemia.[30] Cardiopulmonary bypass procedures, transfusion of aged blood, treatment with some nonsteroidal anti-inflammatory drugs (e.g. Na-salicylate, naproxen and ketorolac) at therapeutic concentrations[31,32] can also hemolyze RBCs causing an increase in the plasma GSH pool. In all these conditions, blood GSSG determination can significantly be altered by hemolysis, these two parameters being directly related (Fig. 4).

To avoid GSSG overestimation due to plasma GSH oxidation, some precautions should be taken. Our data indicate that a rapid sample derivatization is necessary to reduce GSH oxidation. As previously reported,[14] sample manipulation for GSSG determination is easily susceptible to methodological mistakes, depending on the technique utilized: (i) oxidation of thiols in acidified samples; (ii) oxidation of thiols after restoring neutral-alkaline pH; (iii) oxidation of thiols during acid deproteinization; (iv) shift in the GSH/GSSG equilibrium because of irreversible blocking of free thiols; (v) reaction of electrophiles with amino groups. We previously observed[14] that all these pitfalls can be overcome by sample pre-treatment with the thiol blocking agent NEM and its successive extraction after sample acidification. Our new data indicate that this rapid sulfhydryl quenching prevents plasma GSH oxidation also and, consequently, the related GSSG overestimation (Figs. 2 and 3, data obtained 1 min after blood collection). In conclusion, our data suggest the need to perform NEM pre-treatment a few seconds after blood collection to minimize GSSG overestimation due to plasma GSH oxidation.

Acknowledgements

Contract grant sponsor: FIRST 2003 (Fondo Interno Ricerca Scientifica e Tecnologica, University of Milan) and Fondazione Monte dei Paschi di Siena.

References

[1] Meister, A. and Anderson, M.E. (1993) "Glutathione", *Ann. Rev. Biochem.* **52**, 711–760.
[2] Halliwell, B. and Gutteridge, J.M.C. (1999) "Oxidative stress: adaptation, damage, repair and death", In: Halliwell, B. and Gutteridge, J.M.C., eds, *Free Radicals in Biology and Medicine* (Oxford University Press, New York), pp 246–350.
[3] Baynes, J.W. and Thorpe, S.R. (1999) "Role of oxidative stress in diabetic complications: a new perspective on an old paradigm", *Diabetes* **48**, 1–9.
[4] Olinski, R., Gackowski, D., Foksinski, M., Rozalski, R., Roszkowski, K. and Jaruga, P. (2002) "Oxidative DNA damage: assessment of the role in carcinogenesis, atherosclerosis, and acquired immunodeficiency syndrome", *Free Radic. Biol. Med.* **33**, 192–200.
[5] Harrison, D., Griendling, K.K., Landmesser, U., Hornig, B. and Drexler, H. (2003) "Role of oxidative stress in atherosclerosis", *Am. J. Cardiol.* **91**, 7A–11A.
[6] Yorek, M.A. (2003) "The role of oxidative stress in diabetic vascular and neural disease", *Free Radic. Res.* **37**, 471–480.

[7] Butterfield, D.A. (2003) "Amyloid beta-peptide (1–42)-induced oxidative stress and neurodegeneration in Alzheimer's disease brain: mechanisms and consequences", *Curr. Med. Chem.* **10**, 2651–2659.
[8] Nakamura, H., Masutani, H. and Yodoi, J. (2002) "Redox imbalance and its control in HIV infection", *Antiox. Redox Signal* **4**, 455–464.
[9] Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R. and Milzani, A. (2003) "Protein carbonylation in human diseases", *Trends Mol. Med.* **9**, 169–176.
[10] Dalle-Donne, I., Scaloni, A., Giustarini, D., Cavarra, E., Tell, G., Lungarella, G., Colombo, R., Rossi, R. and Milzani, A. (2004) "Proteins as biomarkers of oxidative/nitrosative stress in diseases. The contribution of redox proteomics", *Mass Spectrom. Rev.*, In press.
[11] Griffiths, H.R., Moller, L., Bartosz, G., Bast, A., Bertoni-Freddari, C., Collins, A., Cooke, M., Coolen, S., Haenen, G., Hoberg, A.M., Loft, S., Lunec, J., Olinski, R., Parry, J., Pompella, A., Poulsen, H., Verhagen, H. and Astley, S.B. (2002) "Biomarkers", *Mol. Aspects Med.* **23**, 101–208.
[12] Giles, G.I., Tasker, K.M. and Jacob, C. (2001) "Hypothesis: the role of reactive sulfur species in oxidative stress", *Free Radic. Biol. Med.* **31**, 1279–1283.
[13] Sies, H. (1999) "Glutathione and its role in cellular functions", *Free Radic. Biol. Med.* **27**, 916–921.
[14] Rossi, R., Milzani, A., Dalle-Donne, I., Giustarini, D., Lusini, L., Colombo, R. and Di Simplicio, P. (2002) "Blood glutathione disulfide: *in vivo* factor or *in vitro* artifact?", *Clin. Chem.* **48**, 742–753.
[15] Pastore, A., Federici, G., Bertini, E. and Piemonte, F. (2003) "Analysis of glutathione: implication in redox and detoxification", *Clin. Chim. Acta* **333**, 19–39.
[16] Giustarini, D., Dalle-Donne, I., Colombo, R., Milzani, A. and Rossi, R. (2003) "An improved HPLC measurement for GSH and GSSG in human blood", *Free Radic. Biol. Med.* **35**, 1365–1372.
[17] Mansoor, M.A., Svardal, A.M. and Ueland, P.M. (1992) "Determination of the *in vivo* redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma", *Anal. Biochem.* **200**, 218–229.
[18] Jones, D.P., Carlson, J.L., Mody, V.C., Jr., Cai, J., Lynn, M.J. and Sternberg, P., Jr. (2000) "Redox state of glutathione in human plasma", *Free Radic. Biol. Med.* **28**, 625–635.
[19] Tentori, L. and Salvati, A.M. (1981) "Hemoglobinometry in human blood", *Methods Enzymol.* **76**, 707–715.
[20] Magnani, M., Novelli, G. and Palloni, R. (1984) "Human plasma glutathione oxidation in normal and pathological conditions", *Clin. Physiol. Biochem.* **2**, 287–290.
[21] Curello, S., Ceconi, C., Cargnoni, A., Cornacchiari, A., Ferrari, R. and Albertini, A. (1987) "Improved procedure for determining glutathione in plasma as an index of myocardial oxidative stress", *Clin. Chem.* **33**, 1448–1449.
[22] Anderson, M.E. and Meister, A. (1980) "Dynamic state of glutathione in blood plasma", *J. Biol. Chem.* **255**, 9530–9533.
[23] Halliwell, B. and Gutteridge, J.M.C. (1999) *Free Radicals in Biology and Medicine* (Oxford University Press, Oxford).
[24] Jones, P.D., Carlson, J.L., Samiec, P.S., Sternberg, P., Jr., Mody, V.C., Jr., Reed, R.L. and Brown, L.A.S. (1998) "Glutathione measurement in human plasma. Evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC", *Clin. Chim. Acta* **275**, 175–184.
[25] Usberti, M., Gerardi, G., Micheli, A., Tira, P., Bufano, G., Gaggia, P., Movil, P., Movilli, E., Cancarini, G.C., De Marinis, S., D'Avolio, G., Broccoli, R., Manganoni, A., Albertin, A. and Di Lorenzo, D. (2002) "Effects of a vitamin E-bonded membrane and of glutathione on anemia and erythropoietin requirements in hemodialysis patients", *J. Nephrol.* **15**, 558–564.
[26] Spickett, C.M., Reglinski, J., Smith, W.E., Wilson, R., Walker, J.J. and McKillop, J. (1998) "Erythrocyte glutathione balance and membrane stability during preeclampsia", *Free Radic. Biol. Med.* **24**, 1049–1055.
[27] Devasena, T., Lalitha, S. and Padma, K. (2001) "Lipid peroxidation, osmotic fragility and antioxidant status in children with acute post-streptococcal glomerulonephritis", *Clin. Chim. Acta* **308**, 155–161.
[28] Matteucci, E. and Giampietro, O. (2000) "Oxidative stress in families of type 1 diabetic patients", *Diabetes Care* **23**, 1182–1186.
[29] Sudha, K., Rao, A.V. and Rao, A. (2001) "Oxidative stress in antioxidants epilepsy", *Clin. Chim. Acta* **303**, 19–24.
[30] Simon, E., Paul, J.L., Atger, V., Simon, A. and Moatti, N. (1998) "Erythrocyte antioxidant status in asymptomatic hypercholesterolemic men", *Atherosclerosis* **138**, 375–381.
[31] Reiter, C.D., Wang, X., Tanus-Santos, J.E., Hogg, N., Cannon, R.O., III, Schechter, A.N. and Gladwin, M.T. (2002) "Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease", *Nat. Med.* **8**, 1383–1389.
[32] Orhan, H. and Sahin, G. (2001) "*In vitro* effects of NSAIDS and paracetamol on oxidative stress related parameters of human erythrocytes", *Exp. Toxicol. Pathol.* **53**, 133–140.