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Determination of protein thiolation index (PTI) as a biomarker of oxidative stress in human serum

Daniela Giustarini [a], Federico Galvagni [b], Graziano Colombo [c], Isabella Dalle-Donne [c], Aldo Milzani [c], Anna Maria Aloisi [a], Ranieri Rossi [d, *]

[a] Department of Medicine, Surgery and Neurosciences, University of Siena, Via A. Moro 2, I-53100 Siena, Italy
[b] Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Via A. Moro 2, I-53100 Siena, Italy
[c] Department of Biosciences, Università degli Studi di Milano, Via Celoria 26, I-20133 Milan, Italy
[d] Department of Life Sciences, Laboratory of Pharmacology and Toxicology, University of Siena, Via A. Moro 2, I-53100 Siena, Italy

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ABSTRACT

We have introduced protein thiolation index (PTI), i.e. the molar ratio of the sum of all low molecular mass thiols bound to plasma proteins to protein free cysteinyl residues, as a sensitive biomarker of oxidative stress. According to the original procedure its determination requires a rapid separation of plasma and a specific treatment of samples to stabilize thiols. Here we demonstrate that samples can be collected without use of any anticoagulant to prevent blood clotting and without any stabilization of thiols too. This simplification of the determination of PTI makes its analysis more feasible also in routine clinical laboratories.

We have recently proposed that plasma protein thiol redox state may be a sensitive yet convenient parameter for assessing whole-body oxidative stress. In particular, we introduced the molar ratio of the sum of all low molecular mass thiols (LMM-SHs) bound to plasma proteins (forming, as a whole, *S*-thiolated proteins) to protein free cysteinyl residues (P-SH). We called this new parameter the protein thiolation index (PTI) and demonstrated that it can be used as a suitable biomarker of oxidative stress [1]. In fact, in proteins, −SH groups are the most reactive ones towards oxidants. Additionally, proteins bearing −SH groups or mixed disulfides with LMM-SH circulate in plasma for several days before being removed; therefore, they can be considered a good sensor of cumulative oxidative perturbations. Finally, since PTI is calculated as molar ratio between two oxidative states, it is not influenced by the total content of plasma proteins, which can vary in some pathological conditions. We also demonstrated that PTI can increase with ageing and in patients suffering from alkaptonuria [1]. More recently, PTI was demonstrated to increase in patients with end stage renal disease on maintenance haemodialysis [2] and to be inversely associated with spontaneous intake of calories and proteins in the same patients [3].

For PTI analysis, we developed and validated a spectrophotometric method to detect quickly total *S*-thiolated proteins (PSSX) in plasma samples [1]. The procedure is easy to perform but it requires a pre-treatment of sample to stabilize thiols in blood and the rapid separation of plasma (i.e., blood specimen centrifugation at 12,000*g* for 15 s to be carried out within 1 min from blood sampling), which makes it of difficult application in blood specimens coming from routine clinical laboratories. Samples stabilized by this procedure are stable for months at −20 °C [4].

In this study, we report on a deeper investigation on the pre-analytical procedure required to assess PTI, which demonstrates that whole blood samples can be collected without use of any anticoagulant to prevent blood clotting. Even if PTI can increase with time due to oxidation of thiols to form disulfides, probably the only reactive cysteine (Cys) residue at position 34 in albumin cannot form dimers because of steric hindrance of the protein itself. In addition, we demonstrate that the reaction between low molecular weight disulfides (LMM-SS) and the albumin Cys^{34} -SH group by transsulfuration is very slow. Conditions of storage and possible drawbacks are also investigated.

* Corresponding author.
E-mail address: ranieri@unisi.it (R. Rossi).

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Material and methods

Materials

All the other reagents were purchased by Sigma Aldrich (Milan, Italy), with the exception of mBrB that was from Calbiochem (Milan, Italy). The HPLC solvents were from Carlo Erba (Milan, Italy).

Blood collection

Blood samples were taken from the antecubital vein of healthy people, after oral consent, in the morning after about 12 h of fasting. The study group comprised 55 consenting volunteers (32 females and 23 males, age range: 19–72 years). All the participants reported that they were in good health and none of them had any abnormality on physical examination or in routine laboratory blood tests. Active smokers: 8 of whom 5 smoked <5 cigarettes/day, 3 smoked >5 < 20 cigarettes/day. None of the participants was under antioxidant treatment at the time of the study and all were on free diet. Two aliquots of whole blood (3 ml each) were collected, one in evacuated plastic tubes containing K_3EDTA and the other one in evacuated plastic tubes containing separating gel only. Plasma was obtained from K_3EDTA-treated aliquot by centrifugation at 12,000*g* for 15 s carried out within 1 min from blood collection and then immediately stored at −20 °C as described in the original method [1]. Serum was separated by centrifugation (10 min at 800*g*) of blood specimens left at room temperature for 2 h after collection. Both plasma and serum samples were analyzed using the same method for PTI detection [1]. Briefly, one aliquot (0.05 ml) of either plasma or serum was used for spectrophotometric analysis of P-SH groups by endpoint colorimetric reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [5]. Another aliquot (0.15 ml) of plasma/serum was analyzed for PSSX content by colorimetric reaction with ninhydrin. PTI was calculated as the molar ratio of PSSX to the concentration of free, DTNB-titratable P-SH groups [1].

Method validation and statistics

Data are expressed as mean ± SD. Differences between means were evaluated using Student's *t*-test. A value of $p < 0.05$ was considered statistically significant. Correlation analysis was performed by calculating the Pearson's product-moment correlation coefficient. The agreement between plasma and serum values was evaluated by the Bland-Altman plot [6].

Results and discussion

We have proposed PTI as a new biomarker of oxidative stress a few years ago. Mounting evidence has shown that perturbations in the thiol/disulfide homeostasis and, in particular, an oxidative shift in the thiol/disulfide redox potential in plasma are associated with aging and with several different diseases, such as atherosclerosis, chronic renal failure and neurodegenerative diseases [2,3,7–11]. Cys, homocysteine, glutathione (GSH) and cysteinylglycin (CysGly) exist in plasma in reduced, disulfide, and protein-bound forms, interacting with each other through thiol-disulfide exchange reactions [11]. Cys prevails over the other LMM-SH but the largely more abundant free thiols in plasma are represented by P-SH, with the relative molar ratio of LMM-SH, low molecular mass disulfides (LMM-SS), PSSX and P-SH being about 1:20:20:40 [11]. Albumin provides almost all free thiols in plasma since it is the most abundant protein, with typical concentrations ranging from 0.5 to 0.75 mM [12].

Previously, we validated the method for PTI determination in human plasma, and we supposed that the procedure should include, as main constraint, the rapid separation of plasma and its immediate freezing [1]. Here, PTI was assessed both in plasma and in serum from the same donors (Fig. 1). The obtained values measured in the two matrices were not significantly different, as highlighted by the strong correlation ($r^2 = 0.995$, Fig. 1A) and the 95% limits of agreement calculated by the Bland-Altman plot (Fig. 1B).

This suggests that the blood coagulation process and the consequent deprivation of some proteins do not influence the results. Additionally, a 2-h time lapse, needed to obtain serum, does not lead to protein −SH group oxidation and consequent increase in PTI. We further considered the possibility that samples can be stored for times longer than 3 h and/or at varying temperatures. To study the effect of these variables on PTI levels, serum samples were divided into three aliquots and processed after standing 3 h at 20 °C (~room temperature), 24 h at 20 °C, 24 h at 30 °C or three

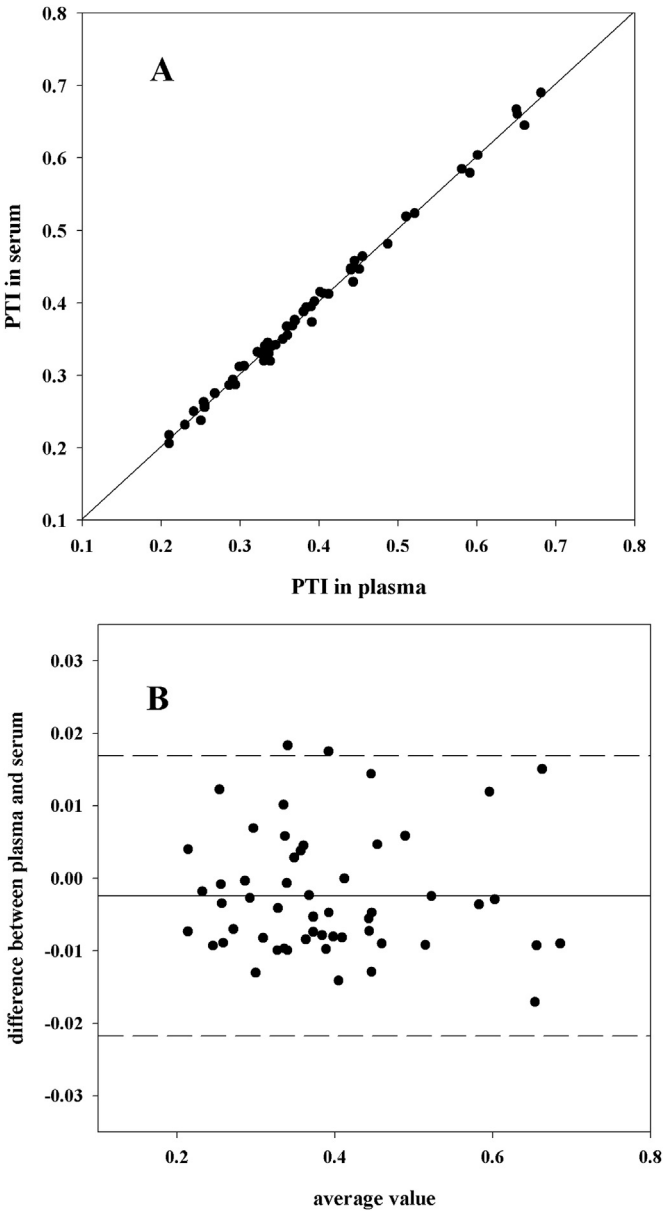

Fig. 1. Correlation between PTI measured in plasma and serum. PTI was measured in plasma and serum of healthy people. Panel A: data set and correlation. Equation regression: y = 0.995x + 0.00175. Panel B: Bland-Altman plot, *n* = 55. The reported values for each individual are the mean of 3 analyses.