Published in final edited form as:

Anal Biochem. 2010 May 15; 400(2): 289–294. doi:10.1016/j.ab.2010.01.032.

A FLUORIMETRIC SEMI-MICROPLATE FORMAT ASSAY OF PROTEIN CARBONYLS IN BLOOD PLASMA

Joy G. Mohanty^{1,*}, Surya Bhamidipaty¹, Michele K. Evans², and Joseph M. Rifkind¹

¹Molecular Dynamics Section, National Inst. On Aging, Baltimore, MD 21224

²Laboratory of Cellular and Molecular Biology, National Inst. On Aging, Baltimore, MD 21224

Abstract

Oxidative stress, originating from reactive oxygen species (ROS), has been implicated in aging and various human diseases. The ROS generated can oxidize proteins producing protein carbonyl derivatives. The level of protein carbonyls in blood plasma has been used as a measure of overall oxidative stress in the body. Classically, protein carbonyls have been quantitated spectrophotometrically by directly reacting them with 2,4, dinitrophenylhydrazine (DNPH). However, the applicability of this method to biological samples is limited by its low inherent sensitivity. This limitation has been overcome by the development of sensitive ELISA methods to measure protein carbonyls. As part of the Healthy Aging in Neighborhoods of Diversity across the Lifespan study, oxidative stress in humans were quantified by measuring blood plasma protein carbonyls using the two commercially available ELISA kits and the spectrophotometric DNPH assay. Surprisingly, two ELISA methods gave very different values for protein carbonyls that were both different from the spectrophotometric method. We have developed a fluorescent semi-microplate format assay of protein carbonyls involving direct reaction of protein carbonyls with fluorescein thiosemicarbazide that correlates ($R=0.992$) with the direct spectrophotometric method. It has a coefficient of variation of 4.99% and is at least 100 times more sensitive than the spectrophotometric method.

Keywords

Protein carbonyl; fluorescence; fluorimetric; assay; human plasma; fluorescein thiosemicarbazide; micro plate format

Introduction

A number of reports suggest that aging is caused by the deleterious and cumulative effects of reactive oxygen species (ROS) [1;2]. When the generation of ROS in the body exceeds the ability to neutralize and/or eliminate them, oxidative stress occurs. This can result from a lack of antioxidant capacity or by an overabundance of ROS generated by stress and/or environmental causes. If not regulated properly, the excess ROS can damage cellular lipids, proteins or DNA, inhibiting normal function [3]. Thus, oxidative stress has been implicated in a growing list of human diseases such as: Alzheimer's[4], Parkinson's[5], cancer [6], diabetes

*Corresponding author, telephone: 410-558-8279, Fax: 410-558-8397, mohantyj@mail.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

[7], macular degeneration [8], multiple sclerosis [9], muscular dystrophy [10], rheumatoid arthritis [11], cardiovascular diseases [12] and the aging process [13]. Oxidative stress can give rise to the formation of protein carbonyl derivatives due to protein oxidation via a variety of physiological and pathological processes [14] causing fragmentation and amine oxidation through metal ion catalysis [15]. Protein oxidation in blood plasma as measured by an increase in plasma protein carbonyls has been used as a measure of the increase in overall oxidative stress in the body as a function of age [16]. This relationship between plasma carbonyls and overall oxidative stress has also been demonstrated for subjects with chronic renal failure [17].

Classically, protein carbonyls have been measured [18] by a direct reaction of carbonyl moieties in proteins with 2,4, dinitrophenylhydrazine (DNPH) followed by the removal of unreacted DNPH by protein precipitation, washing and solubilizing the precipitate, and then reading the absorbance of the DNPH-reacted hydrazones at ~375nm. The DNPH-based assay of protein carbonyls, which directly measures the carbonyls, has been accepted as the method of choice. However, its applicability to biological samples is limited by the low inherent sensitivity of a direct spectrophotometric method [19]. Thus, more sensitive Enzyme-Linked Immunosorbent assays (ELISA) have been developed [20;21] for the measurement of protein carbonyls. Recently, two different ELISA kits for carbonyl determination have become commercially available: *BioCell* Corporation Limited, (previously known as *ZenTech*) of New Zealand and Cell Biolabs Inc. of U.S.A. The principles behind the assays in these two commercial kits are different and based on the published papers of Buss *et. al.* [21] and Alamdari *et. al.* [20], respectively.

Currently we are studying oxidative stress in humans as part of the Healthy Aging in Neighborhoods of Diversity across the Lifespan (HANDLS) study. Blood plasma protein carbonyls are being measured to determine overall oxidative status in these subjects. For this study, protein carbonyls in plasma samples were initially measured using the BioCell ELISA kit. When we compared these results on 23 plasma samples by the second ELISA method available from Cell Biolabs, very different values were obtained (see results section below). To determine which of these measurements were valid, we repeated the carbonyl assay in those samples by the classical direct Spectrophotometric method. This was possible as larger protein amount required for Spectrophotometric method was not a problem with plasma samples. Interestingly, this direct method gave values that were in between that of the two ELISA methods, but did not agree with either ELISA method (see results section below).

To resolve this dilemma, an alternative direct method having adequate sensitivity for biological systems was sought. Recent reports [22;23] have used a highly fluorescent compound, fluorescein 5-thiosemicarbazide (FTC) that specifically reacts with carbonyl groups in oxidized proteins and not in oxidized lipids. The method of Chaudhuri *et al.* [22] was directed at the identification of the individual carbonylated proteins and their quantitation after separation by SDS-polyacrylamide gel electrophoresis followed by fluorescence densitometry of gels. Such a method is impractical for the assay of hundreds of plasma samples. We, therefore, developed a semi-microplate format assay of protein carbonyls using this FTC-carbonyl reaction. This method directly measures the protein carbonyls similar to the classical spectrophotometric assay and gave results comparable to the later method. However, the use of a fluorescent probe resulted ≥ 100 -fold increase in sensitivity.

Materials and methods

Materials

All chemicals unless otherwise mentioned were obtained from Sigma Aldrich. HANDLS blood samples were collected at the source according to an approved protocol (protocol # 2003-314)

for human study and shipped to our laboratory on ice. Upon receipt, plasma was separated and stored at -150°C until the carbonyl measurements could be performed. BioCell ELISA kits for the measurement of protein carbonyls were obtained from Northwest Life Science Specialities, Vancouver, WA, whereas OxiSelect Protein Carbonyl ELISA Kits were obtained from Cell Biolabs, Inc., San Diego, CA.

Spectrophotometric assay of protein carbonyls

The spectrophotometric assay of protein carbonyls in human plasma was assayed following the published protocol [18] with minor modifications. Briefly, 23 frozen plasma samples were thawed, appropriately diluted with phosphate buffered saline (PBS) and the total protein concentration was measured by the BCA assay from Pierce (Rockford, IL). Average total protein concentration in plasma was found to be $\sim 80\text{mg}$ per ml. Diluted plasma samples ($100\mu\text{l}$) corresponding to total protein of $10\text{mg}/\text{ml}$ in PBS was either reacted with $400\mu\text{l}$ of 10mM of DNPH in 2.5M HCl or 2.5M HCl only (blank) for 45 min in the dark while mixing every 10–15min. Following reaction, proteins were precipitated with 0.5ml of 20% cold trichloroacetic acid (TCA), washed once with 1ml of 10% TCA, three times with a 1:1 ethanol ethyl acetate mixture and the precipitate solubilized in its original volume ($100\mu\text{l}$) of 6M guanidine hydrochloride (GuHCl) prior to reading absorbance at $\sim 375\text{nm}$ with corresponding blanks as references. The protein concentration in these samples was measured by BCA assay and the concentration of protein carbonyls was expressed as nmol/mg protein.

ELISA assays of protein carbonyls with commercial kits

Measurements of protein carbonyls in human plasma were performed in triplicate using ELISA kits obtained from BioCell and Cell Biolabs according to the respective company protocols. With BioCell kit, assay was done using their standard protocol with $5\mu\text{l}$ ($\sim 80\text{mg}/\text{ml}$) of each of the plasma samples. With Cell Biolabs OxiSelect Protein Carbonyl ELISA Kit, plasma samples were diluted 8000-fold to $\sim 10\mu\text{g}/\text{ml}$ protein with PBS prior to adsorption onto ELISA plates.

Determining conditions for the complete reaction of carbonyls with FTC

Completion of the reaction of FTC with a sample of oxidized bovine serum albumin (BSA) was monitored by the use of size exclusion chromatography (SEC) on a Shimadzu HPLC system consisting of LC-20AD pumps, RF-10XL fluorescence detector (Ex: 492nm ; Em: 516nm), a BAS UV116A UV-Vis detector (212nm) and Zorbax GF-250 column ($4.6 \times 250\text{mm}$) from Agilent Technologies Inc with 0.1M NaH_2PO_4 pH 7.0 as the mobile phase. Oxidized BSA (OxBSA) was prepared as described by Buss *et. al.* [21] by oxidizing BSA with Fe^{2+} /ascorbic acid as the oxidant and then exhaustively dialyzing it against PBS. FTC stock (100mM) was prepared in dimethylsulfoxide (DMSO) and then diluted in HEPES, pH 6.0 buffer for reaction with protein carbonyls. OxBSA ($100\mu\text{l}$) corresponding to $10\text{mg}/\text{ml}$ in HEPES pH 6.0 was mixed with $100\mu\text{l}$ of 0.2mM FTC, and immediately $10\mu\text{l}$ of it was injected on to the HPLC column for a 0min time point. Thereafter, $10\mu\text{l}$ samples of the reaction mixture were injected into the HPLC column at different time points to monitor the reaction. The HPLC method of separating the FTC-reacted proteins from the unreacted FTC was used to determine the time required for completion of reaction and the washings required to remove unreacted FTC from the sample.

FTC Fluorimetric assay of protein carbonyls

Diluted plasma samples ($50\mu\text{l}$ in 1.5ml tubes) as above in duplicates corresponding to total protein of $10\text{mg}/\text{ml}$ in HEPES, pH 6.0 were reacted with an equal volume of 0.2mM FTC ($50\mu\text{l}$) overnight in the dark. Proteins were precipitated by the addition of 4-volumes of cold 20% trichloroacetic acid (TCA, $400\mu\text{l}$). Following 10min incubation on ice, tubes were

centrifuged ~12000 RPM in a refrigerated microfuge (Eppendorf 5417R) for 10min at 4°C. Supernatants were carefully decanted; precipitates were washed 3 times by vortexing with 1ml of acetone, and centrifuged immediately as above. Finally, acetone supernatant was carefully decanted out and protein precipitates were air dried, solubilized with 50µl of 6M guanidine hydrochloride (GuHCl) and diluted 10-fold (to raise the pH) by the addition of 450µl of 0.1M NaH₂PO₄ pH 7.0. Protein concentration in each of these samples was measured by the BCA assay as above. Solubilizing the protein precipitate first in 6M GuHCl and then diluting 10-fold with 0.1M Sodium Phosphate buffer (pH 7.0) makes it possible to fully dissolve the precipitate and still assay for protein by the BCA assay, which requires that GuHCl be < 4M. We also found improved reliability of the fluorescence measurement by this procedure. The samples were aliquoted 100µl per well in triplicate into a white microtiter plate (Perkin Elmer Optiplate), and fluorescence measured in a Perkin Elmer Victor³V 1420 Multilabel Counter with excitation at 485nm and emission at 535nm. Fluorescence readings from 6 wells for each sample (3 for each of the duplicates) were averaged and nanomoles of FTC-reacted carbonyls were calculated using a standard curve generated from the readings of various concentrations of FTC prepared in a medium similar to that of the samples. For this purpose, a dilution buffer for the FTC standard curve was prepared from a pool of plasma samples that had not reacted with FTC. This pool was precipitated by 20% TCA, the precipitates were dissolved in 6M GuHCl (~10mg protein/ml) and diluted 10-fold with 0.1M NaH₂PO₄ pH 7.0 prior to use. Diluted FTC standards (100µl per well) were put into triplicate wells on the same microtiter plate along with the samples and the fluorescence was read as above. The amount of protein carbonyls in plasma were expressed as nmol/mg protein. For samples with high protein carbonyl content such as OxBSA, greater than 10-fold dilution may be necessary to read FTC fluorescence.

Results and Discussion

Comparison of commercial ELISA kits with Spectrophotometric assay

Measurement of protein carbonyls in 23 plasma samples were performed in triplicate by using two commercial ELISA kits from BioCell and Cell Biolabs as well as the classical Spectrophotometric assay. The mean values with standard error from these assays are shown as bar graphs in Figure 1. As seen in figure 1, the level of protein carbonyls measured by BioCell kit was much lower (10-fold less) whereas the Cell Biolabs kit measured much higher levels of protein carbonyls (~8-fold more) than the level detected by the spectrophotometric assay. The dramatic difference (80 fold) between the two ELISA assays was particularly surprising, since the papers [20;21] where both ELISA assays were developed compared results obtained with plasma samples by their ELISA assays to the spectrophotometric assay.

The paper used to develop the BioCell assay found that their assay gave significantly higher values than the spectrophotometric assay and attributed the difference to uncertainty in the absolute values of carbonyls by both methods and emphasized the validity of their method for measuring differences between different samples. Since the BioCell and Cell BioLabs assays provide good standard curves using oxidized BSA standards provided with the respective kit, they must reflect protein carbonyls in the sample and can be considered valid for measuring differences between plasma samples. However, the paper that developed the Cell BioLabs ELISA assay reported a much closer agreement with the spectrophotometric method and concluded that their method provides an absolute measure of the protein carbonyls in the sample. The 8 fold discrepancy that we observed using the protocol provided in the Cell Biolabs ELISA assay kit may reflect difficulties with the kit and/or the standards provided and not the methodology as developed in the literature [20].

While we have not been able to explain the reason for the major differences between assays (Fig. 1), the possible contributing factors can be preferential adsorption to the plate of certain

pools of protein carbonyls, difficulties in removing unreacted DNPH from the microtiter plates, preferential reaction with antibodies and HRP linkage for certain pools of adsorbed DNPH-reacted proteins [18;20].

Development of a Fluorimetric assay of protein carbonyls in a microtiter plate format

Since the commercial kits available to measure protein carbonyls in human plasma yielded values that were either much higher or much lower than the direct spectrophotometric method, an alternative sensitive method for directly measuring protein carbonyls was explored. FTC was found to be a good alternative as it is fluorescent and is much more sensitive than DNPH as a label for protein carbonyls. This method was developed on the principle that one mole of FTC, a thiosemicarbazide derivative of fluorescein, directly reacts with one mole of carbonyls in samples to produce one mole of thiosemicarbazones [22], which can be measured by reading the fluorescence of the FTC-reacted proteins and converting the fluorescence data to nanomoles by using an FTC standard curve. This method like the spectrophotometric method involves the reaction of one molecule of FTC (instead of DNPH) with one molecule of a protein carbonyl and is therefore a direct measure of the protein carbonyls.

The validity of this method depends on being able to quantitatively react with all protein carbonyls, remove any unreacted FTC and provide valid standards for the FTC reacted with protein carbonyls. To determine the time required for FTC to react with all protein carbonyls and to develop a method to remove unreacted FTC, we used size exclusion HPLC to follow these processes. A 200 μ l reaction mixture of OxBSA was prepared and reacted with FTC as described in the Methods. 10 μ l aliquots of the reaction mixture were analyzed at different times by size exclusion chromatography (SEC) on HPLC with a fluorescence detector to detect fluorescent peaks. In SEC, unreacted FTC is eluted as a broad peak (perhaps due to its strong interaction with the stationary phase) between 5min and 15min (Figure 2 panels A–B), whereas FTC-reacted proteins were eluted around 1.5min to 3.5min (Figure 2 panels B–C). The chromatogram of FTC alone (data not shown) had the same broad band between 5 min and 15 min as in Figure 2 panels A or B with no peak in the time frame of 1.5min–3.5min. Since the unreacted FTC is completely separated from the protein reacted FTC, the time course for the reaction could be determined by integrating the area under the peaks in the chromatogram between 1.5min and 3.5min at different injection times. As shown in Figure 3, the FTC reaction is almost complete after 10 hours. It was, thus, decided to use an overnight reaction for further experiments. Since SEC also detects the free unreacted FTC, we were able to use HPLC to establish a method that completely removed free FTC from the precipitate of FTC-linked proteins. As shown in the panel C of Figure 2, this is accomplished by acetone washing of the TCA precipitate, which completely removed the peak due to free FTC.

For the FTC assay, both the standards and samples were prepared in the same medium. 6M GuHCl was used to solubilize the protein precipitate. Since the optimal fluorescence of fluorescein in FTC [24] is around pH 7.0 the GuHCl-solubilized proteins were diluted 10 fold in the pH 7.0 phosphate buffer. The medium used for the standards was, therefore, 0.6M GuHCl, protein concentration around 1mg/ml and phosphate buffer of pH 7.0.

Since FTC fluorescence depends on the environment such as nature of the proteins, their concentration and pH, we prepared the FTC dilution standards in the media containing similar proteins as in the FTC-reacted plasma samples. For this purpose, a pool of plasma samples that had not reacted with FTC were precipitated by 20% TCA, the precipitates were dissolved in 6M GuHCl (~10mg protein/ml) and diluted 10-fold with 0.1M NaH₂PO₄ pH 7.0 to prepare the dilution medium for various concentrations of FTC. Even though the standards were prepared in the same medium at the same pH and in the presence of GuHCl and the same level of protein, FTC in the standards was not incubated for 24 hours and was for the most part not reacted with carbonyls unlike the FTC in the sample. To check the validity of these standards,

we compared the fluorescence for a set of FTC standards prepared with BSA and with OxBSA, both at 1mg/ml in the buffer diluent after 24 hr incubation. For BSA, there are a minimal number of carbonyls for the FTC to react with. However, for OxBSA a major fraction of the low levels of added FTC will react with carbonyls present in 24 hours. Figure 4 shows that both calibrations agree except for a small deviation for the highest concentration of FTC and this confirms the validity of the standards used in our assay. Although Ahn et al. [25] have reported a decrease in fluorescence for the FTC reacted with carbonyls, this was not in the presence of GuHCl and at the protein levels used in our assay.

In order to be able to measure multiple samples along with the FTC standards at the same time, we used a microplate format to read the fluorescence of standards and samples on a white microtiter plate using a fluorescence microplate reader.

To validate this Fluorimetric assay, protein carbonyls were measured in a series of samples with total protein concentration of 10mg/ml, containing various amounts of oxidized BSA (0%, 1%, 2%, 5%, 10% and 20%) and reduced BSA (prepared as described by Buss *et al.* [21]; assumed to have no protein carbonyls, 0%), by both the FTC method and the Spectrophotometric method. For each sample, the protein concentration was measured by the BCA assay in the same aliquot that was used to measure fluorescence or absorbance and the protein carbonyl content was expressed as nmol/mg protein. The mean values of protein carbonyls for each sample obtained by these two methods are compared in Figure 5. As can be seen, there is an excellent correlation ($R = 0.992$) between the Fluorimetric FTC and the Spectrophotometric methods for the measurement of protein carbonyls.

Precision of the FTC assay calculated from the data presented in Table 1 of repeated measurements of protein carbonyls in 3% OxBSA samples was 4.99%. The equation used for this calculation is presented below.

$$\text{Percent Coefficient of variation (\%CV)} = (\text{Standard deviation, SD}) \times 100 / \text{mean}$$

To compare the sensitivity of FTC fluorimetric carbonyl assay and the DNPH spectrophotometric assay, various dilutions of FTC and DNPH were made with a buffer containing bovine serum albumin (1mg/ml), 0.6M GuHCl and 0.1M NaH₂PO₄ pH 7.0. Fluorescence and absorbance of FTC and DNPH, respectively, at various dilutions are shown in Figure 6. Both curves are linear with respect to concentration, while the detection limit of FTC is about 2 nanomolar (nM) which is about 1000-fold lower than that for DNPH (2 micro molar (μM)). However, since the FTC assay involves a 10-fold dilution step to adjust the pH prior to fluorescence measurement, its sensitivity can be considered as about 100-fold higher in terms of nanomoles of protein carbonyls per milligram of proteins than the DNP assay. For both assays the protein level in the final sample were measured so as to be able to reliably calculate the level of carbonyls in terms of nmol/mg protein.

We have also used the FTC method to measure protein carbonyls in 3% OxBSA at different protein concentrations (10mg/ml diluted down to 10μg/ml with PBS) to determine the lower limit of protein content in samples, which can be reliably measured. Results (data not shown) suggest that the FTC method of protein carbonyl determination is not reliable for samples containing less than 2mg/ml protein. There is a considerable protein loss during TCA precipitation and washing of the protein precipitates that affects the validity of the results in samples with protein levels below 2mg/ml by this FTC method.

To test the validity of this method with plasma samples we measured the protein carbonyls in the same 23 plasma samples as in Figure 1 comparing the FTC method with the spectrophotometric method. As shown in Figure 7, the mean carbonyl content of plasma

samples obtained by the FTC method is slightly lower than the mean value obtained from the Spectrophotometric method; but the difference is not statistically significant ($p=0.08$). A Bland-Altman analysis (using Analyze-it add-in statistical software to MS Excel) of these data shown in Figure 8 also corroborates that the difference seen between the FTC and spectrophotometric methods is not statistically significant. The slightly higher value of protein carbonyl with the spectrophotometric method may be attributed to the presence of unreacted DNPH that is not completely removed due to its low solubility. This is not the case with FTC as unreacted FTC is quantitatively removed by washing with acetone (Figure 2 panel C).

In summary, these results establish that the FTC method is a reliable and sensitive method that can be used to measure protein carbonyls in blood plasma and in presumably other biological samples having protein content 2mg/ml or above. However, for cultured cell or tissue extracts, it would be necessary to remove nucleic acid contamination [18] (as they also contain carbonyls) prior to FTC assay. FTC assay on diluted protein samples can also be performed after concentrating them to 2mg/ml protein or more. In addition, use of a micro plate to measure fluorescence of FTC-linked protein carbonyls will allow processing multiple samples at the same time.

List of abbreviations

ROS	Reactive oxygen species
DNPH	2,4, Dinitrophenylhydrazine
ELISA	Enzyme linked immunosorbent assay
HANDLS	Healthy Aging in Neighborhoods of Diversity across the Lifespan
FTC	Fluorescein thiosemicarbazide
PBS	Phosphate buffered saline
SEC	Size exclusion chromatography
TCA	Trichloroacetic acid
OxBSA	Oxidized (Ox) Bovine serum albumin (BSA)
GuHCl	Guanidine hydrochloride
DMSO	Dimethylsulfoxide
HRP	Horseradish peroxidase.

Acknowledgments

This research was supported entirely by the Intramural Research program of the NIH, National Institute on Aging.

References

1. Knight JA. The biochemistry of aging. *Adv. Clin. Chem* 2000;35:1–62. [PubMed: 11040957]
2. Rikans LE, Hornbrook KR. Lipid peroxidation, antioxidant protection and aging. *Biochim. Biophys. Acta* 1997;1362:116–127. [PubMed: 9540842]
3. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol* 2007;39:44–84. [PubMed: 16978905]
4. Christen Y. Oxidative stress and Alzheimer disease. *Am. J. Clin. Nutr* 2000;71:621S–629S. [PubMed: 10681270]

5. Cohen G. Oxidative stress, mitochondrial respiration, and Parkinson's disease. *Ann. N. Y. Acad. Sci* 2000;899:112–120. [PubMed: 10863533]
6. Upham BL, Wagner JG. Toxicant-induced oxidative stress in cancer. *Toxicol. Sci* 2001;64:1–3. [PubMed: 11606793]
7. Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* 1991;40:405–412. [PubMed: 2010041]
8. Yildirim O, Ates NA, Tamer L, Muslu N, Ercan B, Atik U, Kanik A. Changes in antioxidant enzyme activity and malondialdehyde level in patients with age-related macular degeneration. *Ophthalmologica* 2004;218:202–206. [PubMed: 15103217]
9. Gilgun-Sherki Y, Melamed E, Offen D. The role of oxidative stress in the pathogenesis of multiple sclerosis: the need for effective antioxidant therapy. *J. Neurol* 2004;251:261–268. [PubMed: 15015004]
10. Rodriguez MC, Tarnopolsky MA. Patients with dystrophinopathy show evidence of increased oxidative stress. *Free Radic. Biol. Med* 2003;34:1217–1220. [PubMed: 12706502]
11. Mapp PI, Grootveld MC, Blake DR. Hypoxia, oxidative stress and rheumatoid arthritis. *Br. Med. Bull* 1995;51:419–436. [PubMed: 7552073]
12. Usberti M, Gerardi GM, Gazzotti RM, Benedini S, Archetti S, Sugherini L, Valentini M, Tira P, Bufano G, Albertini A, Di LD. Oxidative stress and cardiovascular disease in dialyzed patients. *Nephron* 2002;91:25–33. [PubMed: 12021516]
13. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. *Science* 1996;273:59–63. [PubMed: 8658196]
14. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. *J. Cell Mol. Med* 2006;10:389–406. [PubMed: 16796807]
15. Stadtman ER, Oliver CN. Metal-catalyzed oxidation of proteins. Physiological consequences. *J. Biol. Chem* 1991;266:2005–2008. [PubMed: 1989966]
16. Mutlu-Turkoglu U, Ilhan E, Oztecan S, Kuru A, Ykac-Toker G, Uysal M. Age-related increases in plasma malondialdehyde and protein carbonyl levels and lymphocyte DNA damage in elderly subjects. *Clin. Biochem* 2003;36:397–400. [PubMed: 12849873]
17. Dursun E, Dursun B, Suleymanlar G, Ozben T. Carbonyl stress in chronic renal failure: the effect of haemodialysis. *Ann. Clin. Biochem* 2005;42:64–66. [PubMed: 15802036]
18. Reznick AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol* 1994;233:357–363. [PubMed: 8015470]
19. Cao G, Cutler RG. Protein oxidation and aging. I. Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine. *Arch. Biochem. Biophys* 1995;320:106–114. [PubMed: 7793968]
20. Alamdari DH, Kostidou E, Paletas K, Sarigianni M, Konostas AG, Karapiperidou A, Koliakos G. High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein. *Free Radic. Biol. Med* 2005;39:1362–1367. [PubMed: 16257645]
21. Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. *Free Radic. Biol. Med* 1997;23:361–366. [PubMed: 9214571]
22. Chaudhuri AR, de Waal EM, Pierce A, Van RH, Ward WF, Richardson A. Detection of protein carbonyls in aging liver tissue: A fluorescence-based proteomic approach. *Mech. Ageing Dev* 2006;127:849–861. [PubMed: 17002888]
23. Fujita H, Hirao T, Takahashi M. A simple and non-invasive visualization for assessment of carbonylated protein in the stratum corneum. *Skin Res. Technol* 2007;13:84–90. [PubMed: 17250537]
24. Hammer M, Schweitzer D, Richter S, Konigsdorffer E. Sodium fluorescein as a retinal pH indicator? *Physiol Meas* 2005;26:N9–N12. [PubMed: 15886427]
25. Ahn B, Rhee SG, Stadtman ER. Use of fluorescein hydrazide and fluorescein thiosemicarbazide reagents for the fluorometric determination of protein carbonyl groups and for the detection of oxidized protein on polyacrylamide gels. *Anal. Biochem* 1987;161:245–257. [PubMed: 2883911]

Figure 1. Comparison of the mean values of protein carbonyls (nanomoles per mg total protein) in 23 plasma samples as measured by using Spectrophotometric assay (Spectro), and commercial ELISA assay kits from BioCell Corporation, NZ (BioCell) and Cell Biolabs, USA (CellBioLabs).

Figure 2.

Size exclusion chromatograms (SEC) of oxidized BSA reacted with FTC (Flow: 1ml/min; Mobile Phase: 0.1M Sodium Phosphate, pH 7.0), Panel A – 10 μ l of reaction mixture injected to HPLC column as soon as FTC was added to oxidized BSA (2 minute passed), Panel B – similar to panel A except that it was run after 24 hour reaction and Panel C – proteins in the reaction mixture after 24hour was TCA precipitated, washed with acetone as described in the Methods, dried, re-dissolved in the original reaction volume of 0.1M Na₂HPO₄, pH 7.0, and 10 μ l injected.

Figure 3. Time-dependent reaction of FTC with oxidized BSA. The ordinate (Fluorescence) is the area under the fluorescent peaks (between retention times 1.5min to 3.5min) as in Figure 2 at different time points.

Figure 4. Comparison of fluorescence values of FTC dilutions after 24 hr incubation in media containing 0.6M GuHCl, 0.1M Sodium phosphate, pH 7.0 with 10% OxBSA, 1mg/ml (squares), and with BSA, 1mg/ml (Circles).

Figure 5.

Correlation between Spectrophotometric assay and the Fluorimetric assay. Protein carbonyl values (nmol/mg total protein) in samples containing 0%, 1%, 2%, 5%, 10% and 20% OxBSA measured by both of these methods.

Figure 6.

Plots of absorbance (A) of DNPB at 375nm and fluorescence (B) of FTC (Ex: 485nm; Em: 535nm) at their various dilutions in medium containing 0.6M GuHCl, bovine serum albumin (1mg/ml) and 0.1M NaH_2PO_4 , pH 7.0. Inserts in A and B show amplified portion of DNPB (0–10 μM) and FTC (1–10nM) curves respectively.

Figure 7. Comparison of the mean level of protein carbonyls (nmol/mg total protein) as measured by FTC method with the values obtained by the Spectrophotometric assay (Spectro) in the same 23 plasma samples as in Figure 1.

Figure 8.
Bland-Altman analysis of the same data as in Figure 7

Table 1

Precision analysis of FTC assay on a sample containing 3% OxBSA mixed with Reduced-BSA containing 10mg/ml protein

Number of attempts	Protein Carbonyl (nmol/mg)	Mean $\pm$ SD	Coefficient of variation (%)
1	0.490	0.480 $\pm$ 0.024	4.99
2	0.453		
3	0.459		
4	0.487		
5	0.524		
6	0.467		
7	0.477		