Free Radical Biology and Medicine 59 (2013) 27–35

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Methods in Free Radical Biology and Medicine

Method for the simultaneous determination of free/protein malondialdehyde and lipid/protein hydroperoxides

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ARTICLE INFO

Article history:
Received 27 June 2012
Received in revised form
25 September 2012
Accepted 27 September 2012
Available online 3 October 2012

Keywords:
Lipid/protein malondialdehyde
Thiobarbituric acid
Lipid/protein hydroperoxides
Oxidative stress
Xylenol orange
Free radicals

ABSTRACT

A simple and sensitive method is presented for the simultaneous quantification (spectrophotometric and spectrofluorimetric) of the main lipid and protein peroxidation products after their initial fractionation: free malondialdehyde (FrMDA), protein-bound malondialdehyde (PrMDA), total hydroperoxides (LOOH), and protein hydroperoxides (PrOOH). FrMDA and PrMDA (released from proteins by alkaline hydrolysis) are measured after the reaction of MDA with thiobarbituric acid (TBA) under acidic conditions, by the specific fluorimetric quantification of the resulting MDA–$(TBA)_2$ adduct chromophore. The measurement of LOOH and PrOOH is based on the reaction of Fe^{3+} (resulting from the reaction of LOOH and PrOOH with Fe^{2+}) with xylenol orange (XO) and the photometric quantification of the resulting XO–Fe complex. The sensitivity of the assays for FrMDA/PrMDA and LOOH/PrOOH is 20 and 100 pmol, respectively. The method was applied successfully on human plasma and can be used for the evaluation of oxidative stress in both basic and clinical research.

Introduction

Lipid peroxidation is a disease-related process characterized by the oxidation-related destruction of biological membranes and other lipid- and protein-containing structures. Because lipid peroxidation is a cascade-like process triggered by a number of reactive oxygen species (ROS), it results in a plentitude of various products. Often these products are highly unstable in a biological environment and react easily with proteins. All this makes the proper measurement of lipid peroxidation exceptionally difficult. The resulting oxidation products are used as indirect indicators of high oxidative stress [1], thus necessitating their accurate determination.

For some time, lipid hydroperoxides and malondialdehyde were identified as some relatively reliable and stable products of lipid peroxidation, formed during this process in general. An array of methods was developed for the measurement of these products. However, many of them are qualitative and the quantitative ones focus on only one indicator; some of them are not indicator-specific and others do not use appropriate controls. This makes it almost impossible to judge the various forms of these lipid peroxidation products.

To overcome these methodological difficulties we developed a simple and sensitive method for the simultaneous quantification of the main lipid and protein peroxidation products: free malondialdehyde (FrMDA), protein-bound malondialdehyde (PrMDA), total hydroperoxides (LOOH; representing mainly lipid and other hydrophobic hydroperoxides), and protein hydroperoxides (PrOOH). The adoption of this method makes it possible to judge the lipid and protein peroxidation process comprehensively and also to judge the results in comparison to previously used more traditional methods. The determination of a set of parameters from a single sample makes the method multidimensional, enabling in our opinion the best determination of lipid peroxidation possible today. The method is easily applicable to human plasma, as tested by us, and gives, therefore, the possibility of adopting it to clinical procedures and research. Moreover, the method has been tested in a variety of organisms (e.g., mouse organs, fungi, bacteria), and it can be used for the indirect evaluation of oxidative stress in basic research.

Background of methodology

The formation of lipid peroxidation products usually is initiated by the abstraction of a hydrogen atom from a lipid methylene group with adjacent double bonds, leading to the formation of conjugated dienes (usually quantified in lipid extracts by second-derivative spectroscopy [2]) and of a carbon-centered radical. To this radical an

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http://dx.doi.org/10.1016/j.freeradbiomed.2012.09.038

oxygen molecule is incorporated, leading to the formation of a lipid peroxyl radical ($LOO^{\bullet}$) and then of a hydroperoxide (LOOH), which is considered an early product indicator of the lipid peroxidation process (enzymatic and nonenzymatic) [1]. $LOO^{\bullet}$ can be further degraded to the final lipid peroxidation products malondialdehyde (MDA) and other monoaldehydes (with 4-hydroxynonenal, HNE, being the most biologically important) [1]. On the other hand, protein oxidation by ROS (extensively reviewed by Stadtman [3]) results in the formation of protein carbonyls and hydroperoxides (PrOOH); the formation mechanism of the latter has been dealt with elsewhere [4,5]. PrOOH can be generated in reactions between protein radicals and oxygen, as in the following general reactions [6]: $PH+X^{\bullet}\rightarrow Pr^{\bullet}+X^{-}+H^{+}$, $Pr^{\bullet}+O_2\rightarrow PrOO^{\bullet}$, and $PrOO^{\bullet}+(H^{+}+e^{-})\rightarrow PrOOH$, where $X^{\bullet}$ is a one-electron oxidant such as a hydroxyl ($HO^{\bullet}$), peroxyl ($XOO^{\bullet}$), or thiyl ($XS^{\bullet}$) radical.

The chemistry and biochemistry of MDA and HNE as well as their quantification have been extensively reviewed [7,8]; MDA is very reactive and forms Schiff-base adducts with lysine residues and cross-links proteins; HNE reacts with lysine primarily via a Michael addition reaction [9] and also with histidine and cysteine as verified by HNE monoclonal antibodies [10]. However, free HNE is not a reliable indicator of lipid peroxidation because it is rapidly metabolized (90–95% of it within 3 min) [8,11,12], whereas protein-bound HNE is a reliable, although qualitative, indicator of lipid peroxidation [13,14]. Protein-bound MDA and HNE can be detected qualitatively, e.g., by monoclonal antibodies [15,16]. MDA can be measured in DNA by monoclonal antibodies as well [17].

Several photometric and fluorimetric methods (new and modified from previous ones) have been reported for the direct quantification of PrOOH, FrMDA, and LOOH separately or in mixture [7,8,18–22], whereas PrMDA is determined only by HPLC [23]. The main choice for MDA determination has been the thiobarbituric acid (TBA) assay (photometric and fluorimetric) [24,25]. The photometric assay has been also used in conjunction with HPLC for the determination of FrMDA and PrMDA (after alkaline hydrolysis) [23]. For LOOH determination, prominent methods are the stoichiometric iodine assay (alone or in conjugation with HPLC) and the nonstoichiometric chemiluminescence, dichlorofluorescein, cyclooxygenase activation, and thiocyanate assays [26], together with the ferrous oxidation in xylenol orange (FOX) method [27], which has also been applied to the determination of PrOOH [28]. Moreover, many of the lipid peroxidation methods do not discriminate FrMDA from LOOH and are restricted in the determination of FrMDA [29].

Determination of PrOOH/LOOH and FrMDA/PrMDA: problems and solutions

PrOOH/LOOH

PrOOH and LOOH are determined by this method (see Fig. 1) using certain modifications of the original FOX assay. This assay is based on the reaction of Fe^{3+} (resulting from Fe^{2+} after its oxidation by hydroperoxides) with xylenol orange (XO) under acidic conditions and the formation of a XO–Fe complex absorbing at 560 nm (Fig. 2A) [30]. The FOX method was initially developed for H_2O_2 analysis in radiolyzed solutions [27], and it was then modified for LOOH determination (in liposomes, plasma, and lipoproteins [31–33]), as well as for PrOOH determination [5,28]. The factors that influence the FOX assay's performance have been extensively reviewed elsewhere [34], with the most crucial being its very narrow optimum pH range 1.7–1.8 [35]. Another important interfering factor is reducing agents (e.g., ascorbic acid, glutathione) in the sample because they will reduce Fe^{3+} back to Fe^{2+}.

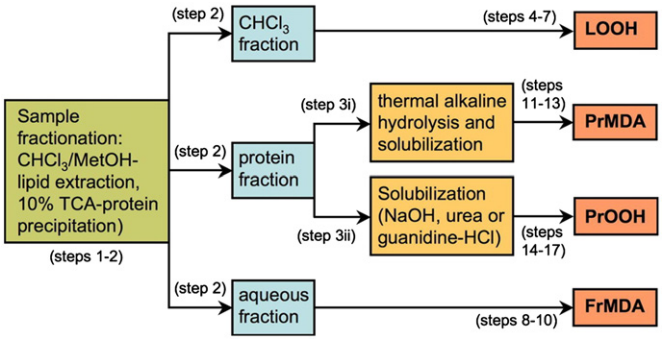

Fig. 1. Method flow chart for the determination of FrMDA, PrMDA, LOOH, and PrOOH. Steps depicted here are described in detail in the text.

The PrOOH assay employed in this method is based on the initial FOX-1 assay [33]. It is used in place of the equally sensitive M-PCA-FOX assay [28,35], with the following modifications: (i) to make the sample treatment compatible with the FrMDA/PrMDA trichloroacetic acid (TCA) assay (see following section), the present PrOOH assay solubilizes the fractionated protein pellet at pH 13 with 0.1 M NaOH (equally as effective as guanidine–HCl used in the M-PCA-FOX assay), followed by equimolar neutralization with HCl. However, whenever alkaline protein solubilization is ineffective because of sample peculiarity, urea and guanidine–HCl (8 and 6 M, respectively at pH 1.8) can be used, although both interfere with the assay (Table 1) possibly because of scavenging of the intermediate protein alkoxyl radicals [28]. In support of this, it was found that the absorption coefficient of the XO–Fe complex depends on the concentration of urea or guanidine–HCl; at 8 M urea, the slope decrease is 60%, whereas at 2–4 M urea it is ~20% and similar to that of 3–6 M guanidine-HCl. (ii) To avoid autoxidation of Fe^{2+}, this component is separated from the XO component in the initial FOX assay reagent and is prepared in 5 mM H_2SO_4. (iii) The optimum assay pH range 1.7–1.8 [35] is attained by decreasing by 2× the final concentration of H_2SO_4 (to 12.5 mM) in the initial FOX assay [33]. (iv) Sorbitol is omitted from the present FOX assay as it is more likely to cause complications [36]. All these assay modifications ensure better stabilization of the optimum assay pH when using samples having buffering capacity (such as proteins) and introduce two reagent blanks ($\pm Fe^{2+}$) and an additional sample control (without Fe^{2+}) for increasing the specificity of the assay (e.g., from interfering substances in samples, such as ascorbic acid, or from any 560-nm-absorbing sample components).

The assay for LOOH employed in this method is a modification of the FOX-2 assay [37]. It fractionates LOOH in $CHCl_3$:methanol (MetOH) [38] with concurrent fractionation of proteins (by precipitation with 10% TCA), thus removing lipids also from lipoproteins (in samples such as blood plasma, brain, and intestine). By doing so, the assay quantifies LOOH in MetOH instead of propanol because MetOH is very effective in solubilizing the $CHCl_3$-extracted lipids and with minimum assay interference (Table 1). This fractionation step removes interference from (i) H_2O_2 present in samples (thus omitting the use of catalase as an additional control) and (ii) free amino acid hydroperoxides (such as ValOOH, resulting from $HO^{\bullet}$ attack on valine [4]).

Because peroxides such as monocyclic peroxides and serial-cyclic peroxides may also give a positive FOX assay response [39], we performed a comparative control experiment between cumene/*tert*-butylhydroperoxide and benzoyl peroxide. It was found that the modified FOX-2 assay shows ~250-fold higher specificity for these hydroperoxides over benzoyl peroxide (data not shown). The high specificity of the present FOX assay for hydroperoxides has also been confirmed independently by a

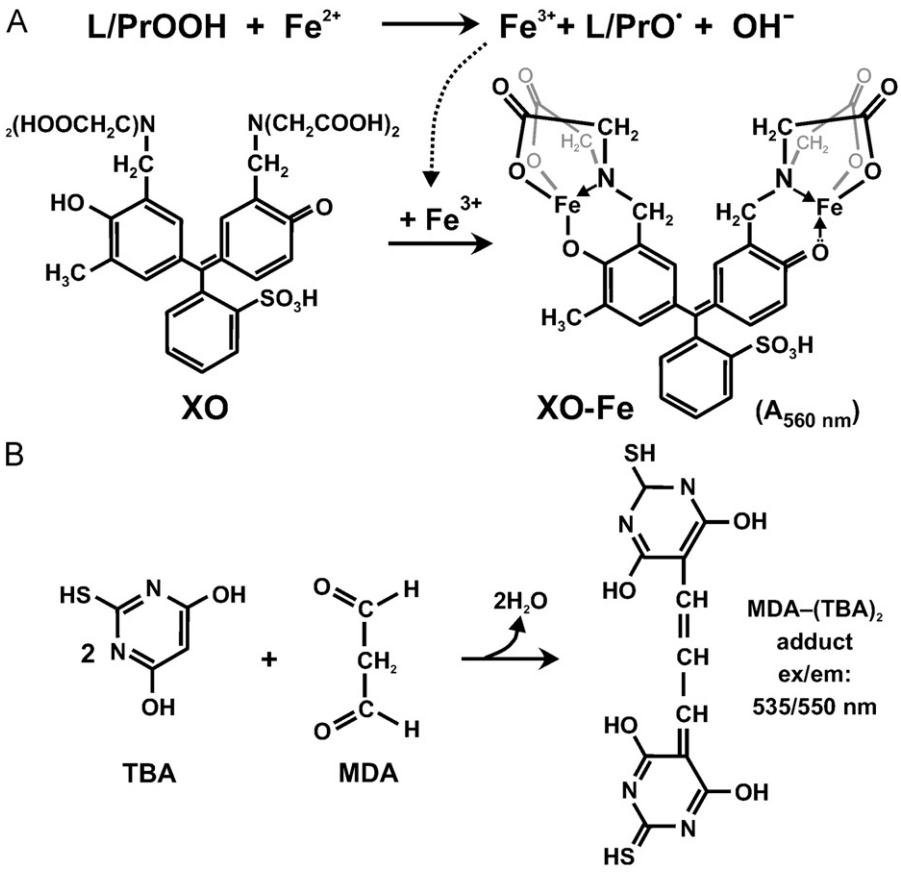

Fig. 2. Reaction mechanisms of the assays used for the determination of (A) LOOH/PrOOH and (B) FrMDA/PrMDA.

Table 1
Interference of various reagents with the method.

Reagent	Standard curve slope[a]
FrMDA assay	
ddH_2O/50 mM phosphate buffer (pH 7.0) ± 10% TCA, or extraction solution	100
PrMDA assay	
0.1 M NaOH (± heating at 60 °C)[b]	100
12 M formamide (in 5 mM phosphate, pH 1.8)	80
8 M urea (in 5 mM phosphate, pH 1.8)	40
6 M guanidine–HCl (in 5 mM phosphate, pH 1.8)	35
10, 30% formic acid	95, 50
LOOH assay	
ddH_2O or 5 mM phosphate buffer (pH 7.0)	100
10, 20% acetone[c]	75, 65
5–25, 60% dimethyl sulfoxide[c]	55, 30
10–30, 50% acetonitrile[c]	30–50, 60
10–100% MetOH/EtOH[c]	50
1 mM EDTA	0
NaCl/KCl (saturated)	60/80
PrOOH assay	
0.1 M NaOH[b]	100
3–6 M guanidine–HCl (in 5 mM phosphate, pH 1.8)	75
2, 4, 8 M urea (in 5 mM phosphate, pH 1.8)	90, 80, 40
10, 20, 30, 70% formic acid	80, 55, 35, 15
12 M formamide (in 5 mM phosphate, pH 1.8)	0
1 mM EDTA	0

[a] Percentage of the control standard curve slope (designated as 100%), which was derived from the corresponding assays performed only with their required reagents.
[b] NaOH (0.1 M) used for protein pellet solubilization does not interfere with the method.
[c] Resolubilization effectiveness (100%) of the $CHCl_3$-extracted LOOH pellet among the indicated solvents (used at 100% v/v) is best achieved with both MetOH and EtOH.

comparative reactivity study of its FOX-2 version (performed in methanol instead of sorbitol) with the use of various organic peroxides and hydroperoxides [31].

Moreover, we modified the FOX-1/2 assay versions for PrOOH/LOOH, respectively, by the use of either the hydrophobic tributylphosphine (TBP) or the more polar tris(2-carboxyethyl)phosphine (TCEP) (both in place of their triphenyl analogue TPP [34,40]) as assay hydroperoxy group specificity controls, because phosphines reduce hydroperoxides to their corresponding alcohols ($R_3P + ROOH \rightarrow R_3P{=}O + ROH$) [41]. Organic peroxides are known to also react with phosphines (only slightly with TBP) but at a much slower rate (e.g., in a period of 3–4 days) than with hydroperoxides and mainly at high temperatures (50–70 °C) [42]. We further improved assay specificity by introducing additional controls to eliminate any reactivity of sample constituents with TBP/TCEP. Taking into account sample availability and degree of desired specificity, the present method allows two modified FOX-1 assay versions (± TBP/TCEP) for the quantification of LOOH and PrOOH.

FrMDA/PrMDA

FrMDA and PrMDA are determined with this method by a modified spectrofluorimetric TBA assay, which is based on the reaction of TBA with MDA and the formation of a fluorescing (ex/em 535/550 nm) MDA–$(TBA)_2$ adduct (Fig. 2B) [24,25]. The rationale and methodology of the general TBA assay have been discussed extensively elsewhere [43,44] and have been criticized for low specificity and artifact formation because only a fraction of the MDA measured was generated in vivo [1,44]. Previous versions (fluorimetric and photometric) of the TBA assay are not MDA-specific for the following main reasons: (i) much of the MDA

incorporated into the MDA–$(TBA)_2$ adduct may be formed from LOOH reacting with TBA during the heating stage of the assay [45,46], and these secondary reactions are affected markedly by metal ions (Fe and Cu), chelating agents, H_2O_2, and various antioxidants [47,48]. (ii) Other monoaldehydic lipid peroxidation products such as hexanal, heptanal, octanal, nonanal, nonenal, and HNE (which together with MDA can be quantified by GC–CI/MS [49,50]) react with TBA, although to a lesser degree, forming near 535-nm-absorbing chromophores [51–53]. Other TBA assay versions measure the MDA–$(TBA)_2$ adduct photometrically after HPLC fractionation [23] and qualitatively (the PrMDA) by an immunochemical assay [16]. However, a more reliable quantification approach of the MDA–$(TBA)_2$ adduct uses its specific fluorescence at ex/em 535/550 (measured also after HPLC fractionation) [54]. Furthermore, MDA is not exclusively derived from polyunsaturated fatty acids, because, e.g., radiation and iron-dependent damaged amino acids, carbohydrates, peptides, proteins, and nucleic acids can release intermediates that form a MDA–$(TBA)_2$ adduct [50].

The MDA-specific spectrofluorimetric TBA assay applied in this method is based on the following modifications of the fluorescent TBA assay [24]: (i) it quantifies the MDA–$(TBA)_2$ adduct (extracted in isobutanol) more specifically by the use of a sample control (without TBA), because sample species other than the MDA–$(TBA)_2$ adduct may exhibit nearly the same fluorescence [55]. (ii) It measures FrMDA and PrMDA separately. Moreover, this assay is a much simpler MDA-specific quantitative method than the cumbersome GC–CI/MS–TBA [49,50] and HPLC–fluorimetric [55] assays.

Method outline

In this method (outlined diagrammatically in Fig. 1) we quantify simultaneously the lipid peroxidation products FrMDA, PrMDA, and LOOH and the protein peroxidation product PrOOH after their fractionation, thus offering a more complete assessment of lipid peroxidation in organisms. Specifically, FrMDA is fractionated from PrMDA and PrOOH by TCA–protein precipitation, followed by the thermal-alkaline hydrolysis of PrMDA [23] to release the PrMDA for further quantification—the alkaline hydrolysis of PrMDA was verified on synthesized bovine serum albumin (BSA)–MDA as described elsewhere [23,56], by comparing its ex/em spectra against control FrMDA (data not shown). Moreover, FrMDA is fractionated from LOOH by the extraction of LOOH in chloroform ($CHCl_3$:MetOH 2:1 [38]) and its subsequent concentration by vacuum evaporation. The fractionated FrMDA and PrMDA are then reacted with TBA to form the fluorescent MDA–$(TBA)_2$ adduct, and the fractionated LOOH and PrOOH are reacted with Fe^{2+} to form the chromophore complex XO–Fe.

Materials

Instrumentation

Balance (Kern, Cat. No. 770/65/6J);
Benchtop centrifuge (Hermle, Model Z206A);
Centrifuge tubes, 15 ml (ISC BioExpress, Cat. No. C-3394–1);
Double-beam spectrophotometer (Shimadzu, Model UV-1800);
Glass tubes, 15 ml;
Microcentrifuge;
Micropipettes (adjustable volume), 2.5 μl, 10 μl, 20 μl, 100 μl, 200 μl, and 1 ml, and tips (Eppendorf Research);
Microcuvette for absorbance measurements, 12.5 × 12.5 × −45 mm external dimensions, 4 mm internal window, and 9 mm bottom (9/B/9/Q/10 quartz, 1.16 ml);
Microcuvette for fluorescence measurements, quartz (Starna SOG/Q), 45 × 4 mm, 0.5 ml, with its FCA4 adapter;
pH meter (Metrohm, 827 pHlab);
Refrigerated microcentrifuge (Eppendorf Research, Model 5417R);
Spectrofluorimeter (Shimadzu, Model RF-1501).

Reagents

Acetone;
Acetonitrile (ACN);
Ammonium ferrous sulfate (FAS), $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, or Fe^{2+} (Sigma, Cat. No. 215406);
Butanol-1 (Merck, Cat. No. 101990); **caution,** *highly flammable;*
Butylated hydroxyanisole (BHA; Sigma Cat. No. B-1253);
Chloroform, $CHCl_3$ (Merck Cat. No. 1.02445); **caution,** *highly flammable;*
Cumene hydroperoxide (CumOOH; Sigma, Cat. No. C-0524); **caution,** *oxidizing, corrosive;*
Ethanol (EtOH; Merck Cat. No. 159010); **caution,** *highly flammable;*
Ethylenediaminetetraacetic acid (EDTA; Merck Cat. No. 34033918); **caution,** *irritant;*
Formamide (Merck, Cat. No. 344205);
Guanidine–HCl (Sigma, Cat. No. G4505);
Malonaldehyde bis(dimethyl acetal) (or 1,1,3,3-tetramethoxypropan) (Sigma, Cat. No. 10,838-3);
MetOH (Sigma–Aldrich, Cat. No. 34860); **caution,** *highly flammable;*
Hydrochloric acid, HCl (concentrated 37%, or 12 M) (Fluka, Cat. No. 84415); **caution,** *corrosive;*
Sodium hydroxide, NaOH;
Sodium phosphate, Na_2HPO_4 (Merck, Cat. No. 30412);
Sulfuric acid, H_2SO_4 (concentrated 96%, or 18 M) (Merck, Cat. No. 112080); **caution,** *corrosive;*
TBA (Sigma, Cat. No. T-5500);
TCA (Merck, Cat. No. 641730); **caution,** *corrosive;*
TBP (Sigma, Cat. No. 247049); **caution;** *corrosive, highly flammable;*
TCEP (Sigma, Cat. No. C-4706); **caution,** *corrosive;*
Urea (Sigma, Cat. No. U1250);
XO (Sigma, Cat. No. X-3500).

Reagent setup

- FOX reagent: prepare fresh by dissolving 15.2 mg XO (final 2 mM) in 5 ml 0.5 M H_2SO_4 and bringing to final 10 ml with ddH_2O. The reagent must be prepared fresh before use.
- 5 mM H_2SO_4: dilute 3600 × the commercial H_2SO_4 concentrated stock using ddH_2O.
- 8 mM Fe^{2+} reagent: prepare fresh by dissolving 31.2 mg FAS in 10 ml 5 mM H_2SO_4. The reagent must be prepared fresh before use.
- 5 mM CumOOH: dilute stock (5.52 M) to 5 mM with absolute MetOH. The reagent is stable for a few hours at room temperature.
- 5 mM H_2O_2: prepare a 5 mM H_2O_2 stock solution by diluting the commercial 30% H_2O_2 solution with ddH_2O, using the extinction coefficient for H_2O_2 at 240 nm (43.6 M^{-1}) [57].
- 80 mM TCEP: prepare fresh by dissolving 23 mg TCEP in 1 ml ddH_2O. The reagent must be prepared fresh before use.
- 80 mM TBP: prepare fresh by diluting commercial TBP stock (4 M) 50 × with MetOH. The reagent must be prepared fresh before use.

- 100% TCA: prepare by dissolving 10 g TCA with ddH_2O to a final 10 ml. The reagent is stable at 4 °C for a few days.
- 0.6 mM MDA: dilute commercial stock (6 M) to 0.6 mM with ddH_2O. The reagent must be prepared fresh before use.
- TBA reagent: prepare fresh by mixing equal volumes of the following solutions A and B. (A) TCA–HCl solution: prepare fresh by mixing 12.5 ml 100% TCA (final 83%) and 2.5 ml concentrated HCl (final 2 N). (B) TBA solution: prepare fresh by dissolving 25 mg TBA in 1 ml 0.2 M NaOH (TBA final 2.5%). *Note. TCA–HCl and TBA solutions are stable for a few hours at room temperature. TBA reagent must be prepared before use.*
- TBA solvent: prepare fresh by mixing equal volumes of TBA reagent solution A and 0.2 M NaOH (prepared by dissolving 0.16 g NaOH in 20 ml ddH_2O).
- 0.1 M BHA: dissolve 18 mg BHA in 1 ml absolute EtOH. The reagent is stable for a few hours at room temperature.
- P_i–BHA buffer: prepare 10 mM phosphate buffer, pH 7.0, containing 0.5 mM BHA. The reagent is stable at 4 °C for a few days.
- Extraction solution: prepare 15 ml 10 mM phosphate buffer, pH 7.0, containing 0.5 mM BHA. To this solution add 5 ml MetOH and 2 ml 100% TCA. The reagent is stable at 4 °C for a few days.
- 0.1 M NaOH: prepare fresh by dissolving 0.16 g NaOH in 40 ml ddH_2O. The reagent is stable at 4 °C for a few days.

Construction of standard curves

LOOH assay standard curve

From the 5 mM CumOOH, prepare a series of CumOOH standards (0.5 to 4 μM, in MetOH) and mix 925 μl of each with 50 μl FOX reagent and 25 μl Fe^{2+} reagent. As reagent blank use 925 μl MetOH in place of the CumOOH solution. After 30 min incubation at room temperature, the absorbance of the mixtures is measured at 560 nm against the reagent blank.

FrMDA assay standard curve

From the 0.6 mM MDA, prepare a series of MDA standards (0.1 to 1 μM, in extraction solution). Mix 250 μl each standard with 50 μl TBA reagent and 3 μl 0.1 M BHA. As a reagent blank use 250 μl extraction solution in place of the MDA solution. After 20-min incubation at 100 °C, let the tubes cool, add to each 300 μl butanol, vortex vigorously, centrifuge at 20,000 g for 5 min, transfer the butanol upper phase into the quartz microcuvette, and measure fluorescence units (FU) at ex/em 535/550 nm against the reagent blank (with the Shimadzu Model RF-1501 spectrofluorimeter set at low sensitivity).

PrMDA assay standard curve

From the 0.6 mM MDA, prepare a series of MDA standards (0.1 to 1 μM, made in 0.1 M NaOH). Mix 250 μl each standard with 50 μl TBA reagent and 3 μl 0.1 M BHA. As reagent blank use 250 μl 0.1 M NaOH in place of the MDA solution. After 20-min incubation at 100 °C, let the tubes cool, add to each 300 μl butanol, vortex vigorously, centrifuge at 20,000 g for 5 min, transfer the butanol upper phase into the quartz microcuvette, and measure FU at ex/em 535/550 nm against the reagent blank (using the spectrofluorimeter settings of the FrMDA assay).

PrOOH assay standard curve

From the 5 mM H_2O_2, prepare a series of H_2O_2 standards (0.5 to 4 μM, made in a mixture of 0.1 M HCl and 0.1 M NaOH or in the alternative protein pellet solvent 6 M guanidine–HCl or 8 M urea, both made in 5 mM phosphate with pH adjusted to 1.8 with HCl, see step 3.ii), and mix 925 μl each with 50 μl FOX reagent and 25 μl Fe^{2+} reagent. As reagent blank use 925 μl 0.1 M HCl and 0.1 M NaOH (6 M guanidine–HCl or 8 M urea) in place of the H_2O_2 solution. After 30 min incubation at room temperature, the absorbance of the mixtures is measured at 560 nm against the reagent blank.

Method procedure

The steps of the method are presented as follows and are depicted diagrammatically in Fig. 1.

Sample treatment

1. The method is described for human plasma and it is applicable to any biological sample type. Blood was collected from normal human subjects (eight and seven middle-age males and females, respectively) after overnight fast (using EDTA as anticoagulant) and centrifuged immediately at 3000 g for 10 min to separate the clear plasma.
   Dilute 0.4 ml plasma with 0.8 ml P_i-BHA buffer (*do not centrifuge it*) and measure its protein concentration [58] to express FrMDA and LOOH per plasma protein. To facilitate subsequent step 2, split the diluted plasma into two 0.6-ml portions and transfer each to a 2-ml microcentrifuge tube. Both tubes are then treated as in step 2.
   Note. Homogenize other sample types in P_i-BHA buffer, centrifuge at 20,000 g for 10 min at 4 °C, measure protein concentration in the resulting clear supernatant [58] (to express FrMDA and LOOH per protein) and proceed to step 2.
   Note. If human plasma and homogenates from other biological samples will not be analyzed immediately they should be stored at −80 °C. To minimize autoxidation, samples should be mixed with the lipid antioxidant BHA in a proportion of 1 ml sample and 20 μl 2 mM BHA (prepared in 100% ethanol).
2. Extract lipids (and LOOH) in each of the two 1-ml diluted plasma samples (or in 1 ml supernatant from other sample types, see notes in step 1) by mixing with 1 ml $CHCl_3$:MetOH 2:1 [38] followed by vigorous vortexing, then add to the resulting mixture 0.15 ml 100% TCA (final 10% TCA, not counting the $CHCl_3$ volume) and vortex briefly, incubate for 20 min in an ice-water bath, and centrifuge at 20,000 g for 10 min. After centrifugation, three layers will be formed in the microcentrifuge tube: an upper aqueous, a middle disk (of precipitated proteins), and a bottom chloroform layer. *If no clear layers are formed, dilute the sample further and adjust accordingly the volumes of the added lipid fractionation reagents.* Collect first the aqueous (nonprotein) fraction and then the bottom chloroform layer (*pushing away carefully the middle protein disk in its periphery with the tip of a micropipette*) and finally the protein disk pellet, and combine the corresponding fractions that resulted from the two diluted plasma samples. Subsequently, measure the volume of the combined aqueous fractions (to quantify FrMDA in the combined fraction as described in step 8), concentrate/isolate the lipids as a pellet in the combined chloroform fractions by vacuum-drying (to quantify LOOH as described in step 4), and wash the combined protein disk pellets with 1 ml 10% TCA (using a microtube fitting pestle–homogenizer) and re-pellet by centrifugation (to quantify PrMDA and PrOOH as described in steps 3i/11 and 3ii/14/16, respectively).
   Note. The optimum protein concentration range by TCA precipitation is 0.1–10 mg ml^{-1} as determined with BSA.
   Note. If the starting sample is high in lipid content (e.g., blood, brain, intestine, adipose tissue), measure the volume of the collected upper aqueous layer, reextract it with 1/2 volume

$CHCl_3$, combine the resulting $CHCl_3$ layers, and concentrate/isolate their lipid content by vacuum-drying. Alternatively, dilute the initial sample volume and repeat step 2.
Note. The lipid extraction step 2 also allows the use of large volumes of samples that are low in LOOH concentration.

3. Treat the resulting protein pellet as follows.
   i. *For PrMDA determination,* solubilize the pellet in a minimum volume (e.g., 0.5 ml) of 0.1 M NaOH (using a microtube fitting pestle–homogenizer) and incubate at 60 °C for 30 min (to hydrolyze PrMDA from the protein–MDA complex [23]). Then, *cool the mixture immediately in an ice-water bath,* collect the clear protein solute after centrifugation (at 20,000 g for 5 min), measure its protein concentration [58] so as to express PrMDA per protein amount (or in nM), and proceed to step 11.
   ii. *For PrOOH determination,* solubilize the pellet in a minimum volume of 0.1 M NaOH, neutralize the solute with 1/10 volume 1 M HCl, centrifuge, and measure protein concentration in the resulting supernatant to express PrOOH per protein (or in μM), and proceed to step 14.

Note. The maximum protein concentration range for effective alkaline solubilization is at least 10 mg ml^{-1} (determined with BSA).
Note. The 60 °C heating step is not required for the construction of the PrMDA standard curve (see Reagent setup).
Note. The protein solubilization by alkalinization step (for PrOOH determination) does not result in a noticeable PrOOH decomposition as tested with the organic hydroperoxide analogue CumOOH. However, neutralization with HCl should be performed as soon as possible.
Note. When alkalinization does not solubilize the protein pellet effectively, it can be solubilized with the least interfering choice (Table 1) between 6 M guanidine–HCl and 8 M urea (both prepared in 5 mM phosphate adjusted to pH 1.8 with HCl).

LOOH assay with TBP or TCEP (when sample size is not limited)

4. Dissolve the plasma lipid pellet (obtained in step 2) in up to 3.6 ml absolute MetOH and mix with the appropriate reagents as shown in Table 2.
5. After the 30-min incubation period, measure the absorbance value of the tube mixtures at 560 nm. Then, calculate net absorbance from the absorbance difference (Sample − TBP/TCEP + Fe) − (Sample + TBP/TCEP + Fe), from which the absorbance difference (Sample − TBP/TCEP − Fe) − (Sample + TBP/TCEP − Fe) is subtracted. This net absorbance is converted to CumOOH concentration equivalents using the corresponding standard curve (see Reagent setup).

Note. Sample size in this step (and in subsequent steps 6/7, 14/15, and 16/17) can be reduced by half or more using absorbance microcuvettes of sample volume 0.5 or 0.25 ml.

LOOH assay without TBP or TCEP (when sample size is limited)

6. Dissolve the plasma lipid pellet (from step 2) in 2 ml absolute MetOH and mix with the assay reagents as shown in Table 3.
7. After the end of the 30-min incubation period, measure the absorbance value of the tube mixtures at 560 nm, and calculate the net absorbance from the absorbance difference (Sample + Fe) − (Reagent blank + Fe), from which the absorbance difference (Sample − Fe) − (Reagent blank − Fe) is subtracted. This net absorbance is converted to CumOOH concentration equivalents using the corresponding standard curve (see Reagent setup).

FrMDA assay

8. Mix 250 μl of the aqueous phase obtained from step 2 (if more dilution needed use the extraction solution) with the reagents as shown in Table 4.
9. Incubate tubes in step 8 for 20 min at 100 °C, cool their content in an ice-water bath, and add to each 300 μl butanol.

Table 3.
LOOH assay reagents/procedure (without TBP or TCEP).

Reagent	Reagent blank + Fe	Sample + Fe	Reagent blank − Fe	Sample − Fe
Sample (μl)	–	925	–	925
MetOH (μl)	925	–	925	–
FOX reagent (μl)	50	50	50	50
Fe^{2+} reagent (μl)	25	25	–	–
5 mM H_2SO_4 (μl)	–	–	25	25
Incubate at room temperature for 30 min				

Final concentrations of reagents in the assay mixture: 92.5% MetOH, 100 μM XO, 12.5 mM H_2SO_4, 200 μM Fe^{2+}.

Table 4.
FrMDA assay reagents/procedure.

Reagent	Reagent blank	Sample blank	Sample
Sample (μl)	–	250	250
Extraction solution (μl)	250	–	–
TBA reagent (μl)	50	–	50
TBA solvent (μl)	–	50	–
0.1 M BHA (μl)	3	3	3

Final assay reagent concentrations: 7% TCA, 0.21% TBA, 1 mM BHA.

Table 2.
LOOH assay reagents/procedure (with TBP or TCEP).

Reagent	Sample − TBP/TCEP + Fe	Sample + TBP/TCEP + Fe	Sample + TBP/TCEP − Fe	Sample − TBP/TCEP − Fe
Sample (μl)	900	900	900	900
80 mM TBP/TCEP (μl)	–	25	25	–
MetOH/ddH_2O (μl)	25	–	–	25
Incubate at room temperature for 15 min				
FOX reagent (μl)	50	50	50	50
Fe^{2+} reagent (μl)	25	25	–	–
5 mM H_2SO_4 (μl)	–	–	25	25
Incubate at room temperature for 30 min				

Final concentrations of the reagents in the assay mixture are 92.5% MetOH, 2 mM TBP or TCEP, 100 μM XO, 12.6 mM H_2SO_4, and 200 μM Fe^{2+}. Incubation times for this and for subsequent steps 6, 14, and 16 are *minimum,* and the final assay reaction mixtures can stay at room temperature even for an hour without substantial color intensity drifting of the formed chromophore. Incubations in this step (and in step 14) with the TCEP/TBP and FOX reagents are not affected by light.

Then, vortex the tubes for 1 min and centrifuge (at 10,000 g for 3 min) to separate the upper organic (butanol) phase. Subsequently, measure the FU of this organic phase at ex/em 535/550 nm (with the Shimadzu RF-1501 spectrofluorimeter set at low sensitivity).
Note. If MDA fluorescence signal exceeds the measuring range of the spectrofluorimeter in use, either extract the MDA–$(TBA)_2$ adduct in the assay mixture with a higher (than 300 μl) butanol volume or dilute the sample further and repeat step 8.

10. Obtain the net FU value by subtracting from the sample FU value the FU values of the sample and reagent blanks. Then, convert the net FU value to MDA concentration from the corresponding standard curve (see Reagent setup).

PrMDA assay

11. Mix 250 μl protein solute obtained from step 3i (for more solute dilution use ddH_2O) with the appropriate reagents as shown in Table 5.
12. Incubate the tubes in step 11 at 100 °C for 20 min, cool them in an ice-water bath, and add to each 300 μl butanol. Then, vortex vigorously for 1 min, centrifuge (at 10,000 g for 3 min) to separate the upper organic (butanolic) phase, and measure its FU at ex/em 535/550 nm (using spectrofluorimeter settings in step 9).
Note. In the case of strong MDA fluorescence signal see the note for step 9.
13. Subsequently, subtract from the sample FU value the FU values of the sample and reagent blanks and convert the resulting net FU value to MDA concentration from the corresponding standard curve (see Reagent setup).

PrOOH assay with TBP or TCEP (when sample size is not limited)

14. The protein solute (from step 3ii) is mixed with the reagents shown in Table 6.
15. Measure the absorbance of the tube mixtures at 560 nm. Calculate the final sample net absorbance from the absorbance difference (Sample − TBP/TCEP + Fe) − (Sample + TBP/TCEP + Fe), from which the absorbance difference (Sample − TBP/TCEP − Fe) − (Sample + TBP/TCEP − Fe) is subtracted. Then, convert the net absorbance to H_2O_2 concentration equivalents using the corresponding standard curve (see Reagent setup).

PrOOH assay without TBP or TCEP (when sample size is limited)

16. Mix the protein solute from step 3 (if diluted further use ddH_2O) with the reagents shown in Table 7.
17. Measure the absorbance value of the tubes at 560 nm. Calculate the final sample net absorbance from the absorbance difference (Sample + Fe) − (Reagent blank + Fe), from which the absorbance difference (Sample − Fe) − (Reagent blank − Fe) is subtracted. This net absorbance is converted to H_2O_2 concentration equivalents using the corresponding standard curve (see Reagent setup).

Method timing, planning, and statistical variation

Timing

The timing listed in method subheadings is the time required for processing 10 samples. Sample treatment, steps 1–3, 90 min; LOOH assay, steps 4–7, 100 min; FrMDA assay, steps 8–10, 40 min; PrMDA assay, steps 11–13, 40 min; PrOOH assay, steps 14–17, 60 min.

Planning

For handling many samples (e.g., 10), the method does not need to be executed on the same day. The required reagents for treating all samples in steps 1 and 2 are prepared first, ending up with the upper aqueous fraction (Fraction A), middle protein disk

Table 5.
PrMDA assay reagents/procedure.

Reagent	Reagent blank	Sample blank	Sample
Sample (μl)	–	250	250
ddH_2O (μl)	250	–	–
TBA reagent (μl)	50	–	50
TBA solvent (μl)	–	50	–
0.1 M BHA (μl)	3	3	3

Final concentrations of the reagents in the assay mixture are 7% TCA, 0.21% TBA, 1 mM BHA.

Table 7.
PrOOH assay reagents/procedure (without TBP or TCEP).

Reagent	Reagent blank + Fe	Sample + Fe	Reagent blank − Fe	Sample − Fe
Sample (μl)	–	925	–	925
MetOH (μl)	925	–	925	–
FOX reagent (μl)	50	50	50	50
Fe^{2+} reagent (μl)	25	25	–	–
5 mM H_2SO_4 (μl)	–	–	25	25
Incubate at room temperature for 30 min				

The final concentrations of the assay reagents are 100 μM XO, 12.6 mM H_2SO_4, 200 μM Fe^{2+}.

Table 6.
PrOOH assay reagents/procedure (with TBP or TCEP).

Reagent	Sample − TBP/TCEP + Fe	Sample + TBP/TCEP + Fe	Sample + TBP/TCEP − Fe	Sample − TBP/TCEP − Fe
Sample (μl)	900	900	900	900
80 mM TBP/TCEP (μl)	–	25	25	–
MetOH/ddH_2O (μl)	25	–	–	25
Incubate at room temperature for 15 min				
FOX reagent (μl)	50	50	50	50
Fe^{2+} reagent (μl)	25	25	–	–
5 mM H_2SO_4 (μl)	–	–	25	25
Incubate at room temperature for 30 min				

Final assay reagent concentrations: 2 mM TBP/TCEP, 100 μM XO, 12.6 mM H_2SO_4, 200 μM Fe^{2+}.

Table 8
Human plasma Fr/PrMDAs and L/PrOOHs.

Parameter	By the method	From indicated reference
FrMDA[a] (nM)	69 ± 9	43 ± 7 [23]
PrMDA[a] (nM)	230 ± 19	382 ± 49 [23]
Total MDA (nM)	300 ± 21	320 ± 120 [54]
LOOH[a] (μM)	2.9 ± 1.0	2.6 ± 1.5 [40]
		3.02 ± 1.85 [59]
		3.5 ± 2.05 [29]
		6.7 ± 2.7 [60]
PrOOH[a] (μM)	5.0 ± 1.0	NA[b]

[a] All values are the mean (of 15 samples) ± SE after checking for equality of error variances between values of males and females (Levene test, SPSS, Inc. [16]) with two-way ANOVA (to identify significant differences between values) and with the parametric post hoc multiple comparison test (Bonferroni test, $p < 0.05$). Similar statistical differences were also found in plasma from subjects covering the 20–70 year age span (data not shown).
[b] Not available.

(Fraction B), and bottom chloroform fraction (Fraction C), which can be handled as follows:

Fraction A: this must be measured the same day by the FrMDA assay. For this, the TBA reagent must be made fresh. However, the reagents for making the TBA reagent (i.e., the TCA–HCl and TBA solutions) are stable for a few hours at room temperature.

Fraction B: the isolated protein disk (used for PrOOH and PrMDA determination) can be stored for a day at −20 °C (at −80 °C for up to 1 week) without any noticeable artificial peroxidation. The protein disk can be alkaline-solubilized, e.g., the next day, to measure (a) PrOOH (after protein solute neutralization with HCl) and (b) PrMDA after MDA release from proteins (by alkaline hydrolysis at 60 °C). For PrMDA determination reagent preparation cautions are followed as for Fraction A. For PrOOH determination, the TCEP, FOX, and Fe^{2+} reagents are prepared fresh before use.

Fraction C: the LOOH chloroform fraction is evaporated the same day of Fraction C fractionation, and the resulting lipid pellet can be stored for a few days at −20 °C and for 1 week at −80 °C without any noticeable artificial peroxidation. For LOOH determination, the TCEP, FOX, and Fe^{2+} reagents are prepared fresh before use.

Statistical variation

To get the minimum statistical variation of the parameters quantified by this method, human plasma samples are analyzed the same day of blood collection (followed by the immediate addition of the lipid antioxidant BHA, 1 mM final, to the blood sample). The parameters determined by this method in each plasma sample are derived from the analysis of at least three successive dilutions, and their mean value is calculated. The same procedure should be followed with fresh samples from other organisms. The variation of the mean derived from the dilutions of the same sample for each measured parameter is >3%. The day-to-day (interday) coefficient variation (% ratio of the standard deviation to the mean) of the same sample (kept in different storage tubes at −80 °C in the presence of BHA) when measured within a 1-week period is >5% (see also notes of Table 8).

Calculations and expected results

The method quantifies simultaneously and specifically the main lipid peroxidation products FrMDA/PrMDA and LOOH (with sensitivity 20 and 100 pmol, respectively) as well as the protein hydroperoxidation product PrOOH (at 100 pmol). The method was applied in human plasma to measure FrMDA, PrMDA, LOOH, and PrOOH (Table 8). Specifically, the plasma total MDA (FrMDA plus PrMDA) value is similar to those reported elsewhere [23,54] and in agreement that the total plasma MDA is mostly composed of PrMDA [23]. The method also measured plasma PrOOH at 5.0 ± 1.0 μM. Plasma total LOOH is on the same order as the values reported elsewhere [29,40,59,60]. However, the total hydroperoxide value (LOOH) measured by our method in human plasma is expected to be much higher than each of the specific lipid hydroperoxides detected after separation by HPLC methods. The reason for this is that our method measures the sum of the various LOOHs present in the sample and possibly other hydrophobic hydroperoxides of nonlipid origin. The usage of human samples demonstrates the potential of our method to simultaneously measure free and protein-bound MDA and lipid– and protein–OOH in clinical research and also its potential application in basic research in general.

Caveats

When MDA is present in the sample at high concentrations the extraction of the formed MDA–$(TBA)_2$ adduct by the butanol volume stated in the method may not be complete. This problem can be solved ether by increasing the volume of butanol or by diluting the sample before analysis (see note in step 9). The PrOOH/LOOH and FrMDA/PrMDA assays were tested for interference from reagents that may possibly be in various sample treatments used by researchers. Such reagents may be certain protein pellet solvents (formamide, urea, guanidine–HCl, formic acid, and NaOH) and organic solvents (acetone, ACN, DMSO, EtOH, MetOH) that can be used for the solubilization of the $CHCl_3$-fractionated LOOH (Table 1). Possible interference can be eliminated or minimized by choosing the least interfering reagents and/or minimizing their interference by sample dilution.

Acknowledgment

This work was financially supported by the Greek Ministry of Education, University of Patras, Greece. The authors express their gratitude to Dr. Janusz (Jan) Gebicki (Department of Biological Sciences, Macquarie University, Australia) for his very useful suggestions.

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