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

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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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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+}.

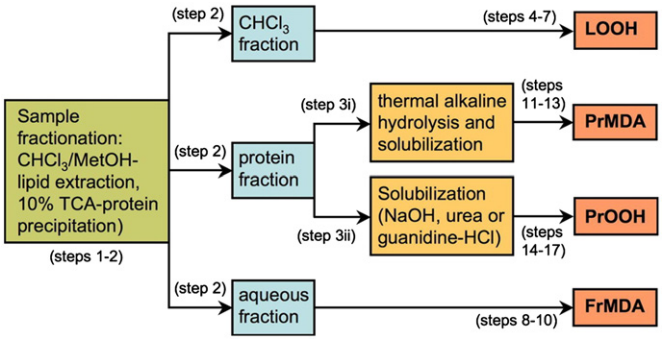

Fig. 1. Method flow chart for the determination of FrMDA, PrMDA, LOOH, and PrOOH. Steps depicted here are described in detail in the text.

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