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Spectrophotometric Measurement of Hydroperoxides at Increased Sensitivity by Oxidation of Fe^{2+} in the Presence of Xylenol Orange

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The method, developed by modifying the FOX methods described by Wolff (*Methods Enzymol.* **233**, 182–189, 1994), involves the oxidation of Fe^{2+} by peroxides at low pH in the presence of both the ferric-complexing dye xylenol orange and sucrose, the amplifier of the reaction. The method proved to be a convenient, simple and efficient assay for the direct measurement of both water and lipid soluble peroxides. In fact it improves by about 60% the sensitivity of the FOX1 method for water soluble peroxides, and by 7–8 times that of the FOX2 method for lipid soluble peroxides. It allows the detection of 0.1 µM peroxide in the test solution. The method is suitable to measure the lipid hydroperoxides present in phosphatidylcholine liposomes and in human LDL. The data obtained allowed us to define a mathematical expression to calculate the lipid hydroperoxide content of liposomes knowing their oxidation index.

Keywords: Hydrogen peroxide, lipid hydroperoxide, LDL, spectrophotometric method, xylenol orange

Abbreviations: BHT, butylated hydroxytoluene; Cu-OOH, cumene hydroperoxide; DMSO, dimethyl sulfoxide; DPPC, dipalmitoyl phosphatidylcholine; FOX, ferrous oxidation-xylenol orange; LDL, low density lipoprotein; LOOH, lipid hydroperoxide; Mes, morpholineethanesulphonic acid; PC, phosphatidylcholine; PBS, phosphate-buffered saline.

INTRODUCTION

Oxidative stress is suggested to contribute to pathological processes in aging and many diseases.[1] It is associated with the formation of lipid hydroperoxides (LOOH) that are degraded by transition metals to initiate the so-called LOOH-dependent lipid peroxidation. The observations that lipid peroxidation both of model liposomes[2,3] and biological materials[4–6] is mainly due to this process render important the assessment of the lipid hydroperoxide content of a sample.

Methods for measuring lipid hydroperoxide were developed; however many of them are either nonspecific or can be inconvenient and

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problematic.[7–9] Wolff described two simple and sensitive spectrophotometric methods[10–14] developed by modifying a method originally described by Gupta[15] which involves the oxidation of Fe^{2+} by peroxides at low pH in the presence of the ferric-complexing dye xylenol orange. The two methods, referred to as FOX1 and FOX2, are applied, respectively, to the measurement of hydrophilic hydroperoxides present in the aqueous phase and to lipophilic hydroperoxides present in the lipid phase. The FOX2 method, for its characteristics, would be of the two the more useful allowing the quantification of lipid soluble hydroperoxides in biological samples such as present in lipoprotein, membranes and fats. However it has a much lower sensitivity (ε_{560} ROOH $= 4.46 \times 10^4\,M^{-1}\,cm^{-1}$) compared to the FOX1 method (ε_{560} HOOH $= 2.24 \times 10^5\,M^{-1}\,cm^{-1}$). Besides, its sensitivity does not significantly differ from that of a similar method previously developed by Cavallini *et al.*[16] where acetic acid and chloroform substitute for sulfuric acid and methanol and KSCN is used as dye instead of xylenol orange.

The aims of this report were to improve the sensitivity of Wolff's methods for the determination of hydroperoxides, in particular of the lipid soluble ones, and to verify the suitability of the new method for their determination in model liposomes and in biological material.

MATERIALS AND METHODS

Chemicals

Xylenol orange [*o*-cresolsulfonaphthalein-3′-3″-bis-(methyliminodiacetic acid sodium salt)], ammonium ferrous sulfate, H_2O_2, cumene hydroperoxide (Cu-OOH), *t*-butyl hydroperoxide, di-cumyl peroxide, benzoyl peroxide, butylated hydroxytoluene (BHT), morpholineethanesulphonic acid (Mes), dipalmitoyl phosphatidylcholine (DPPC) and all other chemicals, of the highest grade available, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Egg phosphatidylcholine (PC) was from Lipid Products (Redhill, UK). Methanol (HPLC grade) was obtained from Carlo Erba (Italy).

Preparation of Liposomes

The standard sonicated egg yolk PC liposomes were prepared as previously described.[17] The phospholipids (about 18 mg), dried under nitrogen, were added with 6 ml distilled water, vortex mixed for 10 min and stored at 4°C for 1 h. This suspension was subsequently sonicated with a titanium probe sonicator (Labsonic 2000) for different times to obtain liposomes with a different oxidation index.[18] The vesicle dispersion was then transferred to a small-volume extrusion apparatus (produced by Avestin, Ottawa, Canada) which allowed the extrusion of large unilamellar vesicles through standard 19 mm polycarbonate filters with 0.1 µm pore size. Usually samples were subjected to 37 passes through two filters mounted in tandem in the mini-extruder, as recommended by Hope *et al.*[19] This procedure makes possible the preparation of large unilamellar vesicles with an average diameter close to the pore size. The PC and DPPC liposomes containing Cu-OOH were prepared by extrusion technique without prior sonolysis. In these cases different volumes of 5 mM Cu-OOH were added to the phospholipid before drying. All extrusion procedures were conducted at 20°C except for DPPC which was extruded at 50°C, 10°C above the gel–liquid crystal transition temperature. The phospholipid content of the liposome suspensions was determined by the method of Marinetti.[20]

Measurement of Peroxidation Index

Lipid peroxidation was measured by determining the oxidation index of the liposomes.[18] The samples containing 100–150 µg phospholipid were extracted with 1 ml butan-l-ol. Phases were

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separated by centrifugation at 3000 rpm for 10 min and the 200–300 nm ultraviolet spectrum of the upper organic phase was recorded against appropriate blanks containing all reagents but liposomes. The oxidation index ($A_{233\,nm}/A_{215\,nm}$) was determined.

Preparation and Extraction of LDL

Blood was collected from fasted healthy volunteers by venipuncture into sampling vials containing EDTA. Plasma was prepared by centrifugation at 2000*g* for 10 min at 4°C. LDL was isolated by a very fast ultracentrifugation according to Himber *et al.*[21] Briefly, plasma 0.9 ml was added to a centrifuge tube containing KBr (0.4451 g) adjusting the density of plasma to 1.3 g/ml. This was then overlaid with 2.1 ml of 150 mM NaCl and centrifuged for 2 h at 10°C. LDL orange colored band was recovered by suction, adjusted to a density greater than the LDL density (1.1210 g/ml) by adding solid KBr and centrifuged for 2 h at 10°C at 440,000*g*. LDL was dialyzed and passed through a Pharmacia PD10 Sephadex column equilibrated with PBS buffer (5 mM potassium phosphate, pH 7.4, containing 150 mM NaCl) to remove EDTA and other interfering compounds. The cholesterol content was determined and adjusted to 1 mg/ml with PBS buffer. Lipid fraction was extracted from 1 ml LDL according to Bligh and Dyer.[22] The extracts, evaporated under argon, were added with 1 ml distilled water and vortex mixed for 10 min.

Measurement of Hydroperoxide with the Wolff's FOX Assays

FOX1 Fifty µl of test sample was added to 950 µl FOX1 reagent (100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 100 mM sorbitol, 25 mM H_2SO_4), vortexed and incubated for 40 min at room temperature. The absorbance was read at 560 nm.[14]

FOX2 Fifty µl of test sample was added to 950 µl FOX2 reagent (100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 90% HPLC grade methanol, 4 mM BHT, 25 mM H_2SO_4), vortexed and incubated for 40 min at room temperature.[14] The absorbance was read at 580 nm, wavelength where we observed the maximum absorbance.

RESULTS AND DISCUSSION

Composition and Sensitivity of the FOX1A Assay

First we tried to ameliorate the FOX1 method, which has a high sensitivity for hydrophilic hydroperoxides[10,13,14] but was not considered suitable for the detection of lipid soluble hydroperoxides.[11,12,14] In fact, when variable volumes of either 12 or 1.2 mM Cu-OOH in ethanol were assayed for their hydroperoxide content by the FOX1 method different results were obtained (Figure 1A). The possibility that the ethanolic solvent effected the detection method was studied. When a fixed amount of Cu-OOH was determined with the standard FOX1 method in the presence of further added volumes of ethanol an impressive loss of sensitivity was noticed (Figure 1B). These results may be explained considering the molecular mechanism responsible for the high sensitivity of the FOX1 method. This method is based on a chain reaction which occurs when the oxyl radicals, generated as a consequence of the reduction of hydroperoxides by ferrous ions, react with sorbitol.[14] Sorbitol scavenges oxyl radicals efficiently and generates hydrogen peroxide and hydroperoxyl radicals which propagate the ferrous oxidation step. The chain reaction in the presence of sorbitol is very effective and the yield of ferric ions is 15 mol per mole H_2O_2[10,14] whereas it is much shorter in the presence of methanol, ethanol (3 mol ferric ion per mole H_2O_2).[11,12,14] The effect exerted by ethanol, introduced as a vehicle in the FOX1 assay, is consistent with its competition with sorbitol for the oxyl radicals, competition which causes a far

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lower color enhancement (Figure 1). This observation is also consistent with the low extinction coefficient for Cu-OOH of the FOX2 reagent (ε_{560} $4.46 \times 10^4\,M^{-1}\,cm^{-1}$)[11,12,14] where methanol substitutes sorbitol as amplifier.

To improve the FOX method we assayed other oxyl scavengers to find an amplifier of the Fe^{2+} catalyzed chain reactions which produces a length of radical chains longer than sorbitol. Of the compounds tested at 100 mM concentration, sucrose produced the highest enhancement of color yield relative to the unmodified system (Table I). To find the most suitable conditions to minimize the competition between sucrose and

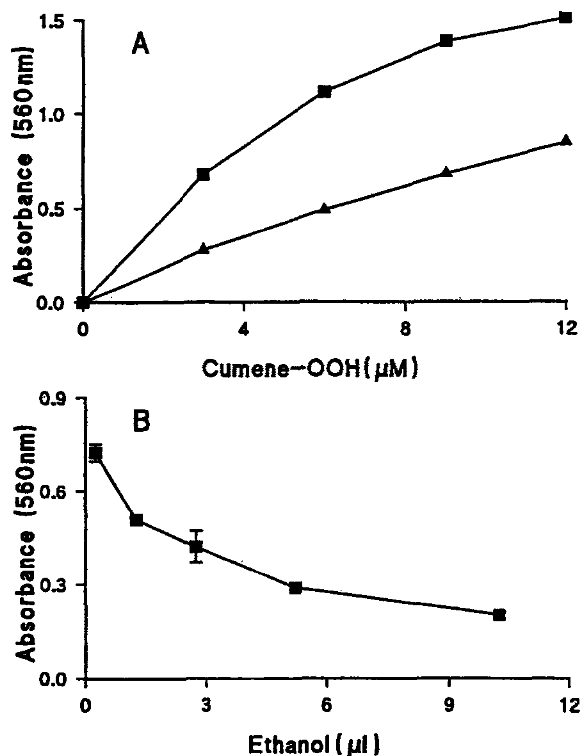

FIGURE 1 The effect of ethanolic solvent on the FOX1 reagent. Panel A. Various volumes of either 12 mM (■) or 1.2 mM (▲) Cu-OOH in ethanol were incubated with FOX1 reagent to give the stated final concentrations in 1 ml assay. Panel B. Cu-OOH (0.25 µl of 12 mM solution in ethanol, 3 µM in 1 ml assay) was incubated with FOX1 reagent in the absence and presence of increasing volumes of ethanol. Absorbance was read at 560 nm after 40 min incubation at room temperature. Data are the mean ± SD of triplicate determinations.

the alcoholic vehicle of organic hydroperoxides, we studied the influence of sucrose concentration on ethanol effect. As expected the inhibition was greatly decreased at higher sucrose concentrations (Figure 2A). For this reason sucrose 400 mM was utilized as amplifier in the formulation of the new FOX1A assay. The new FOX1A reagent contains 250 µM xylenol orange, 625 µM ammonium ferrous sulfate, 1 M sucrose, 62.5 mM H_2SO_4; 0.4 ml of the reagent are added to 0.6 ml of test sample. After an incubation of 40 min at room temperature the samples are centrifuged at 5000*g* for 10 min and the absorbance of the supernatant is monitored at 580 nm, the wavelength that gives the maximum absorbance in our experimental conditions (Figure 3A). Solvents normally utilized to solubilize organic peroxides were assayed for their interference with the FOX1A method (Figure 2B). The inhibitions, although much lower than those observed with the FOX1 reagent, cannot always be ignored and appropriate controls must be run. However, the choice of a suitable volume and type of solvent allows not to greatly decrease the sensitivity of the method.

The new FOX1A assay binds ferric ion with the same extinction coefficient as Wolff's FOX assays (ε_{580} $1.5 \times 10^4\,M^{-1}\,cm^{-1}$)[10–14] and the formation of the complex is not affected by ethanol addition (result not shown). By contrast the extinction coefficient of FOX1A method for H_2O_2 is 60%

TABLE I Effect of radical scavengers on the reaction of H_2O_2 (1 µM) with 100 µM xylenol orange and 250 µM Fe^{2+} in 25 mM H_2SO_4

Assay condition	Absorbance (560 nm)
Control	0.026 ± 0.005
Sucrose	0.385 ± 0.002
Mannitol	0.213 ± 0.003
Formic acid	0.148 ± 0.005
Acetic acid	0.033 ± 0.003
Sorbitol	0.254 ± 0.005

The concentration of the compounds under test was 100 mM. Absorbance was read at 560 nm after 40 min incubation at room temperature. Data are the mean ± SD of triplicate determinations.

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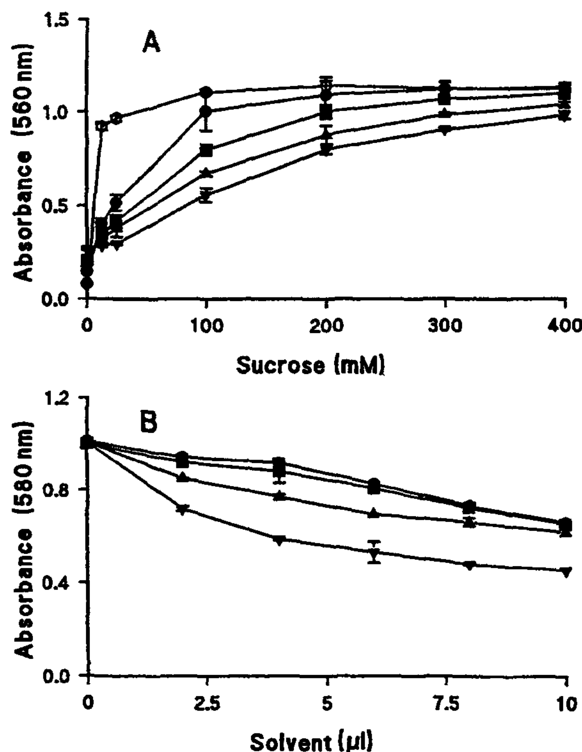

FIGURE 2 Effect of sucrose and solvents on H_2O_2 determination. Panel A. The effect of increasing concentrations of sucrose, in the absence (○) and presence of 1 µl(●), 2 µl(■), 3 µl(▲), 5 µl(▼) ethanol, on the reaction of 3.5 µM H_2O_2 with 100 µM xylenol orange and 250 µM Fe^{2+} in 25 mM H_2SO_4 was assayed. Absorbance was read at 560 nm after 40 min incubation at room temperature. Panel B. The effect of increasing volumes of methanol (●), ethanol (■), butanol (▲) and DMSO (▼) on the reaction of 3 µM H_2O_2 with 100 µM xylenol orange and 250 µM Fe^{2+} in 25 mM H_2SO_4, 0.4 M sucrose was assayed. Absorbance was read at 580 nm after 40 min incubation at room temperature. Data are the mean ± SD of triplicate determinations.

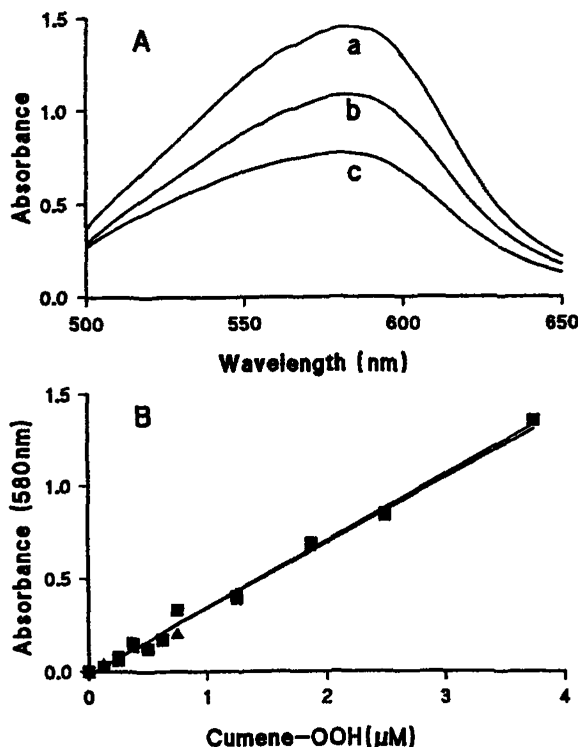

FIGURE 3 Detection of Cu-OOH by FOX1A reagent. Panel A. Spectral characteristics of the FOX1A reagent in the presence of 4 µM H_2O_2 (line a), 3 µM Cu-OOH (line b), 2 µM Cu-OOH in DPPC liposomes (line c) after 40 min incubation. Panel B. Detection of increasing concentrations of Cu-OOH in the absence (■) or presence of DPPC liposomes (▲) by the FOX1A reagent. Absorbance was read at 580 nm after 40 min incubation at room temperature. Data are the mean ± SD of duplicate determinations.

higher than that of FOX1 (ε_{580} $3.6 \times 10^5\,M^{-1}\,cm^{-1}$ versus ε_{560} $2.24 \times 10^4\,M^{-1}\,cm^{-1}$): with the new reagent approximately 24 mol of Fe^{2+} are oxidized to Fe^{3+} per mole of H_2O_2. The high apparent extinction coefficient for H_2O_2 in FOX1A permits the measurement of approximately 60 pmol of H_2O_2 in a 0.6 ml sample, assuming the availability of a spectrophotometer capable of detecting an absorbance of 0.02 AU with accuracy. Thus, 0.1 µM H_2O_2 in the test solution may be detected by FOX1A method compared to the 2 µM detectable by Wolff's FOX1.[14] The new method, as shown in Table II, maintains the specificity for acyl- and alkylhydroperoxides showing a lower reactivity towards the endoperoxides.

Comparison of FOX1A and FOX2 Methods in the Detection of Organic Peroxides

We compared the newly formulated FOX1A method with the Wolff's FOX2 method specifically developed for the detection of organic hydroperoxides.[11,12,14] In the original FOX2 method 90% methanol substituted sorbitol to

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TABLE II Reactivity of various peroxides to FOX1A assay

	Percentage reactivity (relative to H_2O_2)
Hydrogen peroxide	100%
Hydrogen peroxide + ethanolic vehicle	98%
t-Butyl hydroperoxide in ethanol	98%
Cumene hydroperoxide in ethanol	98%
Di-cumyl peroxide in ethanol	1%
Benzoyl peroxide in ethanol	5%

100% refers to an apparent extinction coefficient of $3.6 \times 10^5\,M^{-1}\,cm^{-1}$ for H_2O_2 in the FOX1A reagent. The organic peroxides were in 2.5 µl solvent.

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ensure both the amplification of Fe^{2+} oxidation and the solubilization of the lipophilic sample.

The extinction coefficient for Cu-OOH of FOX1A method is similar to that for H_2O_2 (ε_{580} $3.6 \times 10^5\,M^{-1}\,cm^{-1}$) (Figure 3) and much higher than that of the FOX2 method (ε_{560} $4.46 \times 10^4\,M^{-1}\,cm^{-1}$).[11,12,14] The FOX1A method gave a similar spectrum and extinction coefficient also when Cu-OOH was incorporated within saturated DPPC liposomes indicating that compartmentation of the organic hydroperoxide within membranes does not decrease its accessibility to the reactants (Figure 3).

We studied also the effect of BHT in the FOX1A reagent on the detection of Cu-OOH within DPPC liposomes. This experiment was conducted to verify whether BHT, whose presence is necessary to inhibit further peroxidation when unsaturated liposomes are studied, may affect the sensitivity of the FOX1A method. The presence of BHT (0.5 mM) in the FOX1A reagent, named FOX1B, did not significantly decrease the extinction coefficient for Cu-OOH (result not shown) suggesting that this antioxidant does not react with the hydrosoluble radical chain reactions on which is based the detection method. The very low ε of the original FOX2 method,[11,12,14] by contrast, may be partly due to the scavenging by BHT of the methanol radical that should amplify Fe^{2+} oxidation. To support this hypothesis we observed that the detection of Cu-OOH by FOX2 method, in the absence of BHT, gives higher color development (result not shown).

To assess the usefulness of the new reagent FOX1B first we utilized it to detect the LOOH present in oxidized PC liposomes. For comparison the results obtained with FOX2 method were also reported. PC liposomes sonicated for different times to generate increasing amounts of LOOH[3] were used; the degree of oxidation was inferred by the oxidation index. For each liposome preparation, different amounts of liposomes were analyzed. From Figure 4A and B it is evident that both FOX1B and FOX2 detect LOOH only when the amount of PC liposomes in the assay is higher than a limiting value that represents a lower limit to the applicability of the methods. The value depends on the oxidation index of the liposomes; however we suggest not to use less than 20 and 30 µg phospholipid/assay for FOX1B and FOX2 respectively. Above these values invariably a direct proportionality between the liposome content (µg phospholipid) and the absorbance was observed (Figure 4A and B). When the slopes (A/mg phospholipid) of the curves obtained with the different liposome preparations were related to the oxidation index of the liposomes, two first order curves were obtained (Figure 4C). From their observation it is evident that both methods are unsuitable to detect LOOH when the oxidation index of PC liposomes is lower than 0.135 and that FOX1B method has a sensitivity 7–8 times higher than FOX2. By using the equation of the curve obtained with the FOX1B method ($y = 76.2x - 10.3$), it is possible to estimate the LOOH content of an unknown sample whose oxidation index is higher than the limiting value 0.135. In fact,

$$A/\text{mg phospholipid of sample liposome in 1 ml assay} = 76.2 \times \text{Oxidation index of sample liposome} - 10.3.$$

The value obtained, divided by the extinction coefficient of the FOX1B method ($3.6 \times 10^5\,M^{-1}\,cm^{-1}$), gives the mmol of LOOH per mg phospholipid of the liposome under test. This

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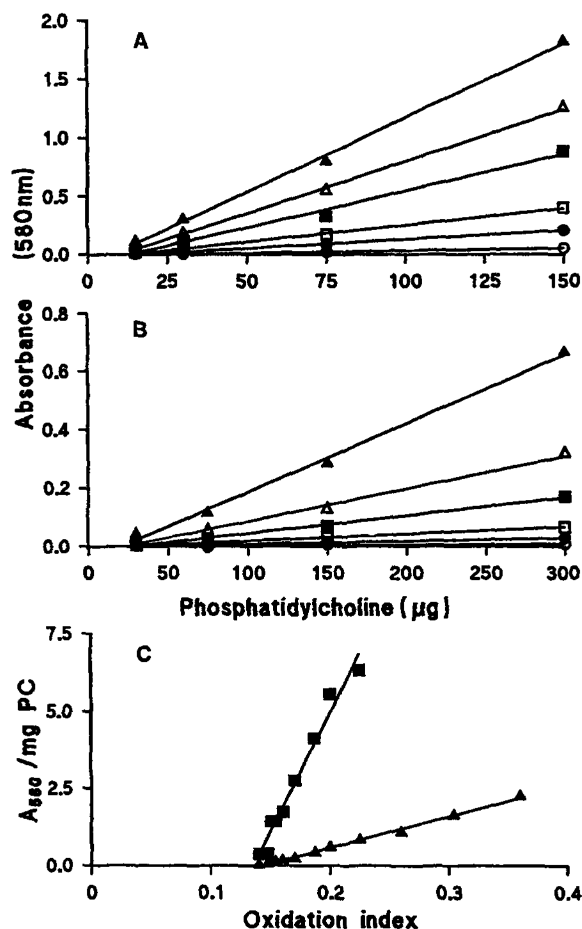

FIGURE 4 Detection of LOOH in sonicated PC liposomes by FOX1B and FOX2 methods. Increasing amounts of liposomes with different oxidation index (0.147 ○, 0.154 ●, 0.16 □, 0.187 ■, 0.225 △, 0.304 ▲) were assayed for their LOOH content by the FOX1B and FOX2 methods. Absorbance was read at 580 nm after 40 mm incubation at room temperature. Data are the mean ± SD of duplicate determinations. Panel C. The slopes of the curves obtained either with FOX1B (■) (Panel A) or FOX2 (▲) (Panel B) were plotted against the oxidation index of the liposomes utilized.

procedure, without excluding the direct and precise quantification by the FOX1B method, may be useful to obtain a quick estimate of the LOOH content of an unknown sample, allowing its further rapid processing.

Then we utilized the new reagent FOX1B to detect the LOOH present in human LDL. We chose this biological material as a precise measurement of its LOOH content would appear critical to the scrutiny of the oxidative stress hypothesis of atherosclerosis. To ascertain the authenticity of LDL lipid hydroperoxides we utilized the following strategy: (a) we assayed LOOH on the LDL total lipid fraction extracted according to Bligh and Dyer[22] and (b) we used a FOX1B reagent without ferrous ion to discriminate between the background signal generated by ferric ions eventually present in the sample and that generated by ferrous oxidation by LOOH. The color developed during the assay was almost totally (>95%) ascribable to the oxidation of ferrous ion and was directly proportional to the amount of lipid extract. When the hydroperoxide content was expressed on a per cholesterol basis, then LDL were found to contain approx. 2.5 ± 1.6 nmol/mg of cholesterol ($n = 10$). This is in good agreement with values obtained by Nourooz-Zadeh *et al.*[23] (2 nmol LOOH/mg cholesterol) using the FOX2 reagent and by Zamburlini *et al.*[24] (2 ± 1 nmol/mg cholesterol) using chemiluminescence modified by a photon counting luminescence technique. The usefulness of the FOX1B assay for the evaluation of oxidative stress *in vivo* is currently under investigation; promising results have been obtained in the detection of total plasma peroxide, confirming the higher sensitivity and the lower incidence of the experimental error with this method, compared to the FOX2 one.

Interferences in the Measurement of Peroxides

The method detects free Fe^{3+} and compounds that either back-reduce it to ferrous (ascorbic acid) or form strong complexes with it e.g., desferrioxamine, EDTA, acidic phospholipids interfere with the reaction. Water soluble interfering substances may be removed by gel filtration as described in the Materials and Methods section. Chain breaking antioxidants, such as α-tocopherol, behave like BHT and thus do not interfere.

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