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Determination of total plasma hydroperoxides using a diphenyl-1-pyrenylphosphine fluorescent probe

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ABSTRACT

Plasma hydroperoxides (HPs) are widely accepted to be good indicators of oxidative stress. By means of the method proposed here, which uses diphenyl-1-pyrenylphosphine (DPPP) as a fluorescent probe, all types of plasma HP were determined. The limits of detection and quantification of the method were 0.08 and 0.25 nmol of cumene hydroperoxide (CHP) equivalents in 40 μl of plasma, respectively. The method is satisfactory in terms of precision (5.2% for 14.5 μM CHP eq., $n = 8$), and the recoveries were 91% and 92% after standard additions of 26 and 52 μM CHP, respectively. The selectivity of the proposed method is higher than 96%. Moreover, optimization of the reaction conditions and the addition of ethylenediaminetetraacetic acid (EDTA) disodium salt and 2,6-di-*tert*-butyl-4-methylphenol (BHT) prevented the formation of HP artifacts during the analysis. Therefore, the proposed method is useful for simple and quantitative determination of total plasma HPs.

Oxidative stress plays an important role in the development of many pathologies, including cancer [1,2] and cardiovascular [3,4] and neurodegenerative diseases as well as other physiological processes such as aging [5,6]. Among the different methods used for the assessment of oxidative stress, the determination of lipid oxidation products in plasma has been widely accepted as a good indicator of oxidative imbalance [7]. However, there is growing evidence that proteins are also a major target of reactive oxygen species, and the resulting oxidative damage can lead to loss of their biological function. Furthermore, some protein oxidation products are good biomarkers to predict neurological disorders and age-related diseases [6,8]. Therefore, due to the biological relevance of oxidative damage regardless of its lipid or protein origin, the overall measurement of oxidation products by means of accurate and simple methods is of interest.

Routine analysis techniques to estimate oxidative stress in biological samples include iodometric assays [9], spectrophotometric determination of conjugated dienes [7,10], determination of thiobarbituric acid reactive substances values [11], and measurement of carbonyl content [12]. However, these methods are usually criticized for their lack of sensitivity and/or specificity. More sensitive methods, such as determination of hydroperoxides (HPs)[2] using luminol chemiluminescence [13] or HP activation of cyclooxygenase [14], have also been proposed. However, the application of these methods is sometimes limited because they are often complex and require sophisticated instrumentation [13]. Alternatively, plasma lipid and protein HPs can be determined by the formation of colored metal complexes with thiocyanate or xylenol orange. These methods are satisfactory in terms of sensitivity and simplicity [15,16], but the procedures are subject to interference caused by chelators, ferric iron, some redox compounds, or the presence of other chromophores. The ferrous oxidation–xylenol orange (FOX) method is commonly used for all kinds of biological sample, but plasma samples contain many compounds that can react with the dye and thus interfere with determination. Hence, it is common to use specific reducing agents for HPs to discriminate the background signal from authentic HPs [16,17].

However, diphenyl-1-pyrenylphosphine (DPPP) is a nonfluorescent molecule that specifically reacts with HPs to form DPPP oxide, which then emits fluorescence at 380 nm (excitation wavelength = 353 nm). In fact, the use of DPPP has been proven to allow

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[2] *Abbreviations used:* HP, hydroperoxide; FOX, ferrous oxidation–xylenol orange; DPPP, diphenyl-1-pyrenylphosphine; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; BHT, 2,6-di-*tert*-butyl-4-methylphenol; PBS, phosphate-buffered saline; GdnHCl, guanidine hydrochloride; CHP, cumene hydroperoxide; TPP, triphenylphosphine; URP, unoxidized rat plasma; ORP, oxidized rat plasma; LDL, low-density lipoprotein; RSD, relative standard deviation.

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selective and very sensitive determination of lipid HPs in biological samples using flow injection and high-performance liquid chromatography (HPLC) postcolumn methods [18,19]. In addition, it has recently been reported that lipid and protein HPs can be determined by this fluorescent probe using simple batch methods [20].

The aim of this study was to set up and validate a modified version of this method based on the DPPP fluorescent probe for simple, sensitive, and selective determination of total lipid and protein HPs in plasma due to their clinical relevance as biomarkers of oxidative stress.

Materials and methods

Materials

Ethylenediaminetetraacetic acid (EDTA) disodium salt solution, 2,6-di-*tert*-butyl-4-methylphenol (BHT), phosphate-buffered saline (PBS, 0.01 M, pH 7.4), guanidine hydrochloride (GdnHCl), 80% cumene hydroperoxide (CHP), and triphenylphosphine (TPP) were purchased from Sigma Chemical (St. Louis, MO, USA). DPPP was purchased from Cayman Chemical (Ann Arbor, MI, USA). Methanol and *n*-butanol were of HPLC grade and purchased from Panreac (Barcelona, Spain). Bidistilled water was obtained using a Milli-Q Gradient System (Millipore, Billerica, MA, USA). Positive displacement pipettes were used throughout the study.

Final procedure for determination of total plasma HPs

The entire procedure was conducted under subdued light conditions, and 1.5-ml microtubes with a safe-lock were used to avoid evaporation. First, 40 μl of plasma was mixed with 160 μl of a solution that contained 0.125% EDTA and 6 M GdnHCl in PBS. Then, 100 μl of 4 mM BHT in methanol was immediately added. After vortexing for 1 min, 100 μl of 400 μM DPPP and 4 mM BHT in butanol were added, and samples were vortexed again for 1 min. These solutions were prepared extemporaneously. The final concentrations of EDTA, BHT, and DPPP were 0.05%, 2 mM, and 100 μM, respectively. The head-space of the microtubes was flushed with nitrogen and immediately closed, and then samples were incubated at 40 °C for 3 h under constant agitation. The reaction was stopped by placing the samples in an ice bath for 20 min. Then, 1 ml of 6 M GdnHCl in PBS was added and samples were vortexed for 1 min. After that, 100 μl of the resulting solution was thoroughly mixed for 2 min with 1 ml of butanol, and samples were then centrifuged at 1500*g* for 10 min at 4 °C. Next, 100 μl of the supernatant was immediately transferred to 96-microwell plates, and fluorescence was determined in a FLUOstar Optima fluorimeter (BMG Labtech, Ortenberg, Germany) at 30 °C using the 360 ± 10-nm and 380 ± 10-nm fluorescence filters for excitation and emission, respectively. The signal was consecutively measured at intervals of 2 min for 10 min. Because the signal was observed to be stable, the average of the measurements was used for calculations.

Sample collection

Blood samples were extracted by heart puncture from healthy Sprague–Dawley rats (6–8 months old) fed with 2014 Teklad Global 14% Protein Rodent Maintenance Diet (Teklan Harlan, Madison, WI, USA). Samples were collected in heparin tubes as anticoagulant and immediately centrifuged at 1500*g* for 15 min at 4 °C for plasma separation. Plasma samples used for method development were pooled and stored in aliquots at −80 °C until analysis to avoid oxidation (unoxidized rat plasma, URP). Plasma samples used to determine the precision and recovery of the method were stored in the dark at 4 °C and analyzed within 24 h after extraction (fresh URP).

Hypercholesterolemic blood samples were obtained from eight Dunkin–Hartley guinea pigs (4 weeks old) from Harlan Interfauna Ibérica (Barcelona, Spain). Animals were fed with 2040 Teklad Global Diet for guinea pigs (Teklan Harlan) for 1 week of acclimation. Subsequently, blood samples from fasting animals were obtained from the saphenous vein and collected in heparin tubes. Animals were switched to a hypercholesterolemic experimental diet, the composition of which was as follows: protein, 18.8%; fat, 17.1%; carbohydrates (nonfiber), 45.6%; cellulose, 12.0%; cholesterol, 0.25%; mineral mix, 5.5%; and vitamin mix, 1.0%. The fat mix content of the diet was olive oil/palm-kernel oil/safflower oil (1:2:1.8), and the carbohydrates were added as starch/sucrose (1:1.34). The mineral and vitamin mix was designed to meet all of the nutritional requirements of guinea pigs [21]. After 4 weeks, blood samples from fasting animals were obtained from the saphenous vein and collected in heparin tubes. Plasma from initial and final blood samples was separated by centrifugation as described earlier and stored at −80 °C until analysis. To assess the hypercholesterolemic effect of the diet, plasma low-density lipoprotein (LDL) cholesterol was determined fluorimetrically using enzymatic kits from BioVision (Milpitas, CA, USA). All of the procedures were approved by the University of Barcelona's animal care and use committee.

Preparation of oxidized plasma samples

Oxidized rat plasma (ORP) samples were obtained by thermal oxidation of URP. A determined amount of URP was diluted with an equal volume of 6 M GdnHCl in PBS and incubated at 80 °C for 1 h under continuous magnetic stirring (500 rpm). ORP samples were used immediately for analysis.

Reaction kinetics

Reaction time was studied by mixing 40 μl of diluted ORP samples (equivalent to 20 μl of plasma) with 160 μl of 6 M GdnHCl in PBS containing 0.063% EDTA (final concentration in the medium of 0.025%), 100 μl of 2 mM BHT in methanol, and 100 μl of 400 μM DPPP and 2 mM BHT in butanol. Then, samples were incubated at 40, 50, and 60 °C, and plasma HPs were determined as described earlier. All sample kinetic studies were conducted in triplicate.

Effect of antioxidant addition

The influence of EDTA and BHT addition on the reaction was assessed by using a two-factor four-level (4 × 4) experimental design. First, 40 μl of ORP samples (equivalent to 20 μl of plasma) was diluted with 160 μl of 6 M GdnHCl in PBS containing 0%, 0.0625%, 0.125%, or 0.25% EDTA (final concentrations in the medium of 0%, 0.0255, 0.05%, or 0.1%, respectively). Then, 100 μl of 0, 2, 4, or 8 mM BHT in methanol was added (final concentrations in the medium of 0, 0.5, 1, or 2 mM, respectively). Samples were incubated at 40 °C for 3 h, and plasma HPs were determined as described earlier. The experiment was replicated four times.

Effect of sample amount

The optimal amount of sample volume was studied by determining the HPs of different amounts of ORP. Equivalent plasma volumes of 5, 10, 20, 30, 40, and 50 μl were made up to 100 μl with 6 M GdnHCl in PBS. Then, 100 μl of 6 M GdnHCl in PBS containing 0.2% EDTA and 100 μl of 4 mM BHT in methanol were added. Finally, 100 μl of 400 μM DPPP in butanol containing 4 mM BHT was added, and samples were then incubated for 3 h at 40 °C. Plasma HPs were determined as described earlier. The final concentrations of EDTA, BHT, and DPPP were 0.05%, 2 mM, and 100 μM, respectively. Studies were conducted in triplicate.