

Simple and specific test for measuring lipid peroxides in plasma

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Abstract

The specificity of an iodometric assay for measuring lipid peroxides in lipoproteins was tested, compared with the fluorimetric thiobarbituric acid assay, and adopted for detecting lipid peroxide in plasma samples. Oxidation of low density lipoproteins in vitro by Cu^{2+} , lipoxidase, and phagocytosing polymorphonuclear leucocytes was sensitively detected by the iodometric assay. Unlike the thiobarbituric acid assay, neither non-lipid substances commonly present in plasma, nor platelet or polymorphonuclear leucocyte by-products interfered with the iodometric assay. The iodometric assay measured a normal mean (SD) plasma lipid peroxide concentration of $10.8 (2.1) \mu\text{M}$; $n = 63$. Two weeks after the start of a high cholesterol diet in rabbits ($n = 5$), a sixfold increase in plasma lipid peroxide concentrations was measured by iodometric assay.

The specificity of a simple and sensitive iodometric test of lipid peroxidation was superior to that of the thiobarbituric acid assay. This iodometric assay should therefore provide a much more accurate assessment of lipid peroxide in plasma samples.

Despite the prevailing belief that lipid peroxides, in general, and oxidised low density lipoprotein, in particular, exist only extracellularly,¹ quantifiable plasma lipid hydroperoxide concentrations have been reported in different clinical conditions.²⁻⁴ Most of these findings have been viewed with suspicion, largely because of the unspecificity of the tests used. The most widely used, simple, and sensitive thiobarbituric acid assay has been criticised for its lack of specificity. Improvements, such as carrying out the assay from precipitated protein fraction,² or lipid extract of plasma,⁵ optimisation of peroxidation and prevention of peroxide decomposition during the acid-heating stage of incubation,⁶ have done little to persuade workers of its clinical reliability.⁷ Sophisticated techniques such as separation of the different lipid peroxides by high performance liquid chromatography, coupled with sensitive chemiluminescence detection,⁸ are likely to be free of artefacts but not applicable for routine clinical use.

As lipid peroxides are increasingly thought to have a pathogenic role in many disorders a specific and reliable test for their concentrations in plasma is greatly needed.

Recently, a simple spectrophotometric test of iodine formation, the iodometric assay, using a commercially available reagent, was suggested for the measurement of lipid peroxides in lipoprotein solution.⁹ We adapted this assay for plasma and compared the specificity of this test with the commonly used thiobarbituric acid assay.

Methods

Sixty three healthy volunteers (26 men and 37 women, aged from 19 to 36 years) were tested for plasma lipid hydroperoxide concentration. Plasma lipid peroxides were also measured in five New Zealand white male rabbits weighing 3.2-3.5 kg, before and two weeks after starting a high cholesterol diet.

SEPARATION OF PLASMA

Blood samples were taken through a 21 gauge needle and a "butterfly" cannula from the antecubital vein of healthy volunteers. The two syringe technique was used: the first 2 ml was used for routine laboratory measurements and the subsequent 2 ml was anticoagulated with EDTA (1.5 mg/ml blood) and used for lipid analysis.

PLATELET SUSPENSION

Blood was drawn into 3.8% trisodium citrate (1 v citrate plus 9 v blood), centrifuged ($220 \times g$ for 10 minutes), and the supernatant platelet rich plasma (PRP) separated. Part of the PRP was centrifuged ($13.500 \times g$ for three minutes) to obtain platelet free plasma (PPP). Platelets were sedimented from PRP by centrifugation ($2000 \times g$ for 10 minutes), washed in Tyrode solution containing apyrase, and resuspended either in phosphate buffered saline (PBS) or in PPP (3×10^8 platelets/ml).

LEUCOCYTE SUSPENSION

Human polymorphonuclear leucocytes (PMN) were isolated from anticoagulated (with heparin) (5 IU/ml) venous blood.¹⁰ The preparation contained more than 96% polymorphonuclear leucocytes. Cells were suspended in either phosphate buffered saline (PBS) or PPP (2.5×10^7 /ml).

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Table 1 Oxidation of low density lipoprotein in buffered saline *in vitro*

Stimulus	Incubation at 37°C (h)	Lipid peroxides	
		TBARS (nmol/mg LDL protein)	LPO
None	6	1.34	6.4
Cu ²⁺ (5 µM)	6	4.65	64.3
Lipoxygenase (100 µg/ml)	5	6.42	76.4

Data are means of three parallel measurements.

INCUBATION OR STIMULATION OF CELLS

Cells were incubated in PBS or PPP in a humidified carbon dioxide incubator at 37°C for 30 minutes. Platelets were activated by thrombin. Five IU/ml of thrombin was added to the stirred platelet suspension; stirring was stopped 30 seconds later to prevent the formation of big platelet aggregates. Cells were put into the incubator for 30 minutes, centrifuged, and lipid peroxides were measured from the cell-free supernatant. Polymorphonuclear leucocytes were stimulated by adding 10 µl of a 2.5% suspension of polystyrene microspheres (3.0 µm; Polysciences, UK) to 1 ml cell suspension. After 30 minutes of incubation cells and beads were sedimented by centrifugation and the clear supernatant was used for the measurement of lipid peroxidation.

LOW DENSITY LIPOPROTEIN

Low density lipoprotein was isolated by ultracentrifugation,¹¹ dialysed at 4°C for 24 hours against 2 × 1000 v of 0.15 M NaCl containing 0.01% EDTA, and then sterilised by passage through a 0.45 µm filter. Before oxidation, low density lipoprotein was dialysed against buffered saline to remove EDTA.

ASSAYS FOR LIPID PEROXIDES

Thiobarbituric acid reactive substance

Lipid peroxidation was assessed by the fluorimetric measurement of thiobarbituric acid reactive substances (TBARS), as first described by Yagi,² and modified by us.¹² The incubation medium contained 20 µl plasma sample, 0.5 ml 0.67% TBA in 0.1 M TRIS-buffer, 0.5 ml 20% acetic acid, 10 µl Fe³⁺ and 10 µl butylated hydroxytoluene (BHT), to give 0.1 M and 20 µM final concentrations, respectively. This medium (pH 3.4) was heated in a dry heating block at 95°C for 60 minutes. After cooling with tap water samples were centrifuged and the fluorescence measured at 515 nm and 553 nm excitation/emission, respectively. Fluorescence was converted to nmol malondialdehyde (MDA) from a standard curve generated with 1,1,3,3-tetramethoxy-propane.

Table 2 Measurement of lipid peroxides in buffer or in plasma, incubated with unstimulated or activated blood cells

Cells	Medium	Stimulus	Incubation at 37°C (h)	Lipid peroxides	
				TBARS (µM)	LPO
Platelets	PBS	None	0.5	2.60	6.7
Platelets	PBS	Thrombin	0.5	11.43	6.2
Platelets	Plasma	Thrombin	0.5	17.52	12.2
PMNs	PBS	None	0.5	3.15	6.3
PMNs	PBS	Beads	0.5	29.80	8.4
PMNs	Plasma	Beads	0.5	253.90	197.4

Data are means of at least three parallel measurements.

Iodometric assay

The assay conditions were identical with those described by El-Saadani *et al.*⁹ Test solution (100 µl) was mixed with 1 ml colour reagent of the commercially available kit for the enzymatic determination of cholesterol (CHO-iodide; Merck, Darmstadt, Germany), and 10 µl BHT (20 µM). Samples were allowed to stand for 30 minutes in the dark at room temperature and the absorbance was then measured at 365 nm against the colour reagent as blank. When plasma samples were tested, two identical samples (100 µl each) were placed in separate test tubes containing 10 µl antioxidant BHT, and stored at 4°C for no more than two days. The assay was performed by adding 1 ml colour reagent to one sample and 1 ml saline to the other to exclude the absorbance due to the original yellow carotenoid colour of plasma. Samples were placed in the dark for 30 minutes. After centrifugation (12 000 × *g* for three minutes) absorbance of the supernatants was measured against colour reagent as blank. From the difference of optical densities (colour reagent-saline), lipid peroxide content of plasma was calculated by the use of the molar absorptivity of I₂ measured at 365 nm ($\epsilon = 2.46 \pm 0.25 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

Results

We have confirmed earlier findings⁹ that the presence of malondialdehyde (<100 mM), glucose (<300 mg/dl), haemoglobin (<300 mg/dl), and bilirubin (<20 mg/dl) do not interfere with the iodometric assay. Furthermore, free sialic acid (up to 10 µmol/dl)¹³ and sialoglycoprotein (human fibrinogen, up to 500 mg/dl) did not affect the iodometric measurement. Oxidation of low density lipoprotein by Cu²⁺ and lipoxygenase (Type I; Sigma) was shown clearly by both assays (table 1).

The effects of platelet activation and polymorphonuclear leucocytes on lipid peroxidation measurements are shown in table 2. The activation of platelets by thrombin in buffer resulted in an increased concentration of lipid peroxide, as measured by the thiobarbituric acid assay, but not the iodometric assay. Similarly phagocytosis of polymorphonuclear leucocytes in buffer increased the concentrations of TBARS but had no effect on the iodometric assay. Both the thiobarbituric acid assay and iodometric assay showed a great increase in hydroperoxides when cells were activated in the presence of plasma.

In 63 healthy volunteers the iodometric assay performed directly from the plasma samples measured a normal plasma lipid peroxide concentration (mean 10.8 (SD 2.1)). Lipid peroxide concentrations did not differ significantly between men and women. In one healthy volunteer blood samples were taken at two to three day intervals, always in the morning, and plasma lipid peroxide concentrations were determined. The coefficient of variation of the six measurements was 8.9%. No significant difference was found between the parallel

Table 3 Mean (SD) plasma hydroperoxides in five rabbits before (day 0) and two weeks (14 days) after starting high cholesterol diet

Plasma hydroperoxides (μM)			
Thiobarbituric acid assay		Iodometric assay	
Day 0	Day 14	Day 0	Day 14
2.74 (0.83)	4.72 (0.96)	8.3 (2.1)	48.7 (8.8)
p = 0.02		p = 0.0001	

measurements using different batches of colour reagent.

Plasma samples from rabbits ($n = 5$) were tested for lipid peroxides with both the thiobarbituric acid assay and iodometric assay, before and two weeks after starting a high cholesterol diet. In contrast to the thiobarbituric acid assay, which showed a very moderate increase, the iodometric assay detected a highly significant increase in plasma lipid peroxide concentrations in response to the high cholesterol diet (table 3).

Discussion

Our findings do not support the view that the thiobarbituric acid assay is "notoriously non-specific,"¹⁴ and that it is "far the most misleading assay to use on human plasma."¹⁷ Use of protein precipitate,² or lipid extract of plasma,⁵ the less acidic pH in which polyunsaturated fatty acids can react with TBA,¹⁵ and the presence of Fe^{3+} and antioxidant during the incubation⁶ greatly improve the specificity of the assay. Its specificity, however, is still limited by interference from substances released from activated platelets or leucocytes. The iodometric test is not subject to interference from cell-release products. An additional advantage of the iodometric assay is that water soluble substances commonly present in plasma do not affect the assay. The iodometric assay can therefore be performed directly from the plasma sample, avoiding troublesome protein precipitation or lipid extraction.

Our findings suggest that the iodometric assay is specific for lipid peroxidation, which occurs when polymorphonuclear leucocytes or platelets are activated in a medium containing plasma. Phagocytosing leucocytes induced oxidation of low density lipoprotein both in vitro and in vivo.¹² Recently, platelet-derived reactive oxygen species were shown to oxidise low density lipoprotein and enhanced its uptake by macrophages.¹⁶ Raised lipid peroxide concentrations in plasma after thrombolytic treatment⁴ could also be attributed to the pronounced platelet activation observed in this condition.¹⁷

The lipid peroxide concentration measured

by the iodometric assay in normal plasma is higher than those we³ and others⁵ have reported using the thiobarbituric acid assay. This is because the iodometric assay measures the total amount of lipid peroxides, including triglyceride-, cholesterol ester-, and phospholipid hydroperoxides. Under clinical conditions this is an advantage of the technique, which is clearly shown by the sensitivity of the iodometric assays in detecting a great increase in plasma lipid peroxide in rabbits at an early stage of atherogenesis. The very modest increase in lipid peroxide concentration shown by the thiobarbituric acid assays may have been due to the fact that the thiobarbituric acid assay does not measure the breakdown products of cholesterol hydroperoxide aldehyde.

- Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modification of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989;320:915-44.
- Yagi K. Assay for serum lipid peroxide level and its clinical significance. In: *Lipid peroxides in biology and medicine*. New York: Academic Press, 1982:223-41.
- Stringer MD, Gorog P, Freeman A, Kakkar VV. Lipid peroxides and atherosclerosis. *Br J Med* 1989;298:281-4.
- Davies SW, Ranjadayalan K, Wickens DG, Dormandy TL, Timmis AD. Lipid peroxidation associated with successful thrombolysis. *Lancet* 1990;335:741-3.
- Wade CR, Rij AM. Plasma malondialdehyde, lipid peroxides, and the thiobarbituric acid reaction. *Clin Chem* 1989;35:336.
- Janero DR, Burghardt B. Thiobarbituric acid-reactive malondialdehyde formation during superoxide-dependent, iron-catalysed lipid peroxidation: influence of peroxidation conditions. *Lipids* 1989;24:125-31.
- Gutteridge JMC, Halliwell B. The measurement and mechanism of lipid peroxidation in biological systems. *Trends in Biological Sciences* 1990;15:129-35.
- Yamamoto Y, Brodsky MH, Baker JC, Ames BN. Detection and characterization of lipid hydroperoxides at picomole levels by high-performance liquid chromatography. *Analyt Biochem* 1987;160:7-13.
- El-Saadani M, Esterbauer H, El-Sayed M, Goher M, Nassar AY, Jurgens G. A spectrophotometric assay for lipid peroxides in serum lipoproteins using commercially available reagent. *J Lipid Res* 1989;30:627-30.
- Kovacs IB, Meyrick Thomas RH, Mackay AR, Rustin MHA, Kirby JDT. Increased chemiluminescence of polymorphonuclear leukocytes from patients with progressive systemic sclerosis. *Clin Sci* 1986;70:257-61.
- Havel RJ, Edner HA, Bragdon J. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955;34:1345-53.
- Gorog P. Activation of human blood monocytes by oxidised polyunsaturated fatty acids: a possible mechanism for the generation of lipid peroxides in the circulation. *Int J Exp Pathol* 1991;72:227-37.
- Rodriguez-Iturbe B, Katiyar VN, Coello J. Neuraminidase activity and free sialic acid levels in the serum of patients with acute poststreptococcal glomerulonephritis. *N Engl J Med* 1981;304:1506-10.
- Hackett C, Linley-Adams M, Lloyd B, Walker V. Plasma malondialdehyde: a poor measure of in vivo lipid peroxidation. *Clin Chem* 1988;34:208.
- Ohkawa H, Ohishi N, Yagi K. Reaction of linoleic acid hydroperoxide with thiobarbituric acid. *J Lipid Res* 1978;19:1053-7.
- Aviram M, Danker G, Brook JG. Platelet secretory products increase low density lipoprotein oxidation, enhance its uptake by macrophages, and reduce its fluidity. *Arteriosclerosis* 1990;10:559-63.
- Fitzgerald DJ, Catella F, Roy L, Fitzgerald GA. Marked platelet activation in vivo after intravenous streptokinase in patients with acute myocardial infarction. *Circulation* 1988;77:142-50.