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Applications on the monitoring of oxidative modification of LDL by capillary electrophoresis: a comparison with spectrophotometer assay

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Abstract

The aim of this work is the application of Stocks and Miller capillary electrophoresis (CE) method in order to evaluate the human LDL susceptibility to Cu^{2+}-induced oxidation. Lipid peroxidation determines a change in the relative electrophoretic mobility (REM) of lipoprotein that can be monitored by capillary electrophoresis using uncoated fused silica capillaries and tricine-methylglucamine as electrophoretic buffer.

We have evaluated the differences in the susceptibility to oxidation of LDL subjected to different preparations (dialysis or gel filtration, after ultracentrifugation, to remove EDTA), and different storage (4 °C for 1 week or lyophilization) by measuring REM and lipid hydroperoxides (ROOH) with a spectrophotometer assay. Our results indicate that gel filtration is a more convenient procedure than dialysis for the isolation of LDL and that lyophilised samples are less prone to oxidation than those stored at 4 °C. Moreover, REM appears to be a more sensitive and easier method than spectrophotometer assay both to monitor the oxidative modification of LDL and to evaluate oxidative state of native LDL.

Keywords: Atherogenesis; Low density lipoprotein; Oxidation; Capillary electrophoresis; Relative electrophoretic mobility

1. Introduction

Several studies pointed out that low density lipoproteins (LDL) play an important role in atherogenesis after undergoing oxidative modifications [1–4]. Oxidized LDLs (oxLDLs) are rapidly taken up by monocytes/macrophages via scavenger receptor transforming them into foam cells, which are essential components of fatty streaks and fibrofatty plaques [5–7]. OxLDL can also stimulate chemotractant secretion [8], inhibit endothelium-dependent vascular relaxation [9], induce the expression of adhesion molecules that mediate the interaction of leukocytes with endothelium [10] and exhibit a dramatic cytotoxic effect on cultured cells [11,12]. LDL undergo oxidative modification when incubated in vitro with endothelial, smooth muscular cells, or macrophages or when exposed to Cu^{2+} [13,4]. Copper-induced oxidation of isolated LDL is frequently used as a model in vitro to evaluate the resistance of lipoprotein to oxidation. Following the addition of cupric ions, oxidation of the polyunsaturated fatty acids, primarily linoleic acid and arachidonic acid, was observed, with the appearance of hydroperoxides in the polyunsaturated fatty acyl chains of LDL lipids [3]. This process can be easily detected by monitoring continuous spectrophotometric measurements of conjugated dienes at 234 nm [14]. The hydroperoxides in LDL can decompose to form other oxidized species, some of which can be detected by different photometric methods, such as iodometric assay [15–17], xylenol orange assay (or Fox assay) [18–20] and thiobarbituric acid assay [21]. The oxidation products of fatty acids react covalently with ε-amino groups of apolipoprotein B, neutralising its positive charge

Abbreviations: Apo-B, apolipoprotein B; CE, capillary electrophoresis; Cu^{2+}, cupric ion; C-LDL, cold stored-LDL; D-LDL, dialyzed-LDL; F-LDL, gel filtrated-LDL; L-LDL, lyophilized-LDL; ROOH, lipid hydroperoxides; OxLDL, oxidized LDL; REM, relative electrophoretic mobility

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and increasing the net negative charge of lipoproteins, which can be readily monitored by capillary electrophoresis (CE) under the conditions described by Stocks and Miller [22].

The evaluation of the oxidative characteristics of lipoproteins in vitro has been accomplished by the isolation of the lipoprotein fractions from plasma using ultracentifugation followed by gel filtration or dialysis to remove EDTA and other interfering compounds. In some cases, after isolation, lipoproteins must be stored before the analysis, for this reason appropriate procedures of preservation have been established to reduce proteolysis and oxidation of LDL [23]. In this work, we have employed the Stock and Miller electrophoresis capillary method and the FOX 1B method to monitor the oxidative modification of LDL subjected to different preparation (dialysis or gel filtration) and to different storage (4 °C or lyophilising) and we have compared their capability to evaluate the differences in the lipoproteins oxidation.

2. Materials and methods

2.1. LDL isolation

Blood was collected from five healthy volunteers into sampling vacutainer vials containing EDTA. Plasma was prepared by centrifugation at 2000 × *g* for 10 min at 4 °C. LDL was isolated by ultracentrifugation according to Himber et al. [24] and McDowell et al. [25]. Briefly, 0.9 ml of plasma was added to a centrifugation tube containing KBr (0.4451 g) adjusting the density of plasma to 1.300 mg ml^{-1}. This was then overlaid with 2.1 ml of 150 mmol l^{-1} NaCl and centrifuged at 541,000 × *g* for 2 h at 4 °C. LDL orange coloured band was recovered, mixed (ratio 1:1) with a solution containing KBr and EDTA 1% (until the density 1.063 mg ml^{-1} was obtained), and centrifuged at 541,000 × *g* for 2 h at 4 °C. LDL were either dialysed in phosphate buffer (5 mM potassium phosphate, pH 7.4 containing 150 mmol l^{-1} NaCl for 24 h at 4 °C (D-LDL) or passed through Sephadex PD-10 column equilibrated with phosphate buffer (F-LDL) to remove EDTA and other interfering compounds. For storage experiments LDL were either stored at 4 °C for 1 week in 33 mmol l^{-1} phosphate buffer, pH 7.4 containing 1 mmol l^{-1} EDTA and 0.02% sodium azide (C-LDL) or lyophilised (L-LDL) in presence of 5% sucrose (L-LDL) as described by Edelstein et al. [23]. Before oxidation reaction LDL were passed through Sephadex PD-10.

2.2. Oxidative modification of LDL

LDL (800 μg ml^{-1}) cholesterol was oxidized in phosphate buffer (5 mM potassium phosphate, pH 7.4 containing 150 mM NaCl) at 30 °C in a shacking water bath in the presence of $CuSO_4$ 20 μmol l^{-1}. Aliquots were withdrawn at 0, 1, 3 and 4 h and analysed by capillary electrophoresis and by FOX 1B method.

2.3. Capillary electrophoresis

Lipoproteins modification was evaluated by a Beckman P/ACE 5510 System capillary electrophoresis fitted with a diode array detector. Aliquots of LDL used for electrophoresis were mixed with Na_2EDTA to a final concentration of 10 mmol l^{-1}. We used the Stocks and Miller method [22] with some modifications as briefly described. The dimension of the uncoated fused-silica capillary was 75 μm i.d. and 57 cm length (50 cm to the detection window), and the instrument was run in normal polarity (cathode at the detector end).

The electrophoresis buffer, 50 mmol l^{-1} tricine, 100 mmol l^{-1} methylglucamine, pH 9.7, was freshly prepared before each analysis set. After incubation, LDL samples were introduced in the system at low pressure (3.8 kPa for 2 s). The 100 mmol l^{-1} dimethylsulfoxid (DMSO), used as electroendoosmotic flow (EOF) marker, was injected for 1 s. A voltage of 25 kV was applied, ramping over 0.2 min. The capillary temperature was maintained at 20 °C. Migration of LDL was monitored by absorption at 200 nm and relative electrophoretic mobility (REM) was calculated from the difference between their velocity (distance from the injection point to the detector/migration time) and that of the EOF marker, divided by the field strength (applied voltage/length of capillary).

2.4. Lipid hydroperoxides (ROOH) quantification

Lipid hydroperoxides were estimated according Deiana et al. [20] by utilizing the FOX 1B reagent obtained by modifying the FOX reagent originally developed by Wolff and co-workers [19]. Aliquots of LDL (0.2 ml) were extracted according to Bligh and Dayer [26]. Test samples were added to FOX 1B reagent (100 μmol l^{-1} xylenol orange, 250 μmol l^{-1} ammonium ferrous sulphate, 0.4 mol l^{-1} sucrose, 0.2 mmol l^{-1} BHT, 25 mmol l^{-1} H_2SO_4 final concentration in 1 ml assay). After 40 min of incubation time at room temperature, the samples were centrifuged at 5000 × *g* for 10 min and the absorbance of the supernatant was read at 580 mm. The hydroperoxide content was expressed on a per cholesterol basis.

2.5. Measurement of α-tocopherol

α-Tocopherol in LDL content was measured on high performance liquid chromatography (HPLC) [27]. A detector UV/Vis at 292 nm wavelength was fitted using a sensitivity range of 0.005 AUSF. A Waters spherisorb C-18 column (5 μm, 250 mm × 4.6 mm i.d.) was used. The mobilephase consisted of 6% tetrahydrofuran in methanol running at 1.5 ml min^{-1} as flow rate. The α-tocopherol content was expressed on μmol mmol^{-1} cholesterol.

2.6. Statistical

All data are expressed as mean ± S.D. The difference of results from D-LDL and F-LDL, C-LDL and L-LDL were analysed using the paired Student's-test.

3. Results

The change in electrophoreric mobility is one of the more reliable indicators of LDL modification and can be easily monitored by capillary electrophoresis as described by Stocks and Miller. This method proposes the use of the organic base methylglucamine in the tricine running buffer to improve the resolution peak, by diminishing the protein interaction with the silanol groups on the capillary wall.

The reproducibility of the electrophoretic mobility of LDL was evaluated by six separate injections of the same sample into the same capillary and CV% was <0.5% (data not shown). Subjecting the LDL to lipid peroxidation by copper we obtained a progressive increase in the migration time (as shows in Fig. 1), indicative of a rise in the electronegativity.

Lipoproteins oxidation determines a considerable broadening of LDL peak, presumably due to the breakdown of the particles [22]. In order to assess the appropriate experimental procedure, after ultracentifugation, LDL were subjected either to dialysis or gel filtration to remove EDTA, and their susceptibility to oxidation was evaluated by the incubation of lipoprotein in PBS at 30 °C containing 20 μM Cu^{2+}. Oxidation patterns, evaluated by measuring capillary electrophoresis REM, are greatly different ($P < 0.005$) as shown in Figs. 2 and 3a, while lipid peroxides content does not significantly differ ($P > 0.18$), although hydroperoxides

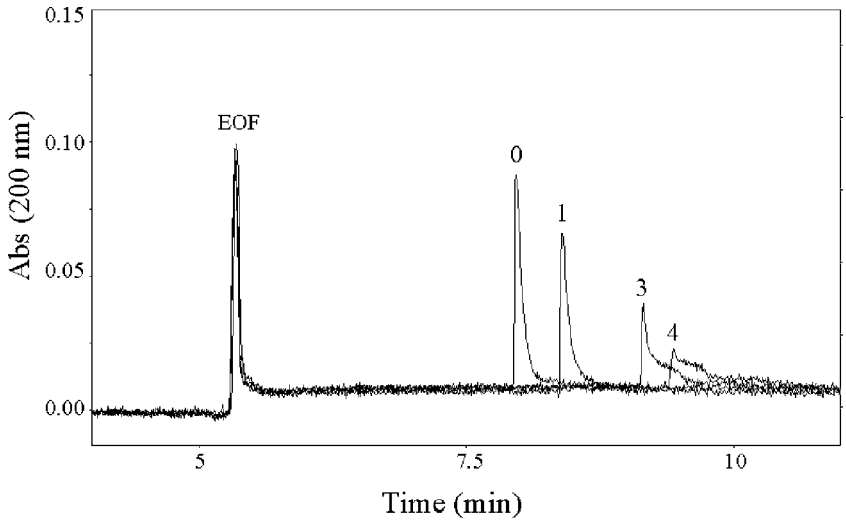

Fig. 1. Monitoring changes in electrophoretic mobility of LDLs by capillary electrophoresis during oxidation copper-induced. The number over the peak gives the timing of the analysed aliquot in hours.

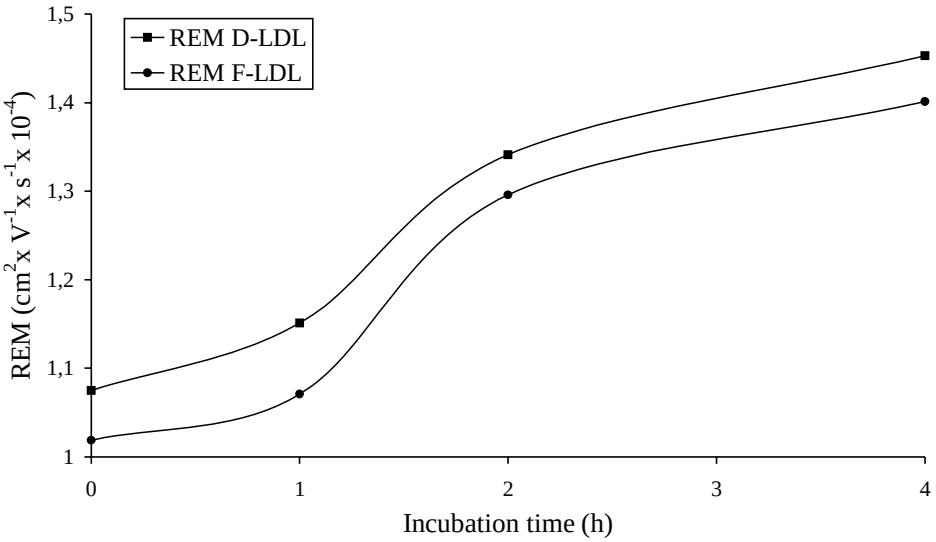

Fig. 2. LDLs REM measured by capillary electrophoresis of dialyzed LDL (D-LDL) and gel filtered LDL (F-LDL).

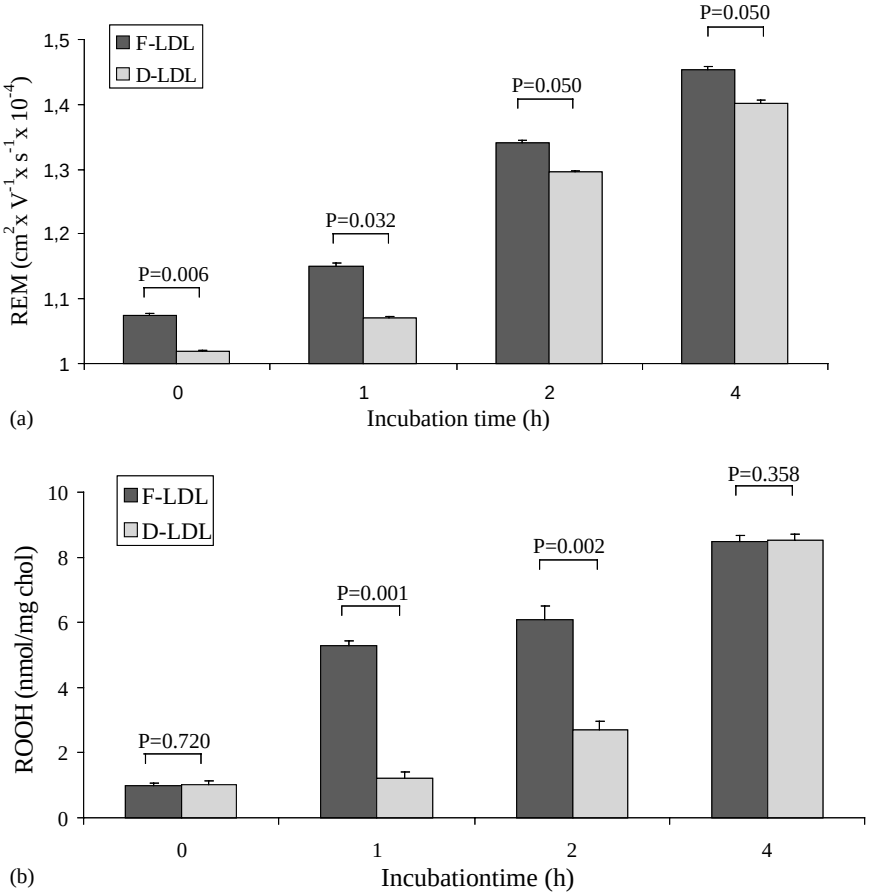

Fig. 3. REM (a) and hydroperoxides (b) values of dialyzed LDL (grey bars) and gel filtered LDL (black bars). Data are expressed as the mean ± S.D. of $n = 5$ different experiments.

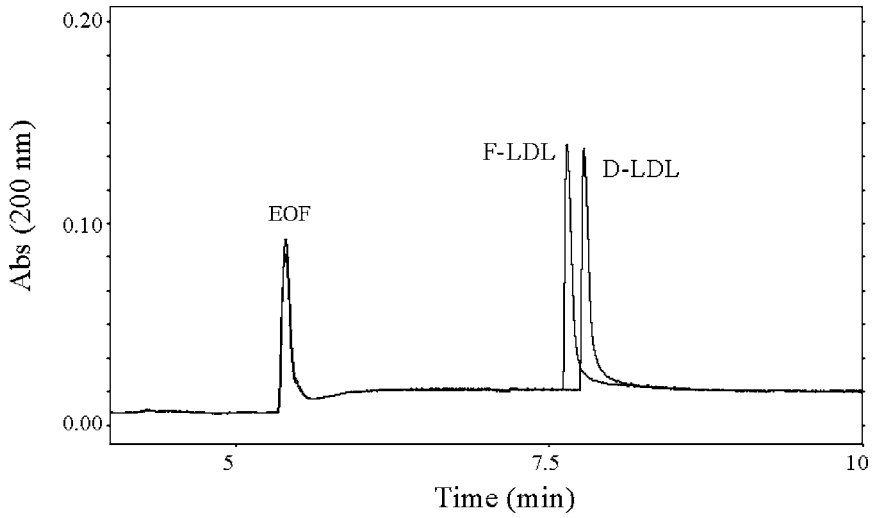

Fig. 4. Electropherograms of native LDL after dialysis (D-LDL) or gel filtration (F-LDL).

content of D-LDL increases more rapidly than that of F-LDL (Fig. 3b). Moreover, even if oxidative state of native LDL is considered very low and difficult to measure, capillary electroforesis appears to be able to detect the differences in the oxidation state at time 0 h ($P = 0.006$) while FOX 1B does not ($P = 0.72$), as shown Figs. 3 and 4. To elucidate on the appropriate storage that gives a better stability, lipoproteins were subjected either to conservation at 4 °C for one week or to lyophilization. Fig. 5 shows REM of homologous C-LDL and L-LDL subjected to lipidic peroxidation by

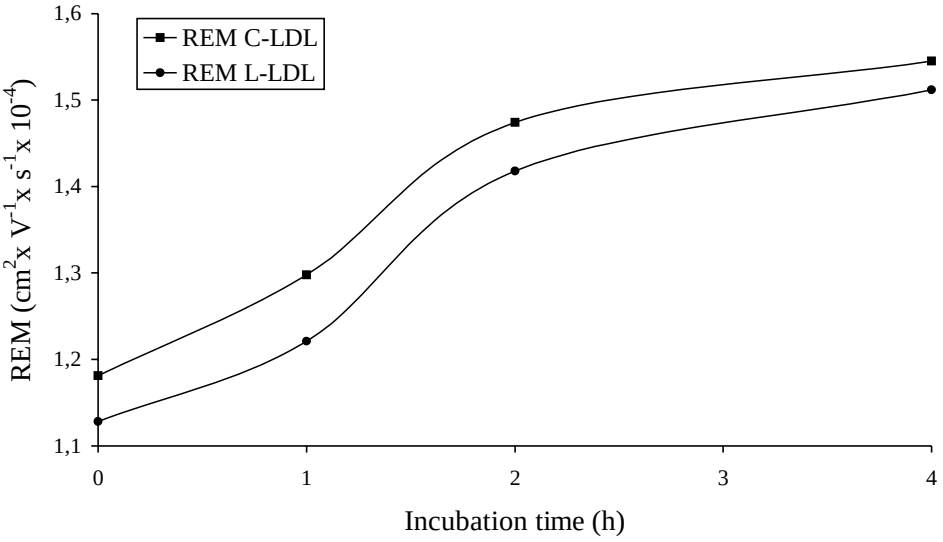

Fig. 5. LDLs REM measured by capillary electrophoresis of cold stored LDL (C-LDL) and lyophilised LDL (L-LDL).

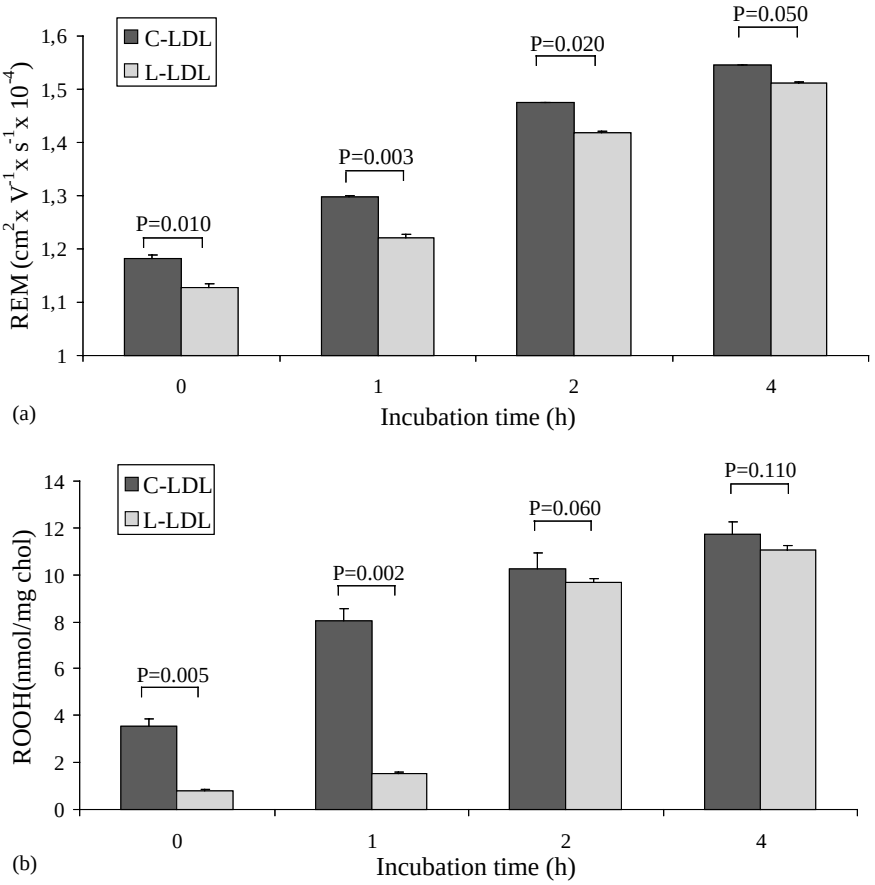

Fig. 6. REM (a) and hydroperoxides (b) values of lyophilised LDL (grey bars) and cold stored LDL (black bars). Data are expressed as the mean ± S.D. of $n = 3$ different experiments.

copper. Lyophilised LDLs are less prone to oxidation than those stored at 4 °C ($P < 0.009$). In this case, both CE and FOX 1B are able to detect the differences of native LDL oxidation state ($P = 0.005$ versus $P = 0.010$, respectively), but only the REM differs significantly during the time run of oxidation ($P < 0.009$), while hydroperoxides do not ($P > 0.15$) Fig. 6a and b). In Fig. 7, an electropherogram of native LDL after lyophilizing and after storing at 4 °C for 1 week is shown. The α-tocopherol content in LDL samples dyalised or gel-filtered is significantly different in the two

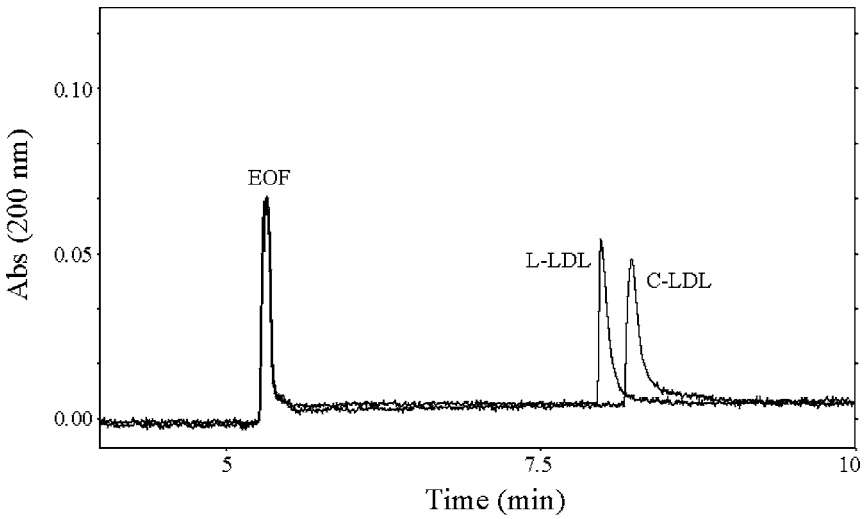

Fig. 7. Electropherograms of native LDL after lyophilization (L-LDL) or cold storage (C-LDL).

forms ($2.55 + 0.63\,\mu g\,mg^{-1}$ and $4.94 + 0.47\,\mu g\,mg^{-1}$ chol, respectively, $P = 0.010$), while the α-tocopherol content in LDL samples cold stored or lyophilised is the same in the two forms ($4.7 + 0.405\,\mu g\,mg^{-1}$ and $5.0 + 0.35\,\mu g\,mg^{-1}$ chol, respectively).

4. Conclusions

Because of the putative role of the oxidation process in atherogenesis, there is a growing interest in the identification of an easy and reproducible method to monitor the LDL oxidation degree. Many investigators have used different photometric methods as iodometric assay, Fox assay, thiobarbituric acid assay or the monitoring of conjugate dienes as index of lipoprotein peroxidation but some of this methods are rather complex or poorly reproducible or sensitive. Stocks and Miller propose the use of capillary electrophoresis to measure the susceptibility of LDL to oxidation copper-induced. Although CE is a technique often used for the quantification of small molecules it gives some complication in the separation and quantification of macromolecules such as proteins or lipoproteins, probably because of their absorption to the capillary wall [28]. The use of methylglucamine, suggested by Dolnick [29] to improve the resolution peak of LDL, was taken up by Stocks and Miller to develop a new method for monitoring the oxidative modification of low density lipoproteins.

The relative electrophoretic mobility of native LDL, in our buffer run conditions, was between $1.02 \times 10^{-4}\,cm^{-2}\,V^{-1}\,s^{-1}$ and $1.18 \times 10^{-4}\,cm^{-2}\,V^{-1}\,s^{-1}$, depending on preparation and storage. Oxidation by copper determines a progressive rise of REM by an increasing of electronegativity derived from the loss of positive surface charges. This is mainly due to the reaction of ε-amino groups of apo(B) lysine residues with aldehydic breakdown products of lipid peroxides [30]. We have utilized this method to investigate the choice of a better experimental procedure to remove EDTA after ultracentrifugation and to evaluate the appropriate conditions of lipoproteins storage. Our data indicate that gel filtration is a more suitable procedure to purify LDL after ultracentrifugation than dialysis, in which we observe an higher susceptibility of the lipoprotein oxidation, in according to Esterbauer [3]. In our experimental condition, we observe that these results are probably affected by the two different procedures utilized to remove EDTA, differently concerning the oxidative state of LDL, as described by Leake [31]. If we consider the obtained result by the storage procedure, the lyophilised lipoproteins are less susceptible to oxidation than those stored at 4 °C, already at the 0 h, even if the cryopreservatives in the buffer are used, as suggested by Edelstein for other lipoprotein species [23]. The explanation of this difference has not well given yet and we need more experimental evidences, about the storage procedures and the antioxidant content, that probably affect the difference observed. Finally, the α-tocopherol content in LDL samples is influenced by the different desalting procedures but not by the different storage procedures. Moreover, capillary electrophoresis appears to be a more sensitive method than FOX 1B assay both to measure the oxidation degree of native LDL and to monitor their susceptibility to oxidation. Besides, automation, small sample volumes used, rapid sample preparation, fast run separation and on-line monitoring suggest capillary electrophoresis as a very suitable assay for the evaluation of the LDL oxidative state in patients.

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