Rapid isolation of low density lipoproteins in a concentrated fraction free from water-soluble plasma antioxidants

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Abstract A rapid method is described for isolation and concentration of plasma low density lipoproteins (LDL) using a Beckman L80 ultracentrifuge equipped with a 70.1 Ti fixed angle rotor. The isolation of LDL achieved by a discontinuous gradient density step (180 min) was followed by a simultaneous purification and concentration step (45 min) using ultrafiltration through a collodium bag under nitrogen. This dialysis/concentration step, in contrast to the standard dialysis techniques in batch or by filtration through short gel columns, prevents oxidation and dilution of the sample. Electrophoresis in agarose and sodium dodecylsulfate-polyacrylamide (SDS-PAGE) gels were used to monitor LDL surface charge, purity, and contamination with plasma proteins. The artifactual oxidation of LDL during isolation and subsequent handling, and thus the ability of LDL preparation for oxidation/antioxidation studies, was assessed by the determination of endogenous hydroperoxides and thiobarbituric acid reactive substances. The dialysis/concentration step by ultrafiltration that allows the obtention of a concentrated and purified LDL preparation was validated by the absence of ascorbate and urate, as measured by HPLC. This method led to LDL preparations free of water-soluble plasma antioxidants that were minimally oxidized and suitable for reliable in vitro LDL oxidation and inhibition studies. The applicability of this methodology was tested by studying the α-tocopherol content of LDL in a Portuguese population of university students.—**Vieira, O. V., J. A. N. Laranjinha, V. M. C. Madeira, and L. M. Almeida.** Rapid isolation of low density lipoproteins in a concentrated fraction free from water-soluble plasma antioxidants. *J. Lipid Res.* 1996. **37:** 2715–2721.

Supplementary key words ultracentrifugation • α-tocopherol • oxidation studies

Several lines of evidence suggest a role for the oxidative modification of LDL in atherogenesis (1). Oxidation of LDL leads to its recognition by the scavenger receptors of macrophages with subsequent lipid accumulation and foam cell formation (2). A corollary is that antioxidants inhibit the development of atherosclerotic lesions in experimental models of atherosclerosis in nonhuman primates (3). Therefore, there has been a great deal of research effort related to lipid oxidation studies in LDL and also to the prevention of this degradative process by antioxidants. Nevertheless, evaluation of LDL oxidation in vivo is a very complex and difficult task and, thus, most of the studies concerning LDL oxidation have been done with isolated LDL (4).

However, LDL preparations are unstable and very prone to oxidation during the isolation procedure, handling, and storage (5). Therefore, the methodology of LDL isolation from human plasma for the in vitro oxidation and inhibition studies is of major importance in order to obtain a reliable LDL fraction. Generally, the traditional time-consuming methodologies yield LDL preparations seeded with lipid hydroperoxides which are thought to play an important role in oxidative modification of LDL and may buffer in vitro oxidation/antioxidation studies; for instance, in particular conditions, LDL oxidation initiated either by Cu^{2+} or hemeproteins is dependent on the presence of preformed lipid hydroperoxides and it may be similar to the oxidative process occurring in vivo (6).

On the other hand, contamination of LDL preparation with water-soluble plasmatic major antioxidants, namely ascorbate and urate, should be avoided as they are effective at very low concentrations (7).

Several methods have been proposed to isolate LDL from human plasma. The sequential buoyant method requires a minimum of 56 h of ultracentrifugation, in-

Abbreviations: HPLC, high pressure liquid chromatography; LDL, low density lipoproteins; MDA, malondialdehyde; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBARs, thiobarbituric acid reactive substances.

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volves multiple steps, and quantitative recovery of the LDL fraction is an issue (8). The improved method of one-step isopycnic density gradient ultracentrifugation (9) still suffers the drawback of the long ultracentrifugation (≈24 h). Chung et al. (10) reported a lipoprotein separation method by density gradient ultracentrifugation in one step, shortening the required time considerably.

In recent years, several rapid methods developed on the basis of ultracentrifugation have been widely used for analytical measurements, particularly in epidemiological and clinical studies (11, 12), but not for preparative purposes.

The aim of the present work was to obtain LDL preparations, obtained from single donor subjects, in a short time, that were very concentrated and free from peroxides, and devoid of plasm antioxidants. Such preparations, therefore, would be suitable for in vitro studies of LDL oxidation and inhibition by antioxidant compounds. LDL preparations obtained by this new methodology are also suitable for endogenous antioxidant determination and consumption analysis. The methodology involves a modification of the method described by Chung et al. (10), using a Beckman L80 ultracentrifuge equipped with a 70.1 Ti fixed angle rotor. The isolation was followed by a concentration step with simultaneous ultrafiltration dialysis under nitrogen. This rapid dialysis/concentration step, relative to other methodologies, optimizes the obtention of a washed and concentrated LDL fraction from a single donor. This isolation procedure reduces the time required to separate LDL to 3 h and yields a concentrated and highly purified LDL preparation within 4 h. The artefactual oxidation of LDL during its isolation and subsequent handling was assessed by the determination of endogenous hydroperoxides, TBARs and the electrophoretic mobility of the isolated LDL fraction. The contamination of the preparation with the main plasma water-soluble antioxidants, ascorbate and urate, was also evaluated by HPLC. The methodology was tested in a screening of vitamin E content in LDL from a Portuguese population of university students with similar diet and style of life.

MATERIALS AND METHODS

Chemicals

α-Tocopherol, uric acid, and SDS-PAGE standards were purchased from Sigma Chemical, Co (St. Louis, MO) and all other chemicals were obtained from Merck (Darmstadt, Germany).

Blood samples

Each blood sample was withdrawn from one healthy normolipidemic adult volunteer, who had fasted overnight, and collected by venipuncture into tubes containing heparin as anticoagulant. Plasma was recovered by centrifugation at 3000 *g* for 15 min at 15°C.

LDL isolation

Density gradient ultracentrifugation. The recovered plasma was adjusted to a density of 1.21 g/ml (with a densitometer DMA 35, Mettler/Paar, Graz, Austria) by adding solid KBr, with gentle stirring, after the previous addition of EDTA (1 mM final concentration). The plasma solution was then distributed into 10-ml polycarbonate centrifuge tubes and a discontinuous density gradient was made by overlaying the plasma solution (2.8 ml) with 6.6 ml phosphate-buffered saline containing 110 mM NaCl, 20 mM phosphate, pH 7.4, and 1 mM EDTA, d = 1.007 g/ml, saturated with nitrogen.

The tubes were ultracentrifuged in a Beckman L 80 ultracentrifuge equipped with a 70.1 Ti fixed angle rotor, at 65000 rpm for 3 h at 15°C with slow acceleration and deceleration. After centrifugation, the tubes were carefully removed from the rotor and placed in the vertical position. The VLDL fraction appears as a white, heavily light scattering band at the meniscus. The yellow-orange LDL fraction stays in the upper half of the tube. The LDL fraction was collected by suction using a long-stem Pasteur pipette. The pipette was introduced through the VLDL fraction with a small air bubble at the end to avoid suction of materials other than the band of LDL.

Ultrafiltration. The LDL fraction was concentrated and simultaneously dialyzed by vacuum filtration through a collodium bag (Sartorius cellulose nitrate ultrafilter of 12,000) in a glass suction apparatus filled with phosphate buffer (20 mM phosphate, 110 mM NaCl, pH 7.4), under nitrogen atmosphere in the dark at 4°C. Thereafter, the LDL solution was filtered through a 0.22-μm pore-size filter (Millipore GS). The protein concentration was determined by the method of Lowry et al. (13) with bovine serum albumin as standard.

All the buffers were prepared in ultrapure water (Milli Q apparatus) and were made free of oxygen by vacuum degassing followed by purging with nitrogen.

Agarose gel electrophoresis

Electrophoresis of LDL preparations was carried out in 0.5% agarose gels in barbital buffer, pH 8.6, at a constant voltage of 220 V, and lipoproteins were stained with Sudan Black 0.1% in ethanol at room temperature.

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The gel was destained by washing in a mixture of ethanol–water 1:1 (v/v). In each agarose strip, the original plasma was applied in one lane as the reference for the LDL band in the samples under analysis.

The oxidized LDL control was obtained by incubation of 1.5 mg of protein in 1 ml of buffer with 10 μM $CuSO_4$, during 24 h, at 37°C.

SDS-PAGE electrophoresis

SDS-PAGE electrophoresis was performed according to Laemmli (14) in a gradient of acrylamide ranging from 3 to 20%. The gels were stained with Coomassie blue R.

Lipid hydroperoxide measurement

Lipid hydroperoxides were measured iodometrically by the method of El-Saadani et al. (15). This assay is based on the oxidative capacity of lipid peroxides to convert iodide to iodine, which is measured at 365 nm. The concentration of hydroperoxides was determined on basis of the molar absorptivity ($\epsilon_{365} = 2.46 \times 10^4\ M^{-1}\ cm^{-1}$).

Fluorometric measurement of thiobarbituric acid reactive substances (TBARs)

The quantification of TBARs was performed as previously described (16). The reaction product was fluorometrically measured using a Perkin-Elmer LS 50 spectrofluorometer with excitation at 500 nm and emission at 550 nm. The TBARs concentration in LDL preparations was estimated on basis of a standard curve prepared from a tetraethoxypropane stock solution (1 mM). The final results were expressed in terms of malondialdehyde (MDA)/mg protein.

HPLC analysis of ascorbate and urate

Ascorbate and urate in the original plasma (**Fig. 1**) and LDL fractions were measured by HPLC (Beckman, System Gold with a Programmable Detector module 166), using a LiCrospher 100 RP-18 (5 μm) column (Merck, Darmstadt, Germany) eluted with 1% acetic acid and UV detection at 265 nm. The column was run at room temperature with a flow rate of 1 ml/min. Sample preparation was performed by adding perchloric acid (for protein precipitation) (17), followed by centrifugation at 14,000 rpm in a Eppendorf centrifuge for 2 min; the supernatant was removed, filtered through a 0.22-μm pore-size filter (Millipore GS), and then injected into the HPLC system. The analytical method used enables simultaneous measurement of ascorbate and urate and their concentrations were calculated on basis of standard curves obtained from ascorbate and urate freshly prepared standards (Fig. 1 inset).

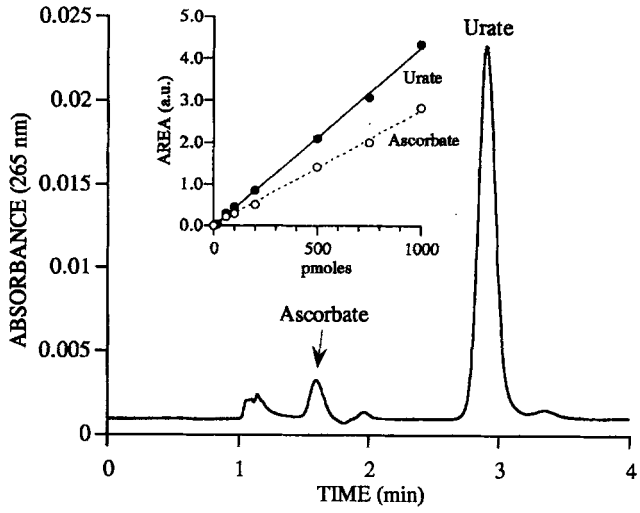

Fig. 1. Typical HPLC chromatogram of ascorbate and urate in human plasma. The inset shows the good correlation between the pics area and the amounts of urate and ascorbate injected.

Analysis of endogenous vitamin E in LDL

Vitamin E concentrations in LDL were measured by HPLC (Beckman, System Gold with a Programmable Detector module 166) with UV detection at 292 nm essentially as previously described (18). Lipid extraction was performed, from 180 μg of isolated LDL, with 1 ml of SDS (10 mM), 2 ml of ethanol, and 2 ml of hexane (19). The organic phase was removed and evaporated to dryness under a stream of nitrogen, and then the film was dissolved in 100 μl of alcoholic reagent (ethanol–isopropanol 95:5, v:v). This extract was injected on a LiChrospher 100 RP-18 (5 μm) column (Merck, Darmstadt, Germany), eluted with a solvent mixture consisting of 65% methanol and 35% alcoholic reagent, at room temperature, with a flow rate of 1.5 ml/min. The vitamin E concentrations were calculated by extrapolation on a standard curve using a freshly prepared stock solution of α-tocopherol.

RESULTS

Purity and contamination of LDL fraction

Purity and contamination of LDL preparations were checked by agarose and SDS-PAGE gel electrophoresis. **Figure 2** shows a typical agarose gel electrophoresis analysis of the isolated LDL preparation (lanes 2 and 5) in parallel with the original plasma (lanes 1 and 4) and an oxidized LDL fraction (lane 6).

The electrophoretic banding patterns of LDL indi-

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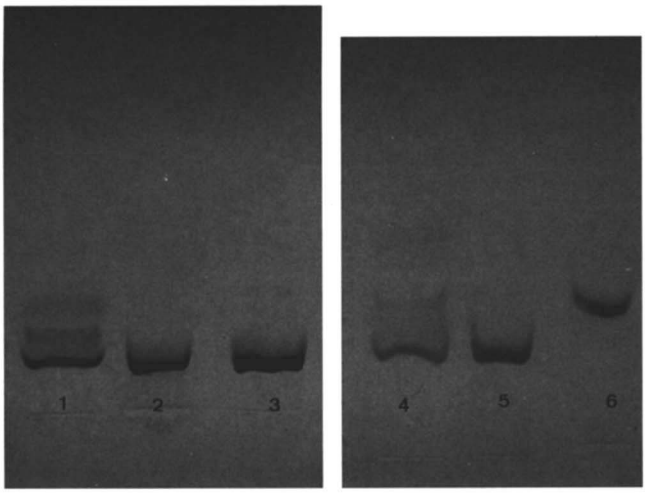

Fig. 2. Agarose gel electrophoresis of isolated LDL preparations, in parallel with original plasma and an oxidized LDL fraction. Lanes 1 and 4, original plasma; lane 2, frozen LDL preparation containing 10% sucrose (−84°C, 1 week, one freeze–thaw cycle); lanes 3 and 5, isolated LDL and lane 6, oxidized LDL (induced by $CuSO_4$ at 37°C).

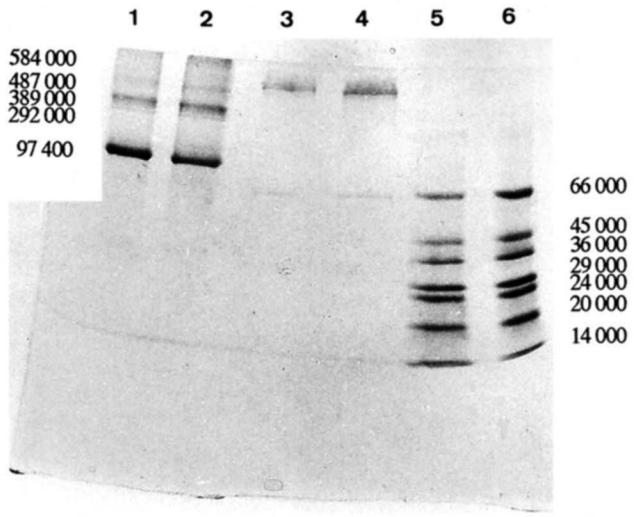

Fig. 3. SDS-PAGE of LDL apolipoprotein B. Lanes 1 and 2, molecular weight markers ranging from 97,400 to 584,000; lanes 3 and 4, LDL samples (20 and 25 μg protein, respectively); lanes 5 and 6, molecular weight markers ranging from 14,000 to 66,000.

cate that the LDL lipoprotein zones are sharply separated with no contamination with other lipoproteins. Moreover, the electrophoretic mobility of isolated LDL is similar to that in original plasma, suggesting that extensive oxidation during the isolation procedure did not occur. In fact, when oxidized (induced by Cu^{2+}), the mobility increases significantly (Fig. 2, lane 6). According to Rumsey et al. (20), LDL preparations frozen in 10% sucrose last for a long time without changes in structure or biological properties. Accordingly, freezing in 10% sucrose (w/v) and storage at −84°C during 1 week did not alter the mobility (Fig. 2, lane 3).

The PAGE-SDS analysis of LDL fraction (**Fig. 3**) indicates absence of significant contamination with plasma proteins and other lipoprotein fractions. According to electrophoretic standards, the LDL fraction, appearing as a single band with a relative molecular mass of 550,000 daltons, is homogeneous; only a small protein contaminant (identified by the relative molecular mass as albumin) was noticed.

Endogenous hydroperoxides

The measurement of the major initial reaction products of lipid peroxidation, the lipid peroxides, is a valuable index of the oxidative status of polyunsaturated fatty acids of LDL lipids. The very low obtained values of less than 5 nmol/mg LDL indicate that the isolated LDL preparations were preserved from oxidation during the isolation procedure and subsequent handling.

TBARs

TBARs and hydroperoxides indicate two stages of lipid peroxidation. The measurement of TBARs as malondialdehyde (MDA) is also widely used as an index of lipid peroxidation in LDL. In contrast with the high MDA values obtained in the oxidized LDL fractions, the values obtained in fresh or frozen preparations (<0.1 nmol MDA/mg LDL protein) were always lower than the detection limit of the method (**Table 1**) which is 0.1 nmol MDA. Typical values of TBARs in carefully isolated LDL were reported as 3.6 ± 1 nmol/mg LDL protein (4).

Urate and ascorbate

The efficiency of removal of plasmatic antioxidants by ultrafiltration was checked by analysis of ascorbate and urate during the LDL isolation procedure. Ascorbate and urate are important and powerful biological antioxidants in human plasma and can delay or protect LDL from the oxidation (21). The simultaneous measurement of ascorbate and urate performed by HPLC is illustrated in a typical HPLC chromatogram (Fig. 2),

TABLE 1. TBARs and relative electrophoretic mobility (REM) in an LDL preparation before and after Cu^{2+}-induced oxidation (Ox-LDL)

	Malondialdehyde	REM
	nmol MDA/mg protein	
LDL	—[a]	1.0
Ox-LDL	35.7	1.3

[a]Lower than the detection limit (0.1 nmol MDA).

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TABLE 2. Ascorbate (ASC) and urate concentrations in LDL preparations collected before and after the ultrafiltration step and also in the original plasma samples of four different subjects as evaluated by HPLC analysis

	Plasma		LDL before Ultrafiltration		Final LDL	
Subjects	ASC	Urate	ASC	Urate	ASC	Urate
	μM		μM		μM	
1	55.00 ± 0.60	410.00 ± 1.50	–[a]	38.80 ± 0.40	–[a]	–[b]
2	34.10 ± 0.20	391.50 ± 0.80	–	37.30 ± 0.10	–	–
3	34.20 ± 0.10	252.90 ± 0.20	–	11.60 ± 0.02	–	–
4	26.20 ± 0.10	231.50 ± 0.50	–	20.20 ± 0.02	–	–

Values represent mean ± SEM of three experiments.
[a]Not detectable (<3 μM).
[b]Not detectable (<0.8 μM).

showing the sharp separation of the two compounds. The concentrations of these antioxidants in the original plasma samples and in LDL preparations just before and after the ultrafiltration step, obtained from four subjects are shown typically in **Table 2.** Ascorbate and urate concentrations in all plasma samples were within the reported physiological range (17, 22). Before the ultrafiltration step, ascorbate was not detectable in any preparation but urate was still significant, although one-tenth of the level in plasma. Neither urate nor ascorbate could be detected in the final LDL preparations after the dialysis/concentration step.

Endogenous LDL vitamin E

Vitamin E in LDL protects against oxidative stress caused by reactive oxygen species, and it is considered the most important lipid soluble chain breaking antioxidant (23). As vitamin E content in LDL is unknown in the Portuguese population, a screening was performed in healthy university students (ages 20–25 years) to prove the usefulness and applicability of this method. Plasma samples were collected from fasting subjects who had been submitted to similar diets at the university cafeterias. The vitamin E values in isolated LDL from 17 students were 11.5 ± 2.6 nmol/mg LDL protein. These values are within the usually reported physiological ranges (8.24–14.92 nmol/mg LDL protein) (4) for other populations and no significant differences are observed between the vitamin E levels of women and men.

DISCUSSION

Lipoproteins have been commonly isolated by ultracentrifugation on the basis of their hydrated density characteristics. Most of the proposed methods are very time consuming and laborious, require a large volume of plasma, and may lead to damage of particles. It has been reported that prolonged ultracentrifugation could result in a partial degradation of apoB and in reduction of the vitamin E content of LDL (5). Esterbauer et al. (24) also reported that the re-isolation of vitamin E-enriched LDL, by ultracentrifugation, led to a significant reduction in its vitamin E content due to the extra ultracentrifugation step. In fact, the lag phase in the oxidative modification of LDL isolated by a time-consuming method is significantly shorter than that for LDL isolated by a rapid method (5). Moreover, the classical time-consuming methods of isolation lead to LDL preparations seeded with peroxides (4) which are unsuitable for reliable in vitro LDL oxidation and inhibition studies.

The method described here is a two-step procedure using a 70.1 Ti fixed-angle rotor and is a modification of the one-step method of Chung et al. (10). The further advantage of this methodology, relative to that of Chung, is to allow the obtention of a concentrated and highly purified LDL preparation suitable for lipid peroxidation studies. This was achieved by including a dialysis/concentration step by ultrafiltration under nitrogen. According to our experience, after ultracentrifugation, a typical dialysis step or a washing step by filtration through short gel columns, as used by several researchers, slightly oxidizes LDL and, conversely to our washing step, dilutes the LDL fraction.

The oxidation degree of the isolated LDL was evaluated by different standard parameters namely relative electrophoretic mobility, SDS-PAGE, lipid hydroperoxides, and TBARs. In fact, as the lipid peroxidation pathway is complex it should be studied on the basis of more than one single index measurement.

The final isolated LDL fraction is electrophoretically pure (Figs. 2 and 3) and shows unchanged electrophoretic mobility in relation to that in original plasma (Fig. 2) suggesting that modification of apoB during the isolation procedure did not occur. Only a small contami-

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nation with serum albumin could be found by SDS-PAGE. When the same LDL preparation was oxidized (induced by Cu^{2+}) its mobility increased significantly (1.3 times) (Fig. 2 and Table 1). This increase in electrophoretic mobility has been attributed to an increase in electronegativity by the blockage of lysine residues in the apoB with loss of positive charge (16).

Moreover, thiobarbituric reactive substances were not detected in the final LDL preparation in contrast with oxidized LDL which showed a high value (Table 1). The sensitivity and use of TBARs to evaluate lipid peroxidation may be a function of the lipid system; for instance, a lipid preparation rich in oleic acid, although extensively oxidized, may produce lower TBARs values (25). However, LDL has a high content of linoleic and arachidonic acids, meaning that LDL is likely to produce larger amounts of TBARs when oxidized. Furthermore, TBARs were measured by fluorescence which improved sensitivity relative to the standard spectrophotometric assay. In our LDL preparations, MDA content was always lower than the detection limit by fluorescence which is 0.1 nmol MDA/mg protein.

Also, the endogenous hydroperoxide content in final isolated LDL is very low (typically <5 nmol/mg LDL protein) similar to that referred to by El-Saadani et al. (15). A very recent paper (26) shows that sensitive HPLC-chemiluminescence techniques could underestimate peroxide levels in LDL. These authors arrived at a mean of 3 nmol peroxides/mg LDL protein, in agreement with the value previously reported by Esterbauer et al. (4). Therefore, values smaller than 5 nmol peroxides/mg LDL protein in our work indicate minimal LDL oxidation of our samples, thus validating the isolation/concentration methodology.

In summary, the results suggest that minimal oxidation of LDL particles occurred during isolation by our fast and reliable method.

On the other hand, this rapid method in two steps permits us to obtain a LDL preparation free from ascorbate and urate, major plasmatic water-soluble antioxidants (Table 2). In fact, although before the ultrafiltration step no ascorbate has been detected, a relatively significant amount of urate is still present which in turn is well removed during this last step (Table 2). Additionally, plasma antioxidants such as bilirrubin can, thus, be assumed to be absent and proteins (whose thiol groups exhibit antioxidant properties) were pelleted during this ultracentrifugation step.

Human blood plasma is well equipped with both chain-breaking and preventive antioxidants to cope with oxidative stress and to prevent peroxidative damage to circulating LDL (27). Ascorbate and urate are the major plasmatic water-soluble antioxidants, and they are effective at concentrations considerably below those normally found in plasma (7). Moreover, there is evidence that vitamin E in LDL is regenerated, after partial oxidation to the tocopheroxyl radical, by ascorbate on the aqueous interface (21). Therefore, LDL preparations for in vitro oxidation studies should be free from plasmatic residues of those compounds.

The methodology presented here has been shown to be suitable also for the measurement of endogenous vitamin E in LDL. This vitamin is the major chain-breaking antioxidant carried in LDL (4) and probably the major defense against oxidative damage in the particles (24). In this regard, the assessment of the LDL endogenous vitamin E content in humans is useful. The student population used is not representative of the Portuguese population for statistical evaluation but was useful to demonstrate the applicability of the method for this kind of study; this study may be relevant in view of the growing evidence from a number of epidemiological studies that there is an association between plasma levels of vitamin E and a low risk of coronary heart disease (28). Volunteer university students (ages between 20–25 years, both sexes) had a similar diet (university cafeterias) and style of life and the levels of vitamin E found in LDL were similar to those found in populations from northern Europe (4).

Among other factors, the susceptibility of LDL to free radical-mediated lipid peroxidation is dependent on its content of lipid peroxides, that can drive propagation reactions, and on its content of α-tocopherol. Hence, after the initiation step of peroxidation, the subsequent processes develop with loss of α-tocopherol. Therefore, it is meaningful that our preparations of LDL obtained by the proposed methodology contain α-tocopherol in higher concentration (11.5 ± 2.6 nmol/mg LDL protein), as compared in normal values. This observation strongly supports minimal oxidation during isolation and dialysis/concentration steps.

In conclusion, this methodology is a rapid and efficient way of obtaining a concentrated and washed LDL fraction from a relatively small volume of human plasma, suitable for peroxidation studies. This method should be preferred to the conventional methods, especially when oxidation/antioxidation studies are to be required. In fact, in this procedure the subsequent two rapid steps of isolation and washing/concentration minimize LDL degradation, as measured by standard procedures for evaluation of LDL oxidation status, and permit us to obtain a preparation free from water-soluble antioxidants. In view of the increasing causal relationship between the oxidizability of LDL and development of atherosclerosis, a methodology that rapidly provides a concentrated and washed LDL fraction suit-

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able for these studies can be envisaged as an important clinical and biochemical tool in the context of atherosclerosis.

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