paper on methodology

# Capillary electrophoresis to monitor the oxidative modification of low density lipoproteins

**Joseph Stocks[1] and Norman E. Miller**

Department of Cardiovascular Biochemistry, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1M 6BQ, United Kingdom

**Abstract** **A procedure has been developed that uses high performance capillary electrophoresis to monitor the changes in the electrophoretic mobility of low density lipoproteins (LDL) resulting from $Cu^{2+}$-catalyzed lipid peroxidation. Using uncoated fused silica capillaries, methylglucamine-Tricine, pH 9.0, as electrophoresis buffer and a field strength of 350 V/cm, separation of native LDL and oxidized LDL could be achieved in 8–10 min. The electrophoretic mobility of native LDL under these conditions was $1.32 \times 10^{-4}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$, and the migration time could be measured with a coefficient of variation of 0.44%. The increase in the electronegativity of LDLs during incubation with 10 μm $Cu^{2+}$ for 0.25–2.0 h resulted in a progressive increase in migration time. Monitoring the absorbance of the migrating LDL particles at a wavelength of 234 nm showed a progressive increase in peak area, which paralleled that in diene conjugation measured spectrophotometrically. Electronegative LDL particles formed by modification with malondialdehyde could also be separated from native LDL particles under these conditions. This new procedure should be useful in studies of factors influencing low density lipoprotein oxidation in vitro and in vivo.**—Stocks, J., and N. E. Miller. **Capillary electrophoresis to monitor the oxidative modification of low density lipoproteins.** ***J. Lipid Res.*** **1998.** 39: **1305–1309.**

**Supplementary key words** apolipoprotein B • malondialdehyde • lipid peroxides • human

Oxidation of low density lipoproteins (LDL) is probably a key event in the development of atherosclerosis (1–3). LDL accumulating in the intimal space are thought to undergo chemical modification as a result of free radical-initiated lipid peroxidation, leading to their enhanced uptake by macrophages and the formation of foam cells. Oxidation of LDL in vitro can be initiated by incubation with transition metal catalysts such as $Cu^{2+}$ ions (1) or by exposure to a free radical-generating agent such as azobis 2-amidino-propane hydrochloride (4). Such chemically modified LDL particles show many of the biological characteristics of LDL that have been modified by endothelial cells or macrophages. Oxidation of LDL is accompanied by an increase in absorbance at 234 nm, due to the formation of conjugated dienes in constituent polyenoic fatty acids, the production of thiobarbituric acid-reacting substances, and the accumulation of lipid hydroperoxides and cholesterol oxidation products (1–3, 5). It is also characterized by an increase in electrophoretic mobility (6). The change in electrophoretic mobility is one of the more reliable indicators of LDL modification.

Conventionally, changes in the electrophoretic mobility of LDL particles are determined in agarose gels using a barbital buffer. We have examined the use of capillary electrophoresis, a technique that is capable of high efficiency separation of charged molecules using microbore capillaries and high field strengths (7). Capillary electrophoresis offers several advantages over conventional electrophoresis methods, including automation, on-line monitoring, rapid separation, and more precise control of operating conditions. In addition, as migration of proteins past the detector is determined by UV absorbance, capillary electrophoresis has the potential to measure simultaneously changes occurring in both the mobility and the absorption spectra of lipoproteins. Here we describe the application of capillary electrophoresis to the rapid and precise measurement of the electrophoretic mobility of native LDL, and the changes in LDL mobility and UV absorption that result from oxidative modification.

## METHODS

### Reagents and materials

Uncoated fused silica capillaries (75 micron i.d.) were obtained from Supelco, Poole, UK. N-methyl-d-glucamine, Tricine, and 1,1,3,3-tetramethoxy-propane were obtained from Sigma, Poole, UK. All other chemicals were obtained from Fisher Scientific, Loughborough, UK.

Abbreviations: apos, apolipoproteins; LDL, low density lipoproteins; MDA, malondialdehyde; PBS, phosphate-buffered saline; EOF, electroendosmotic flow; i.d., internal diameter.

[1]To whom correspondence and reprint requests should be addressed.

### Isolation of LDL

Blood was collected from healthy humans by venipuncture into $Na_2$ EDTA-containing tubes (1 mg/ml), and the plasma was separated by centrifugation for 20 min at 1000 *g* and 4°C. The LDL fraction was prepared on the same day by the single vertical spin technique, as described by Chung et al. (8). Plasma was adjusted to a density of 1.30 g/ml by addition of solid KBr, and 1.5 ml of the solution was placed in a 5 ml Quickseal® ultracentrifuge tube (Beckman), overlayered with 0.19 mm NaCl–1.0 mm $Na_2$ EDTA (d 1.006 g/ml), and centrifuged in a Beckman NVT 90 rotor at 90,000 rpm (585,000 *g*) for 75 min at 20°C. The LDL fraction was removed with a syringe and needle, and then dialyzed overnight at 4°C against phosphate-buffered saline (137 mm NaCl, 3 mm KCl, 10 mm phosphate, pH 7.4) (PBS). Samples were stored under nitrogen at 4°C for up to 3 days before use.

### Capillary electrophoresis

Capillary electrophoresis was performed with a Beckman P/ACE 5510 system fitted with a diode array detector, as described previously (9). Fused silica capillaries were cut to 57 cm (50 cm to the detector), and given a 10-min rinse with 1 m NaOH, followed by several 10-min rinses with deionized water and electrophoresis run buffer. The instrument was run in normal polarity (i.e., cathode at the detector end).

The cathode and anode electrolytes and the capillary run buffer were 40 mm methylglucamine–Tricine, pH 9.0. This was freshly prepared before each set of analyses, degassed by sonication, and filtered through a 45-μm (Millipore) filter before use. LDL samples (10–20 μl) were briefly centrifuged in a microfuge, and then placed in microvials and introduced by low pressure (3.8 kPa) for 3–5 sec. The sample volume injected was 10–15 nl (approximately 3–4 ng LDL protein). An electroendosmotic flow (EOF) marker (7 μm dimethylformamide) was injected for 1 sec. A voltage of 20 kV was applied, ramping over 0.5 min. The capillary temperature was maintained at 20°C. The capillary was rinsed between runs with 0.1 m NaOH for 1 min, and then with buffer for 3 min. Migration of LDL particles was monitored by absorption at 200 or 234 nm.

### $Cu^{2+}$-catalyzed oxidation of LDLs

The LDL sample was diluted with PBS to a concentration 250 μg/ml protein, and $CuSO_4$ was added to a final concentration of 10 μm. The LDLs were then incubated at 37°C in a shaking water bath. Aliquots were withdrawn at intervals, and $Na_2$ EDTA was added to a concentration of 10 mm to stop the reaction. For spectrophotometric measurement of conjugated dienes (10), 100 μl of the sample was diluted with 900 μl PBS, and the absorbance at 234 nm was measured. Initial values were subtracted to give the net increase.

### Modification of LDLs with malondialdehyde

Malondialdehyde (MDA) was prepared by hydrolysis of 1,1,3,3-tetramethoxy-propane with 1 m HCl at 37°C for 30 min, neutralized with 1 m NaOH, and the concentration was determined from the extinction coefficient at 245 nm of 14700 $mol^{-1} \cdot cm^{-1}$ (11). MDA was then added to native LDL (250 μg/ml in PBS) in varying concentrations up to 2 mm, and the mixture was incubated at 20°C for 1 h (12).

### Calculation of electrophoretic mobility

The electrophoretic mobility of the LDL (μ) 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 the capillary), according to the formula:

$$\mu = \left[\frac{I}{t} - \frac{I}{t_e}\right] / \frac{V}{L} \quad cm^2 \cdot V^{-1} \cdot s^{-1}$$

where L = length of capillary (cm), I = length of capillary to the detector (cm), V = voltage, t = migration time of LDL (seconds), and $t_e$ = migration time of the EOF marker.

## RESULTS

### Electrophoresis of native LDL

When native LDL particles were electrophoresed in uncoated silica capillaries containing Tricine, Tris-borate, or sodium borate as electrophoresis buffers over the pH range 8–10, no significant peaks were detected, indicating loss of LDL by adsorption to the capillary wall. However, using the same capillaries and a buffer containing the organic base methylglucamine, good peak resolution of native LDL particles could be obtained. The effect of methylglucamine is illustrated in **Fig. 1**. Increasing the concentration of methylglucamine up to 40 mm resulted in marked improvement in peak resolution of LDL, together with an increase in the migration time of the EOF marker. At 40 mm methylglucamine the EOF marker (dimethylformamide) had a migration time of about 6 min, and the LDL had a migration time of 8–9 min. (The electrophoretic migration of LDL is towards the anode and in the opposite direction to that of the EOF marker, but as the electrophoretic mobility of LDL is less than the EOF, it is carried past the detector.)

Data on the precision of the procedure for measure-

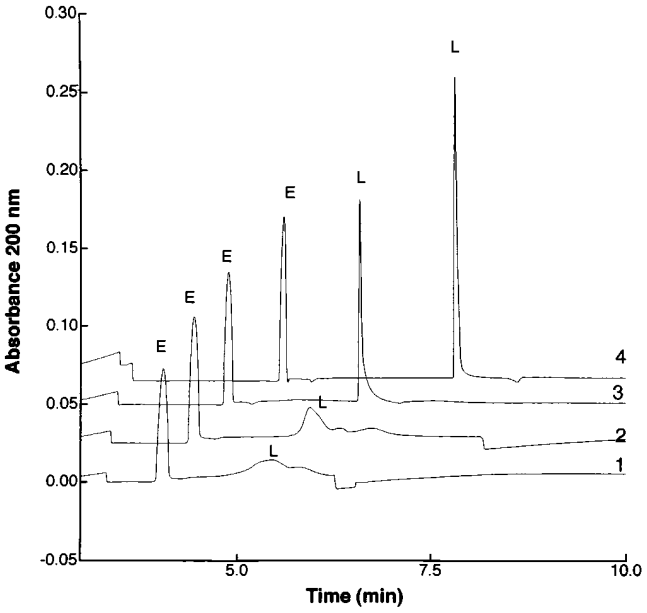


**Fig. 1.** Effect of methylglucamine on the capillary electrophoresis of LDL in uncoated fused silica capillaries. LDL (250 μg/ml) were injected for 3 sec by low pressure into a 57 cm × 75 micron fused silica capillary containing 10 mm (1), 20 mm (2), 30 mm (3), or 40 mm (4) methylglucamine-Tricine buffer, pH 9.0. An EOF marker was also injected (1 sec). Twenty kV (350 V/cm) were applied, and the migration of the EOF marker (E) and the LDL (L) past the detector was monitored at 200 nm.

TABLE 1. Precision of the measurement the electrophoretic mobility of LDL by capillary electrophoresis

| | Mean ± SD | CV (%) |
|---|---|---|
| EOF migration time (s) | 364.6 ± 0.12 | 0.03 |
| LDL migration time (s) | 551.3 ± 2.4 | 0.44 |
| LDL mobility ($cm^2 \cdot V^{-1} \cdot s^{-1} \times 10^{-4}$ | 1.324 ± 0.010 | 0.76 |

LDL was electrophoresed in 57-cm capillaries containing 40 mm methylglucamine buffer at a field strength of 350 V/cm. Migration times of the EOF and LDL peaks (see Fig. 2) were calculated by the system software. Electrophoretic mobility of LDLs was calculated as described in Methods. Results are from 9 separate injections of the same sample over 90 min.

ments of the migration time and electrophoretic mobility of native LDL are given in **Table 1**. Reproducibility is illustrated in **Fig. 2**, in which electropherograms from repeated sequential injections of aliquots of the same LDL sample into the same capillary are overlaid. The procedure is also quantitative, LDL peak areas monitored at a wavelength of 200 nm being directly proportional to LDL protein concentration over the range 50–250 μg/ml (**Fig. 3**).

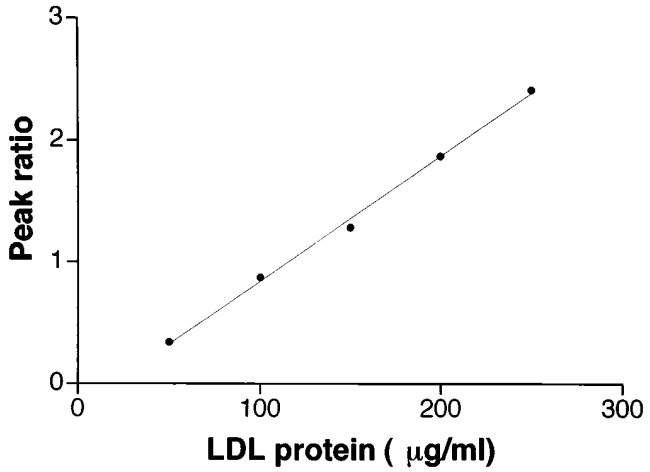


**Fig. 3.** Detector response of capillary electrophoresis of LDLs. LDL at concentrations of 50–250 μg/ml were injected and electrophoresed as in Fig. 2. The areas of the LDL peaks relative to the peak areas of the EOF marker are plotted.

### Changes in LDL electrophoretic mobility during $Cu^{2+}$-catalyzed oxidation

Subjecting LDL to lipid peroxidation by incubation at 37°C in PBS containing 10 μm $Cu^{2+}$ ions resulted in a progressive increase in conjugated dienes, as measured by the increase in absorbance at 234 nm, in accord with previous reports (10). Capillary electrophoresis of aliquots of oxidized LDL sampled over this time period also showed an increase in migration time, indicative of increased electronegativity. Representative electropherograms are shown in **Fig. 4**. The electrophoretic mobility increased from 1.308 to 1.662 × $10^{-4}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$ during a 2-h incubation period. The shift in mobility was detected within 30 min, but the overall change in mobility lagged behind the increase in diene conjugation. During the later stages of oxidation there was significant broadening of LDL peaks, presumably due to the breakdown of the particles. Peak resolution of highly oxidized LDL was not improved by increasing the methylglucamine concentration.

To follow the changes in both electrophoretic mobility and diene conjugates concurrently, electrophoresis was also monitored at 234 nm. At this wavelength there was an increase in peak area in addition to the change in mobility. The increase in peak area measured by the diode array detector paralleled that in diene conjugation measured spectrophotometrically (10). The associations between

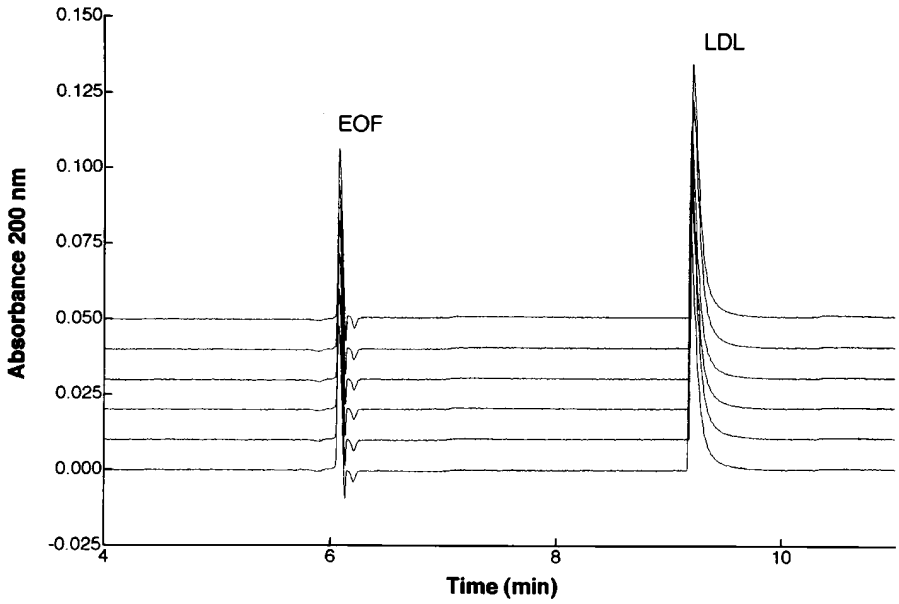


**Fig. 2.** Reproducibility of capillary electrophoresis of LDL. LDL (250 μg/ml) together with the EOF marker were injected into a 57-cm capillary filled with 40 mm methylglucamine-Tricine buffer as described in Fig. 1. Electropherograms from 6 separate injections of the same LDL preparation into the same capillary over 70 min are overlaid.

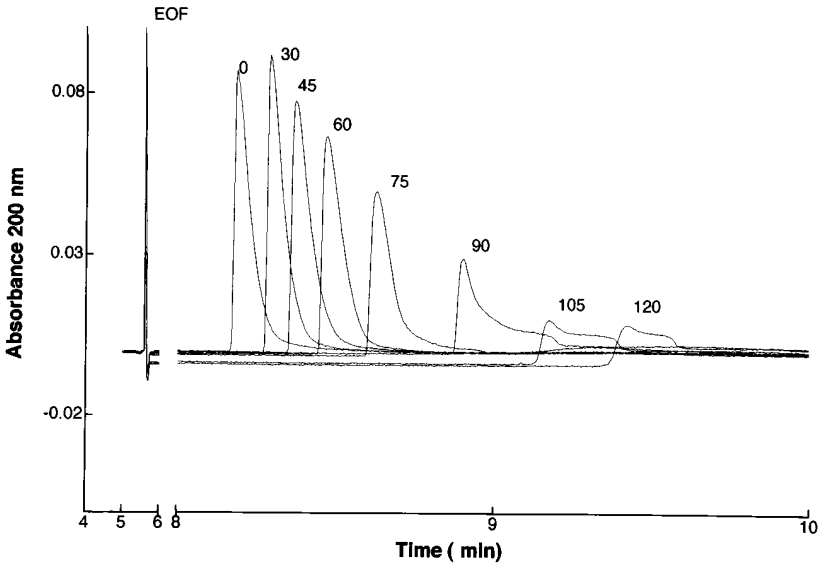


**Fig. 4.** Monitoring changes in the electrophoretic mobility of LDLs by capillary electrophoresis during $Cu^{2+}$-catalyzed oxidation. LDL (250 μg/ml) were incubated with 10 μm $Cu^{2+}$ ions for up to 2 h at 37°C, as described in Methods. Aliquots were withdrawn at 15-min intervals, $Na_2$ EDTA was added, and the samples were electrophoresed as described in Fig. 2. The number adjacent to each peak gives the timing of the aliquot in minutes.

the change in electrophoretic mobility, the increase in diene conjugation measured by capillary electrophoresis, and the increase in diene conjugation measured spectrophotometrically are shown in **Fig. 5**.

Electronegative LDL prepared by modification with MDA also showed an increase in migration time that was proportional to MDA concentration (**Fig. 6**).

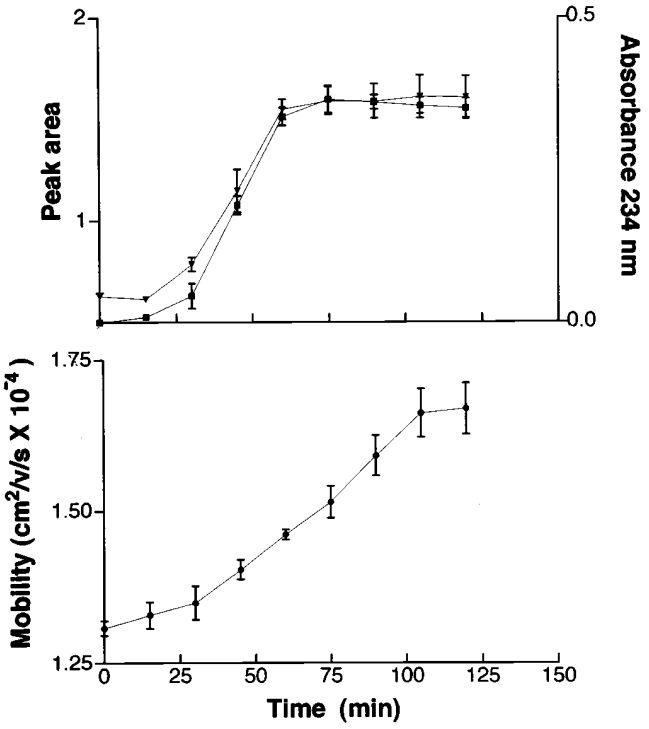


**Fig. 5.** Relationships between changes in the electrophoretic mobility of LDL, absorption at 234 nm measured by capillary electrophoresis, and the formation of conjugated dienes measured spectrophotometrically. Upper panel: diene conjugation was measured by spectrophotometry (■) (8). The increase in absorbance at 234 nm by LDL particles separated by capillary electrophoresis (▼) was followed by determining the areas of the LDL peaks at that wavelength. Lower panel: LDL electrophoretic mobility by capillary electrophoresis (●) was calculated as described in Methods. Results are means ± SD of three experiments. Experimental details were as in Fig. 4.

## DISCUSSION

Recent reports have demonstrated that apolipoproteins (apos) can be separated and quantified by capillary electrophoresis under denaturing conditions, and have described the loss of human serum lipoproteins during capillary electrophoresis, probably by adsorption to the

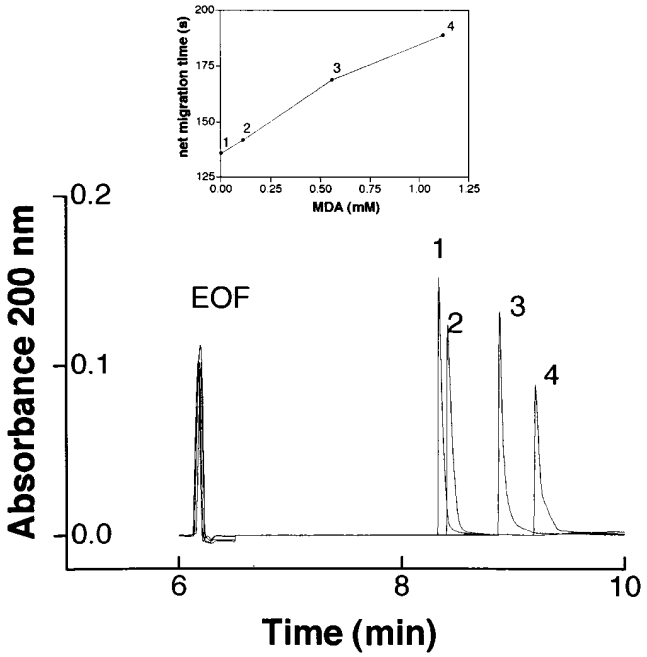


**Fig. 6.** Changes in the electrophoretic mobility of MDA-modified LDL. LDL (250 μg/ml) were incubated for 1 h at room temperature in the absence of MDA (1) or in the presence of MDA at concentrations of 0.11 mm (2), 0.56 mm (3), or 1.10 mm (4), and then separated by capillary electrophoresis in a 57-cm capillary as described in Methods.

capillary wall (9, 13, 14). Hu et al. (15) have demonstrated the separation of LDL apoB and Lp[a] using uncoated capillaries and a denaturing sodium dodecyl sulfate buffer. The present report is the first to describe quantitative capillary zone electrophoresis of intact lipoproteins using fused silica capillaries. Dolnik (16) reported that buffers containing methylglucamine gave the best resolution of human serum proteins in capillary zone electrophoresis. The marked improvement that we found in the peak resolution and recovery of LDL using methylglucamine–Tricine buffer was probably due to shielding of proteins from interaction with the silanol groups on the capillary wall (17).

The procedure described here provides a rapid and sensitive means of measuring the electrophoretic mobility of LDL with a precision (coefficient of variation) of less than 1%. As this is free solution electrophoresis, there is a minimal matrix effect, and the effect of EOF can be directly corrected. Thus, the technique gives absolute electrophoretic mobility, in contrast to slab gels systems which do not correct for EOF or gel retardation effects.

The increase in the electronegativity of LDL that occurs as a consequence of oxidative damage has been well documented (1). Although cleavage of the apoB protein chain occurs in the later stages, the formation of more electronegative LDL results primarily from the loss of positive surface charge. This is due mainly to the reaction of ϵ-amino groups of apoB lysine residues with aldehydic breakdown products of lipid peroxides, such as MDA and 4-hydroxynonenal, to form stable Schiff bases (18), though modification of other amino acid residues may also contribute (19, 20). The progressive increase in the electronegativity of LDL particles that occurs during $Cu^{2+}$-catalyzed autoxidation of LDLs, or during reaction with MDA, can be readily monitored by capillary zone electrophoresis under the conditions described here. Furthermore, the spectral changes [increase in absorption at 234 nm due to the formation of oxidation products of fatty acids and cholesterol (1, 5, 20)] can be monitored simultaneously, and parallel the increase in absorption measured spectrophotometrically. This should be advantageous in situations where various compounds added to inhibit or promote oxidation also contribute significantly to UV absorption, as they can be separated from the LDL particles during electrophoresis. The technique may also be applicable to the investigation of changes in LDL that occur in vivo. Electronegative forms of LDL have been reported in human plasma (21), and LDL showing alterations in size and electronegativity relative to plasma LDL have recovered from aortic lesions of humans (22) and Watanabe rabbits (23).

This work was supported by the British Heart Foundation.

*Manuscript received 10 December 1997 and in revised form 25 February 1998.*

## REFERENCES

1. Esterbauer, H., J. Gebicki, H. Puhl, and G. Jurgens. 1992. The role of lipid-peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **13:** 341–390.
2. Witztum, J. L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88:** 1785–1792.
3. Halliwell, B. 1995. Oxidation of low-density lipoproteins—Questions of initiation, propagation, and the effect of antioxidants. *Am. J. Clin. Nutr.* **61:** S670–S677.
4. Biffanti, S., A. Bonanome, M. Maiorino, F. Ursini, and A. Pagnan. 1994. Assay of low-density-lipoprotein susceptibility to oxidation by means of the free-radical generator AAPH. *Nutr. Metab. Cardiovasc. Dis.* **4:** 137–141.
5. Pinchuk, I., and D. Lichtenberg. 1996. Continuous monitoring of intermediales and final products of oxidation of low density lipoprotein by means of UV-spectroscopy. *Free Radical Res.* **24:** 351–360.
6. Rice-Evans, C., D. Leake, K. R. Bruckdorfer, and A. T. Diplock. 1996. Practical approaches to low density lipoprotein oxidation—whys, wherefores and pitfalls. *Free Radical Res.* **25:** 285–311.
7. Altria, K. D. 1996. Capillary Electrophoresis Guidebook. Methods in Molecular Biology. Vol. 52. Humana Press, Totowa, NJ. 3–97.
8. Chung, B. H., J. P. Segrest, M. J. Ray, J. D. Brunzell, J. E. Hokanson, R. M. Krauss, K. Beaudrie, and J. T. Cone. 1986. Single vertical spin density gradient ultracentrifugation. *Methods Enzymol.* **128:** 181–209.
9. Stocks, J., M. N. Nanjee, and N. E. Miller. 1998. Analysis of high density lipoprotein apolipoproteins by capillary zone and capillary SDS gel electrophoresis. *J. Lipid Res.* **39:** 218–227.
10. Puhl, H., G. Waeg, and H. Esterbauer. 1994. Methods to determine oxidation of low-density lipoproteins. *Methods Enzymol.* **233:** 425–441.
11. Esterbauer, H., R. J. Schaur, and H. Zollner. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biol. Med.* **11:** 81–128.
12. Haberland, M. E., C. L. Olch, and A. M. Fogelman. 1984. Role of lysines in mediating interaction of modified low density lipoproteins with the scavenger receptor of human monocyte macrophages. *J. Biol. Chem.* **259:** 11305–11311.
13. Goux, A., L. Athias, L. Persegol, L. Lagrost, P. Gambert, and C. Lallemant. 1994. Capillary gel electrophoresis analysis of apolipoproteins A-I and A-II in human high density lipoproteins. *Anal. Biochem.* **2188:** 320–324.
14. Proctor, S. D., and J. C. L. Mamo. 1997. Separation and quantitation of apolipoprotein B-48 and other apolipoproteins by dynamic sieving capillary electrophoresis. *J. Lipid Res.* **38:** 410–414.
15. Hu, A. Z., I. D. Cruzado, J. W. Hill, C. J. McNeal, and R. D. Macfarlane. 1995. Characterization of lipoprotein[a] by capillary zone electrophoresis. *J. Chromatogr. A.* **717:** 33–39.
16. Dolnik, V. 1995. Capillary zone electrophoresis of serum proteins: a study of separation variables. *J. Chromatogr. A.* **709:** 99–110.
17. Klausen, N. K., and T. Kornfelt. 1995. Analysis of the glycoforms of human recombinant factor VIIa by capillary electrophoresis and high performance liquid chromatography. *J. Chromatogr. A.* **718:** 195–202.
18. Jurgens, G., H. F. Hoff, G. M. Chisolm, and H. Esterbauer. 1987. Modification of human serum low density lipoprotein by oxidation-characterisation and pathophysiological implications. *Chem. Phys. Lipids.* **45:** 315–336.
19. Giessauf A., B. van Wickern, T. Simat, H. Steinhart, and H. Esterbauer. 1996. Formation of N-formylkynurenine suggests the involvement of apolipoprotein B-100 centered tryptophan radicals in the initiation of LDL lipid peroxidation. *FEBS Lett.* **389:** 136–140.
20. Chang, Y. H., D. S. Abdalla, and A. Sevanian. 1997. Characterization of cholesterol oxidation products formed by oxidative modification of low density lipoprotein. *Free Radical Biol. Med.* **23:** 202–214.
21. Cazzolato, G., P. Avogaro, and G. Bittolo-Bon. 1991. Characterization of a more electronegatively charged LDL subfraction by ion-exchange HPLC. *Free Radical Biol. Med.* **11:** 247–253.
22. Camejo, G., E. Hurt, and M. Romano. 1985. Properties of lipoprotein complexes isolated by affinity chromatography from human aorta. *Biomed. Biochem. Acta.* **44:** 389–401.
23. Daugherty, A., B. S. Zweifel, B. Sobel, and G. Schonfield. 1988. Isolation of low density lipoprotein from atherosclerotic vascular tissue of Watanabe heritable hyperlipidemic rabbits. *Arteriosclerosis.* **8:** 768–777.