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ARTICLE

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A short-run new analytical ultracentrifugal micromethod for determining low-density lipoprotein sub-fractions using Schlieren refractometry

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Abstract We have developed a new analytical ultracentrifugal micromethod for the determination of serum low-density lipoprotein (LDL) subclasses directly from ultracentrifugal Schlieren scans. We have used special software for the analysis of this type of single-spin density-gradient ultracentrifugation. The flotation of LDL patterns was obtained by underlayering a physiological salt solution with serum or isolated lipoprotein fractions raised to a density of 1.3 g/mL in the spinning ultracentrifugation capillary band-forming cell. The repeated analysis of Schlieren curves of the same sample from 10 to 100 μL in the 60–100 min full-speed interval time resulted in quite reproducible results. We obtained quantitative results by measuring the Schlieren areas between the sample curves and the reference baseline curve by using computerised numerical and graphic techniques. The decomposition of the integrated curve was carried out using a nonlinear regression program followed by deconvolution algorithm analysis in order to determine the parameters of the composing Gaussian subclasses. The LDL particle concentrations were calculated from the area under the integral of the Gaussian curve using a calibration data constant. The flotation range of the LDL Schlieren curves in the cell was identified with serum from which LDL had been removed by means of precipitation reagents and with centrifugation of isolated LDL aliquots. With this technique, we measured the concentration of LDL and analysed its polydispersity without the need for preceding sequential isolation of the LDL. On the basis of the Schlieren curves, the LDL samples were either physically polydisperse, having a symmetrical peak within a narrow density range, or were polydisperse, showing an asymmetrical pattern distributed over a broader density region. The described method proved to be useful for a clear and immediate visual presentation of the concentration values of the LDL and for the identification of the heterogeneity of LDL variants without the need for the preparative isolation of that density class.

Key words Analytical ultracentrifugation · Single-spin density-gradient ultracentrifugation · Low-density lipoproteins · Heterogeneity

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Introduction

Plasma lipoproteins are classified on the basis of their density, electrophoretic mobility, and relative lipid and protein content (Lindgren et al. 1972). Three major classes of lipoproteins are found in the blood of a fasting individual: low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL), and high-density lipoproteins (HDL). The LDL typically contain about 60–70% of the total serum cholesterol and both are directly correlated with risk of coronary heart disease (CHD). The HDL normally contain about 20–30% of the total cholesterol, and HDL levels are inversely correlated with CHD risk. The VLDL contain 10–15% of the total serum cholesterol along with most of the triglyceride in fasting serum; VLDL are precursors of LDL, and some forms of VLDL, particularly VLDL remnants, appear to be atherogenic. CHD is the single largest cause of death and the primary contributing factor to CHD is an elevated blood cholesterol level. Since most cholesterol in serum is contained in LDL, the concentration of total cholesterol in most people is highly correlated with the concentration of LDL-cholesterol. Whereas LDL-cholesterol is the major atherogenic lipoprotein and thus is the primary target of cholesterol-lowering efforts, total cholesterol can be used in initial testing for detecting a possible elevation of LDL-cholesterol. LDL-cholesterol

offers more precision for risk assessment and is the primary target of interventions to lower blood cholesterol (Miller et al. 1981; Austin et al. 1990; Genest et al. 1992). The National Cholesterol Education Program (1991) suggests the employment of a direct LDL-cholesterol test for the more accurate assessment and management of patients at risk of CHD. In common terms, high levels of cholesterol, particularly LDL-cholesterol (levels of LDL-cholesterol of 160 mg/dL or greater are classified as "high-risk LDL-cholesterol," those of 130–159 mg/dL as "borderline high-risk LDL-cholesterol," and those <130 mg/dL as "desirable LDL-cholesterol"), are implicated in a vicious process in which blood flow is restricted by cholesterol-related plaque in the vessels and becomes a major component of atherosclerotic plaque lesions. Conversely, HDL-cholesterol may help decrease excess cholesterol from the blood and facilitate regression of plaque, resulting in improved blood flow to vital organs. Genetic predisposition and the ratio of LDL-cholesterol to HDL-cholesterol determines who will develop CHD. A ratio called the cardiac risk factor is obtained by dividing the HDL-cholesterol level into the total cholesterol . The goal is to keep the ratio below 5:1; the optimum ratio is 3.5:1. Recent advances in understanding the metabolism of lipoproteins by the artery wall have yielded new insight into the factors that may be involved in the arterial response. Specifically, certain modifications in the structure of lipoproteins appear to affect their atherogenic potential. The cytotoxicity of LDL seems to result from lipoprotein oxidation. The peroxidation products are bound to LDL and thus modify the particle in a way that decreases the binding and uptake by the LDL receptor. This modified LDL becomes recognised by the scavenger receptor of macrophages with a concomitant accumulation of cholesterol in these cells (Brown and Goldstein 1986; Austin et al. 1988). The denser LDL subfractions had the lowest lag time and the highest rate of oxidation. This indicates that the denser LDL particles are significantly more susceptible to oxidation (Fischer 1983). The density limits of LDL are determined to be between 1.020 and 1.063 g/mL on the basis of ultracentrifugation. Human LDL are now recognised to be heterogeneous macromolecules varying in physicochemical properties, reflecting differences in lipoprotein metabolism and in oxidative susceptibility (Crouse et al. 1985; Swinkels et al. 1989).The heterogeneity of LDL can be demonstrated by various methods, for example analytical ultracentrifugation (Lindgren et al. 1969), density-gradient ultracentrifugation (Swinkels et al. 1987), and gradient gel electrophoresis (McNamara et al. 1987). Density-gradient ultracentrifugation with visualisation of the sub-fractions after staining and gradient gel electrophoresis are the most commonly used methods for analysing LDL heterogeneity. However, the staining procedure in the first method and the lengthy electrophophoresis in the second method may induce denaturation and give rise to artefacts. The standard analytical ultracentrifugation method provides a reliable non-denaturing means for analysing LDL heterogeneity. However, this method also has a drawback because it requires the pre-isolation of lipoproteins by sequential ultracentrifugation using 2–3 runs of 20 h. Stepwise preparative ultracentrifugation, of course, will subject the sample to very long ultracentrifugation times and to exposure of centrifugal force. Computer analysis of both the total LDL and HDL is difficult in many cases owing to corrections, such as flotation versus concentration dependence, the Johnston-Ogston effect, and correction of standard conditions, temperature, density, and the graphic presentation of lipoprotein Schlieren data to gain insight of the profile allowing comparison of serum lipoprotein concentration. It may also be at least partially due to technical difficulties in accurate quantitation of a spectrum of highly heterogeneous particles by sequential flotation ultracentrifugation (Lindgren et al. 1969).

In this paper we describe a single-spin density-gradient analytical ultracentrifugation micro-method for the separation and visualisation of LDL patterns that needs 10–100 μL of sample volume and 100 min of full-speed ultracentrifugation. After validation of its reliability, we studied LDL sub-fraction patterns in serum pools and in individual sera of normal and CHD patients. We think that this simple method may be used in many fields of lipoprotein research.

Materials and methods

Samples

Blood was sampled in collection tubes from healthy subjects ($N=20$) and from CHD patients ($N=30$) after they had fasted overnight. Sera were separated after 40 min clothing time by centrifugation at 1200*g* and 20 °C for 30 min and stored at 4 °C after adding 0.1 g of sodium ethylenediamintetraacetate per litre. In the group of healthy subjects, no-one had a history of myocardial infarction, coronary artery bypass surgery, or angina pectoris. No patient was diagnosed as having gastrointestinal disease, liver function abnormalities, endocrine disorders, or diabetes mellitus. Another group consisted of patients with documented coronary artery disease. All patients had clinical signs or symptoms of ischemic heart disease and coronary angiographs were taken. These patients were reported to have more than 50% diameter-narrowing of one or two major coronary arteries. The characteristics of the population studied are given in Table 1.

Table 1 Characteristics of the population studied

Normal subjects ($N=20$) (men = 12, women = 8)	
Age (years)	41 ± 8
Serum cholesterol	4.17 ± 0.72 mmol/L
Serum triglycerides	1.29 ± 0.81 mmol/L
HDL cholesterol	1.06 ± 0.32 mmol/L
CHD patients ($N=30$) (men = 24, women = 6)	
Age (years)	48 ± 12
Serum cholesterol	4.56 ± 0.92 mmol/L
Serum triglycerides	2.72 ± 0.98 mmol/L
HDL cholesterol	0.86 ± 0.35 mmol/L

Single-spin density-gradient ultracentrifugation using Schlieren refractometry

The runs were carried out in an A65-2 analytical rotor with a type 3180 ultracentrifuge made by the Hungarian Optical Works (Budapest) operating in refractometric Schlieren mode at 546 nm with a high-pressure mercury vapour light source lamp (HBO2000). We applied band-forming, capillary under-layering type single-sector centrepieces furnished with special holes. The 4° band-forming and 12-mm optical path length centrepieces were assembled with negative angle wedge windows (–1°40′) on the bottom of the cell. The setting of the optical system was selected at a constant Philpot angle of 20°. Solid KBr was used to adjust the density of sera or other pre-isolated lipoprotein fractions (obtained by a previous preparative ultracentrifugation isolation step or by precipitation). The samples were adjusted to a density of 1.3 g/mL (0.49 g KBr/mL serum) and 10–100 μL volumes were injected into the holes of the centrepiece from the upper window with a micro-syringe. The cell was assembled and 0.5 mL NaCl ($\rho = 1.006$ g/mL) solution containing 0.1 g/L EDTA was put into the sector of the centrepiece. Runs were performed at 50,000 r.p.m. and 20 °C. When the rotor was accelerated to 2000–4000 r.p.m., the increasing hydrostatic pressure forced the adjusted-density sample from the holes of the centrepiece through capillaries under the physiological solution in the sector. The maximum speed was obtained after 5 min. Photographs of the Schlieren pattern were taken at 10 min intervals from the time that the gradient curves were well visible, i.e. at 60, 70, 80, 90, and 100 min after reaching full speed. The decomposition of the integrated curve was carried out using the BMDP non-linear regression program (Dixin 1981), followed by deconvolution algorithm analysis in order to determine the parameters of the Gaussian subclasses (Medgyessy 1977). A personal computer, computer graphics, and a procedure for minimisation of the sums of non-linear squares were applied as follows. The coordinates of the Schlieren distribution curves of the samples were obtained from the photograph, input into the computer, interpolated using piecewise cubic polynomials, and displayed. The interpolation of the Schlieren distribution curves of the gradient baseline was found in the same way, the curves were subtracted, and the result was integrated exactly for the ordinates of all pixels. Then, this integral curve was displayed on the screen and one Gaussian curve was adapted to its main peak, guessing and inputting the three parameters of the Gaussian (mean value, standard deviation, height of maximum). Next, the minimisation procedure was invoked to improve the guessed parameters. During this computation, only the ordinates of the pixels near the main peak were taken into account. The integral curve was displayed once more, now together with the optimised Gaussian. At the place of greatest difference, a new Gaussian was added, and was adapted by hand. Then, the height of the first Gaussian was reduced somewhat and the optimisation procedure was invoked again, but now the ordinates of all pixels (or of every third pixel) on the x-axis were included in the optimisation. Only a short part of the axis beginning with $x = 0$ was excluded, since the integral curve starts with zero at $x = 0$ and it makes no sense to try to approximate this by a sum of Gaussian curves. In this way the number of Gaussian curves in the sum was raised step by step until the average deviation between the two curves dropped below 0.25. This is about the error with which values of the original curves can be read from the Schlieren photographs. For the success of the method, all three parts of our approach (the stepwise addition of the Gaussian curves, the hand adaptation of the Gaussian curves, and the reduction of the heights of the old Gaussians) were important. The optimisation procedure is specialised to minimise the sums of squares, i.e. in our case:

$$F(\alpha_1, \beta_1, h_1, \ldots, \alpha_N, \beta_N, h_N) = \sum_X \left[y(x) - \sum_{i=1}^{N} h_i e^{-(X-a_i)2/4\beta_i} \right]^2 \qquad (1)$$

where α is the mean value, β is the standard deviation, h is the height of the maximum, h_i is the maximum value of the function, a_i is the place of the maximum value, and β_i is the deviation. Here the summation is taken over all x-values (ordinates of pixels on the screen) and $y = y(x)$ is the integral curve. The optimisation procedure employs gradient vectors (obtained by a finite difference formula) and Gaussian-Newtonian vectors, which are used to define a plane on which the fuctional F is minimised locally. The minimum value of F is called the fitting error.

The LDL particle concentrations were calculated from the area under the integral of the Gaussian curve using a calibration data constant and from these the LDL cholesterol values (Adams and Schumaker 1970) were derived.

Other methods

The preparative isolation of serum lipoproteins was carried out by the single-spin density-gradient ultracentrifugation technique of Chung et al. (1980). LDL was isolated as a faint yellow band in the middle of the tubes, ascertained by measuring cholesterol and triglyceride contents in 0.2-mL fractions isolated from the top to the bottom of the tube by aspiration with a Pasteur pipette. Cholesterol and triglyceride values were determined by enzymatic methods (Burstein and Savaille 1960; Bucolo and David 1973). Heparin and immunoprecipitation reagents were used to control the LDL flotation. Aliquots of supernatant and infranatant serum were taken for Schlieren analysis after adjusting the density of the solutions with solid KBr to 1.30 g/L and underlayering by physiological solution, and the lipoprotein distribution was investigated using Schlieren optics. The divergences in the Schlieren distribution profiles of the lipoproteins were used to follow the interaction of LDL and precipitation reagents. Besides Schlieren optics, ultraviolet absorption optics were used at 254 nm to record the flotation of the LDL band in the cell at the same time, and calibration runs were made with 10–100 μL sera. The measurement of absorbance allows increased sensitivity and high reproducibility. With the absorption optics, the absolute concentration is available at any point rather than a concentration difference with respect to a reference point. Absorption optics are particularly sensitive for detection of macromolecules containing strong chromophores and the strong lamp output at 254 nm can be used to characterise lipoproteins with good signal-to-noise ratios at concentrations as low as 10 μg/mL (Mächtle 1992).

Results and discussion

Differences in the Schlieren diagrams of the serum samples

In order to optimise the method, Schlieren patterns were registered from various aliquots from 10 to 100 μL after reaching full speed. In samples with elevated VLDL concentrations, a small peak or shoulder was observed in the gradient curve corresponding to the density value of floating VLDL in the early phase of the run up to 20 min. Afterwards, the LDL peak emerged in the central region of the cell, whereas the VLDL and or intermediate-density lipoproteins were then compressed against the surface of the sample solution by centrifugation. The LDL peak on the Schlieren diagram was optimum at 80 min after full-speed centrifugation and became clear from the sequential every 10-min photographs between 60 and 100 min. During the 60–100 min interval the Schlieren pattern of the gradient baseline became broader and level (Fig. 1A). A similar pattern was obtained with LDL-depleted serum (Fig. 1B). The Schlieren patterns obtained for serum pools from healthy subjects and from CHD patients are also shown (Fig. 1C, D). A graphical presentation of the evaluation of a Schlieren curve ob-

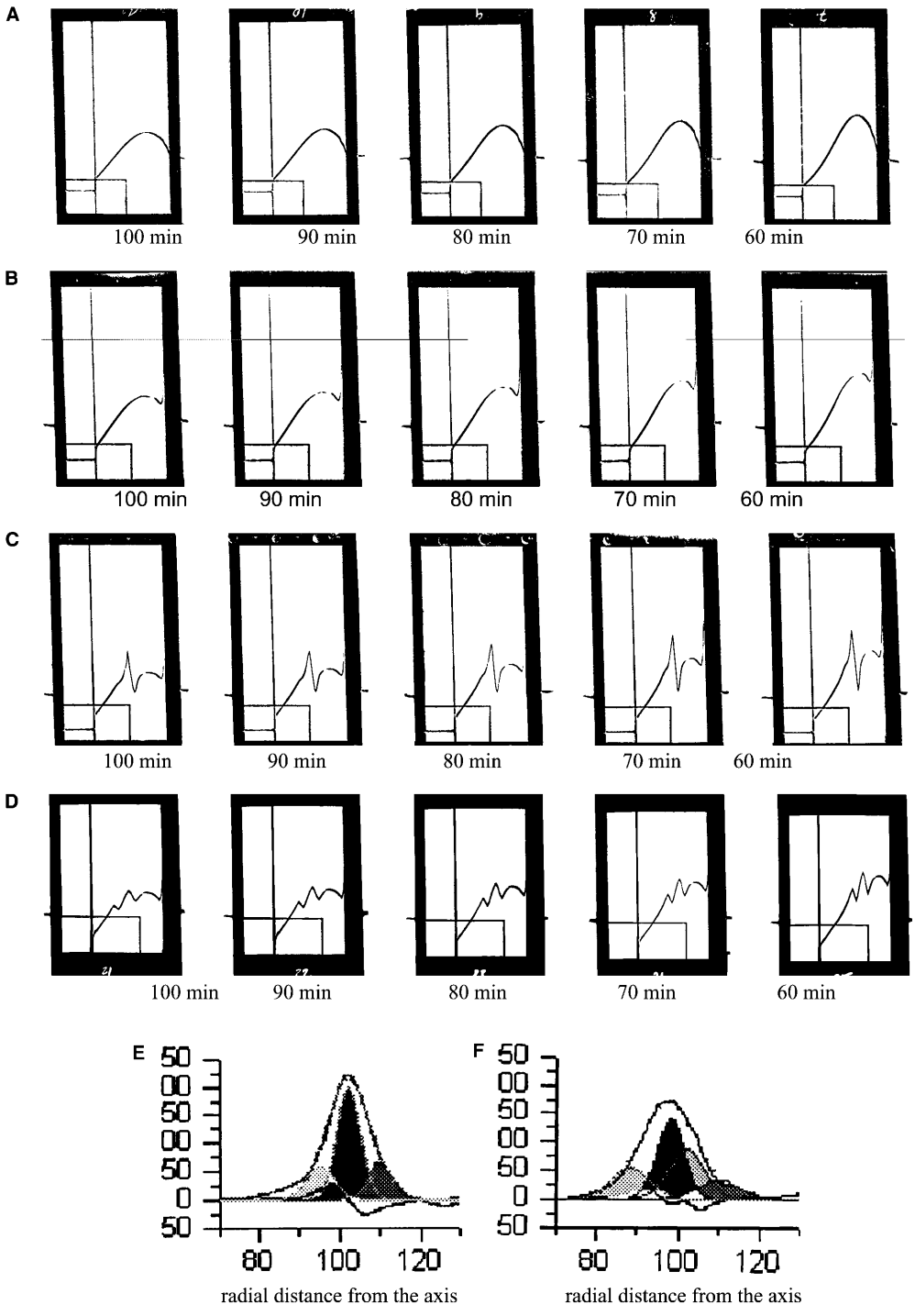

Fig. 1 **A** Schlieren diagrams of density baseline. **B** Schlieren diagrams without low-density lipoprotein distribution; LDL was precipitated with anti-apo-B immunoprecipitating reagent. **C** Schlieren diagrams for the serum pool from healthy subjects. **D** Schlieren diagrams for the serum pool from coronary heart disease patients. **E** LDL concentration according to Y values from healthy subjects' sera ($N=20$) pool. **F** LDL concentration according to Y values from CHD patients' sera ($N=30$) pool. Integration and deconvolution of the measured Schlieren curve to obtain the mass of the different LDL fractions from a Gaussian curve

tained for the serum pool from healthy subjects is shown in Fig. 1E. Figure 1F shows the graphical presentation of the evaluation of a Schlieren curve obtained for the serum pool from CHD patients.

De-convolution analysis of the LDL Schlieren patterns

Using two serum pools, one from healthy subjects' sera ($N=20$) and one from CHD patients' sera ($N=30$), we studied LDL heterogeneity. A characteristic of the flotation pattern is the boundary in the analytical cell, a region in which the concentration varies according to the density baseline from the bottom. Cell scans of LDL were detected at 80 min after reaching full speed (50,000 r.p.m.), utilising previously determined radial positions of LDL class limits after precipitation of LDL with immune and polyanion precipitation reagents as well as by using ultraviolet absorption optics. The refractometric scans of the slides were taken from the

photographs followed by computerised mathematical modelling. We prepared an integrated density curve with correction for the solvent baseline and Gaussian curves were determined by de-convolution analysis. LDL concentrations were calculated from the area under the integral of the Gaussian curve using the calibration data constant. De-convolution analysis of these integrated density curves revealed the presence of three fractions in the healthy subjects' pool (Fig. 1E) and of four fractions in the CHD patients' pool (Fig. 1F). The measured concentrations of LDL sub-fractions are summarised in Table 2.

Reproducibility of the method

When the same sample was analysed in different runs, the Schlieren diagrams of the LDL distribution registered at the 60–100-min intervals of both runs were indistinguishable for the same sample. The Schlieren shapes were identical for all duplicates.

We have estimated the repeatability of the method on the values of standard deviation. Independent t-tests on the data were made (mean: 334 and 335.5, variance: 10675.77 and 10966.94) on the basis of 10 paired t-tests. Thus the computed LDL concentration values in the repeated analysis were within ± 3 CV%. The Schlieren diagrams of the same sample with 50 and 100 μL in duplicate are shown in Fig. 2A and B.

Schlieren diagrams of the individual serum samples

A multiple peak or a polydisperse LDL pattern within the Schlieren samples' curves of LDL was more often found in samples from CHD patients than in controls. In Figs. 3, 4, 5, Schlieren scans of CHD subjects with predominantly nonsymmetrical peaks are shown with the mass of the lipoprotein distributed over a broad density region. Four LDL sub-fractions were observed according to the Gaussian analysis. The concentrations of the LDL sub-fractions are summarised in Tables 3, 4, 5.

Schlieren scans of normal subjects show a predominantly symmetrical peak, where the mass of the lipo-

Table 2 Cholesterol concentrations of the LDL sub-fractions in the pool of normal subjects and of CHD patients

Fraction	Macromolecule concentration (mg/dL)	Cholesterol concentration (mmol/L)
Normolipidemic sample pool ($N=20$)[a]		
1	53.50	0.56
2	120.45	1.31
3	57.65	0.60
Total	239.92	2.61
CHD patients' sample pool ($N=30$)[b]		
1	37.74	0.48
2	88.64	0.96
3	32.10	0.35
4	72.27	0.78
Total	227.24	2.47

[a] In the mathematical evaluation the number of squares is 143, the fitting error is 8.347, and the average deviation is 0.07

[b] In the mathematical evaluation the number of squares is 143, the fitting error is 8.344, and the average deviation is 0.24

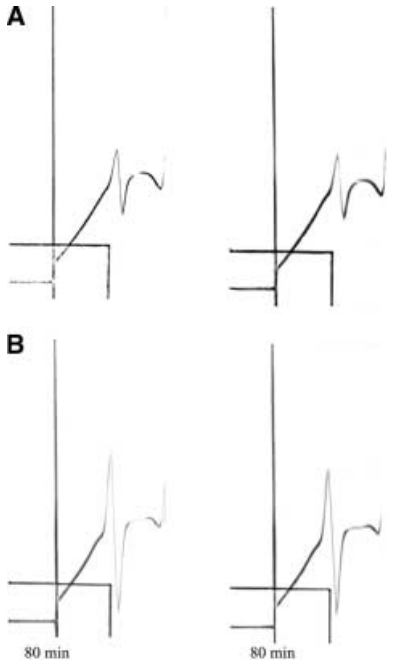

Fig. 2 **A** Sample with 50 μL in duplicate at 80 min after reaching full speed. **B** Sample with 100 μL in duplicate at 80 min after reaching full speed

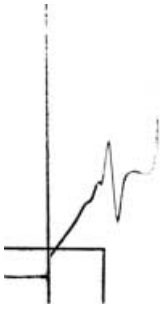

Fig. 3 LDL distribution from a CHD patient at 80 min after full speed obtained from sample 4 sub-fractions

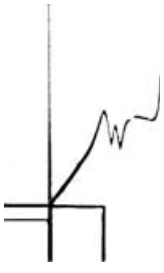

Fig. 4 LDL distribution from CHD samples at 80 min after full speed obtained from sample 4 sub-fractions

Fig. 5 LDL distribution from CHD samples at 80 min after full speed obtained from sample 4 sub-fractions

Table 3 Cholesterol concentrations of the LDL sub-fractions;[a] see Fig. 3

Fraction	Macromolecule concentration (mg/dL)	Cholesterol concentration (mmol/L)
1	33.93	0.37
2	64.24	0.70
3	149.38	1.62
4	24.09	0.26
Total	271.62	2.97

[a] In the mathematical evaluation the number of squares is 162, the fitting error is 7.347, and the average deviation is 0.11

Table 4 Cholesterol concentrations of the LDL sub-fractions;[a] see Fig. 4

Fraction	Macromolecule concentration (mg/dL)	Cholesterol concentration (mmol/L)
1	27.29	0.30
2	92.27	1.06
3	28.26	0.31
4	65.85	0.71
Total	215.90	2.35

[a] In the mathematical evaluation the number of squares is 143, the fitting error is 8.347, and the average deviation is 0.07

proteins is distributed over a smaller density region (see Fig. 6). Three LDL sub-fractions were observed according to the Gaussian analysis. The concentrations of the LDL sub-fractions are summarised in Table 6.

Conclusion

In this report we described a short-run analytical ultracentrifugation method for quantification of the total LDL content and of its sub-fractions from whole serum within 100 min. No pre-isolation of lipoproteins, a time-consuming step, is needed. Because of its rapidity and convenience, lipoproteins probably remain fully native. In the analysis of samples the method appeared to be reproducible. Certainly this method is not suitable for routine analysis. Rather, it can be regarded as a reference method because the risk of denaturing the sample is minimal compared to the commonly used

Fig. 6 LDL distribution in a normolipidemic sample at 80 min after full speed obtain from sample 3 sub-fractions

Table 5 Cholesterol concentrations of the LDL sub-fractions;[a] see Fig. 5

Fraction	Macromolecule concentration (mg/dL)	Cholesterol concentration (mmol/L)
1	35.82	0.39
2	48.17	0.52
3	40.26	0.43
4	281.13	3.06
Total	405.56	4.42

[a] In the mathematical evaluation the number of squares is 161, the fitting error is 7.23, and the average deviation is 0.19

Table 6 Cholesterol concentrations of the consisting LDL sub-fractions;[a] see Fig. 6

Fraction	Macromolecule concentration (mg/dL)	Cholesterol concentration (mmol/L)
1	31.70	0.35
2	131.32	1.45
3	48.10	0.52
Total	211.12	2.35

[a] In the mathematical evaluation the number of squares is 134, the fitting error is 2.431, and the average deviation is 0.13

methods involving density-gradient ultracentrifugation or gradient-gel electrophoresis methods. In normolipidemic serum, three main LDL sub-fractions could usually be identified. According to other researchers, for example, Swinkels et al. (1987) and Fischer (1983), the number of LDL sub-fractions showed a good agreement with our results. The described method proved to be useful for a clear and immediate visual presentation of the concentration values of LDL and for the identification of the heterogeneity of LDL variants without the need for the preparative isolation of that density class. LDL Schlieren curves were more often characterised by a preponderance of less- and more-dense sub-fractions in samples of patients with documented coronary artery disease than in controls.

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