Single spin density gradient ultracentrifugation method for the detection and isolation of light and heavy low density lipoprotein subfractions

Dorine W. Swinkels,[1] Heidi L. M. Hak-Lemmers, and Pierre N. M. Demacker

Department of Medicine, Division of General Internal Medicine, Sint Radboud Hospital, University of Nijmegen, Nijmegen, The Netherlands

Summary A single spin density gradient ultracentrifugation method in a swinging bucket rotor has been applied for the detection and isolation of low density lipoprotein (LDL) subfractions. The visualization of the LDL heterogeneity was facilitated by prestaining the serum with Coomassie Brilliant Blue R prior to density gradient ultracentrifugation for 19.5 hr. A total of 13 human serum pools was analyzed. In each pool, two LDL subfractions, a lighter LDL_1 subfraction, occasionally showing a subdivision into two bands, LDL_{1A} and LDL_{1B}, and a heavier LDL_2 could be clearly distinguished by the banding pattern in the density gradient. Physicochemical characteristics of the isolated LDL subfractions were determined. The simple method for detection and isolation of these subfractions presented here may facilitate future studies on LDL heterogeneity.—**Swinkels, D. W., H. L. M. Hak-Lemmers, and P. N. M. Demacker.** Single spin density gradient ultracentrifugation method for the detection and isolation of light and heavy low density lipoprotein subfractions. *J. Lipid Res.* 1987. **28:** 1233—1239.

Supplementary key words LDL heterogeneity • pooled serum • apoproteins • chemical composition

LDL has usually been regarded as a homogeneous entity despite long standing evidence to the contrary (1–3). The existence of different fractions of LDL was suggested in earlier studies (1–3) and recently confirmed in normal subjects (4–7).

To determine the physicochemical characteristics and metabolic behavior of these LDL subclasses in healthy or hyperlipidemic individuals, there is a need for a simple and accurate isolation method for these subfractions. Until now the preparative methods for isolation of LDL subfractions have made use of sequential flotation (2), zonal ultracentrifugation (8), sequential isolation of LDL followed by density gradient ultracentrifugation (3, 5, 9), and the large-scale density gradient subfractionation method as described by Lee and Downs (6). These methods are all time-consuming and based on arbitrary cuts in the density region of LDL, except for the latter which is sensitive enough to detect minor subfractions but requires a large volume of serum and is less suitable for hypertriglyceridemic serum due to wall-adherence effects of the triglyceride-rich particles.

In this report we describe a density gradient method for the isolation of LDL subfractions from 3.4 ml of serum, which is also suitable for hypertriglyceridemic serum, in a single run of 19.5 hr without a preceding sequential isolation of LDL. Identification and isolation of the subfractions is facilitated by staining the serum with Coomassie Brilliant Blue R prior to ultracentrifugation. The physicochemical characteristics of the LDL subfractions isolated from pooled human sera were also determined.

MATERIALS AND METHODS

Sera

Blood was sampled from subjects (18–81 yr old) visiting the outpatient clinic of our hospital, after fasting overnight and within 2 hr after a light breakfast. Under these conditions chylomicrons may be expected to be absent because serum triglycerides are similar to the fasting values (10). Sera with triglycerides higher than 2.5 mmol/l were excluded, as well as sera from patients who used drugs known to affect lipoprotein metabolism or from subjects with diseases causing secondary hyperlipidemia. Sera were isolated within 2 hr. Serum pools were made by pooling the individual sera of 10 to 18 persons. Concentrations of triglycerides, total cholesterol, and HDL-cholesterol in the 13 pools studied ranged from 1.08 to 2.00 mmol/l, from 4.38 to 7.16 mmol/l, and from 1.00 to 1.17 mmol/l, respectively.

Density gradient ultracentrifugation

The method we used for isolation of LDL subfractions was based on the density gradient ultracentrifugation procedure described by Kuchinskiene and Carlson (11) for the isolation of VLDL subfractions and LDL. The method was modified as follows. Fresh pooled serum (3.4 ml) was pipetted into siliconized polycarbonate centrifuge tubes, capacity 14 ml (MSE, cat. no. 34411-125); then, unless otherwise stated, 20 μl of a freshly prepared 15 g/l aqueous solution of Coomassie Brilliant Blue R (Sigma, no. B-0630) was added. Finally, 0.48 g of KBR was added and dissolved, by stirring with a spatula, in order to bring the density to 1.10 g/ml. The prestained serum was overlayered successively with 2.5 ml of d 1.065 g/ml solution (mixture of appropriate volumes of solutions of d 1.006 and d 1.10 g/ml), 2.5 ml of d 1.020 g/ml solution (mixture of appropriate volumes of d 1.006 and d 1.225 g/ml solu-

Abbreviations: VLDL, very low density lipoproteins, d < 1.006 g/ml or pre-β lipoproteins; LDL, low density lipoproteins, d 1.019–1.063 g/ml or β-lipoproteins; LDL_1, relatively light LDL, consisting of LDL_{1A} and LDL_{1B} (d 1.023–1.029 and 1.030–1.034 g/ml, respectively); LDL_2, relatively heavy LDL of d 1.036–1.041 g/ml; Lp (α), sinking pre-β lipoproteins; HDL, high density lipoproteins, d 1.063–1.021 g/ml or α lipoproteins; apo, apoproteins.

[1]To whom correspondence should be addressed at: Department of Medicine, Division of General Internal Medicine, St. Radboud Hospital, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands.

tion), and finally with 2.9 ml of d 1.006 g/ml solution. Density solutions d 1.006, d 1.10, and d 1.225 g/ml were prepared as described (12, 13). For optimal staining, the density solutions were adjusted to pH 4.5–5.0 with 1 N HCl. The tubes were ultracentrifuged for 19.5 hr including 15 min of acceleration; deceleration of 45 min not included) at 37,000 rpm (g_{av} 160,000) in the IEC SW 41 rotor (no. 488, 6 × 14 ml) at 20°C, in an IEC-B60 ultracentrifuge (Damon/IEC, Needham Heights, MA 02194) or an MSE Prespin 75 ultracentrifuge with an MSE Ti 40 rotor (cat. no. 43127-111).

After ultracentrifugation, photographs of the tubes were taken under optimal illumination. Subsequently, LDL bands were aspirated slowly by means of a rubber-bulb pipette held against the inner side of the tube, just below the meniscus. When the pipette was held in the same position, a critical point was reached at which a continuous flow of fluid was aspirated into the pipette, regularly separated by air segments. This flow was maintained by lowering the pipette opening at the same rate that the fluid was aspirated. In this way colored subfractions could be recovered quantitatively without disturbing the gradient. With some experience, only 10 min is needed for aspirating two LDL subfractions from six tubes. When the serum contained visible amounts of sinking pre-β lipoproteins (LP-α), care was taken not to remove these lipoproteins together with the heavy LDL fraction. Volumes of the isolated fractions were calculated by weighing, after correction for the average densities.

The use of suitable ultracentrifuge tubes appeared to be of utmost importance for obtaining a clear banding pattern, especially in hyperlipidemic sera. Results with IEC polycarbonate Autoclear tubes were unsatisfactory. In contrast to findings of Holmquist (14), surface modification with polyvinyl alcohol did not improve the results. Rather, the polyvinyl alcohol interfered with the staining procedure resulting in a faint green color instead of a strong blue color.

Rebanding of LDL subfractions during a second ultracentrifugation was studied as follows. From an individual known to have three distinct LDL subfractions, LDL_{1A}, LDL_{1B} and LDL_2 were isolated by aspiration after the first run. Subsequently, these fractions were dialyzed overnight against 5 l of saline containing 0.1 g of ethylenediaminetetraacetate per liter, pH 7.4, followed by recentrifugation. The volume of the LDL subfractions was adjusted by the addition of albumin, 40 g/l, to compensate for the serum proteins present in the original serum.

Analytical methods

For gradient gel electrophoresis, commercially available 2–16% polyacrylamide gels were used as in ref. 4. (Pharmacia, Uppsala, Sweden, cat. no. 19-1264-01).

Agarose gel electrophoresis was performed in 0.8% agarose in barbital buffer, pH 8.6, as previously described (15).

The apoprotein composition of the LDL subfractions including apoB-100 and other high molecular weight proteins was studied with SDS gel electrophoresis using 3%/4% discontinuous polyacrylamide disc gels (16). Lipoproteins mixed with SDS-phosphate buffer and dithiothreitol as a reducing reagent were boiled and immediately loaded onto the gels. This resulted in complete delipidation of the apoproteins. Albumin, apoA-I, and apoE were determined by rocket immunoelectrophoresis. ApoA-I and apoE were purified by Sephacryl S-200 column chromatography (Pharmacia, Uppsala, Sweden). ApoE was further purified by preparative flat-bed isoelectric focusing (17). Monospecific antisera were raised in rabbits and the rocket immunoelectrophoresis was performed as described (17). LDL subfractions were analyzed without further dilution (Lowry protein range 800 to 1000 mg/l) against a serum pool of known apoprotein concentrations in suitable dilutions.

Total cholesterol, unesterified cholesterol, phospholipids, and triglycerides were determined by enzymatic, commercially available reagents (Boehringer-Mannheim, FRG, cat. no. 237574, 310328 and Sera Park, Miles, Italy, cat. no. 6639, respectively). The protein content of the LDL was determined by the method of Lowry et al. (18).

A digital density meter (Anton Paar K. G., Graz, Austria) or a pycnometer was used for measurement of relative densities and for calibration of the density solutions at 20°C.

Wilcoxon's paired test was used to test differences for significance. All results are expressed as mean ± SD.

RESULTS

Profile of density gradient and the distribution of cholesterol along the gradient

The profile of the density gradient was determined after ultracentrifugation of a d 1.006 g/ml solution, instead of serum, on two different days **(Fig. 1)**. From fractionation mark 6 downwards, the gradient appears to be rather linear. The layering procedure was reproducible as judged by the small standard deviation of the measured densities along the gradient. The distribution of cholesterol along the gradient in the tube after ultracentrifugation of prestained and nonstained sera on two different days revealed three peaks, representing VLDL, LDL, and HDL (Fig. 1). No subdivision of LDL and HDL into clear subfractions could be observed because of the relatively large pooled fractions (0.5 ml) in which cholesterol determination was performed. Boundaries and density distribution of stained and nonstained cholesterol-containing lipoproteins were similar (Fig. 1).

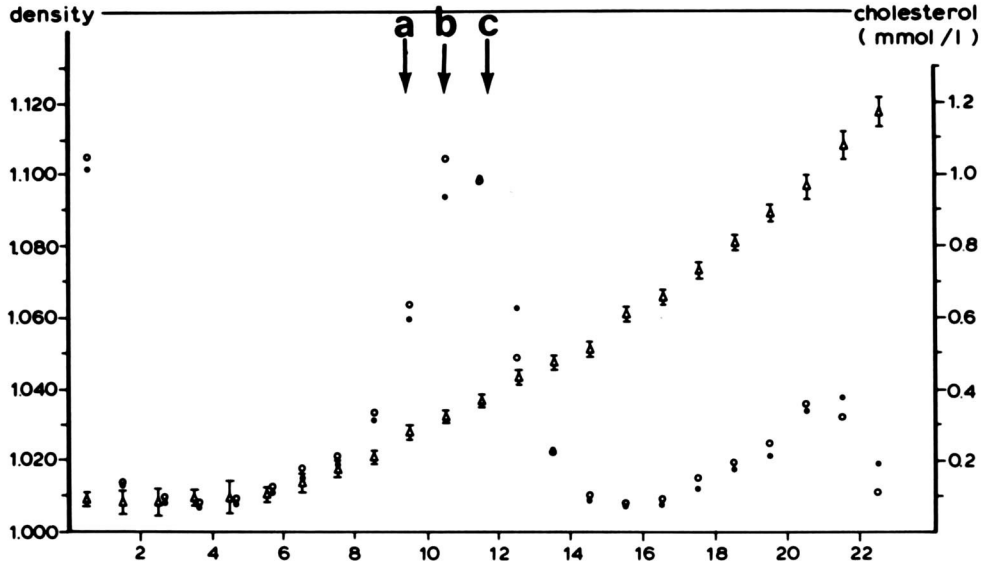

Fig. 1. Profile of the density gradient and distribution of cholesterol along the gradient after density gradient ultracentrifugation. The numbers on the horizontal axis indicate fractionation marks. The relative density was determined in pooled fractions after ultracentrifugation of a solution with a density of 1.006 g/ml instead of pooled serum (△). Cholesterol in the various fractions was determined after density gradient ultracentrifugation of prestained (○) or nonstained serum (●). The data shown are the means of the duplicate results on two different days. The arrows indicate the middle position of the LDL subfractions: LDL_{1A} (a), LDL_{1B} (b), and LDL_2 (c).

Banding pattern of LDL subfractions

In all pooled sera two heavily stained bands could be seen separated in the LDL region by a clear interface of approximately 1.5 mm (between d 1.033 ± 0.003 and d 1.038 ± 0.002 g/ml) **(Fig. 2)**. Even without staining, this subdivision of LDL into two subfractions was usually visible. When stained, the light LDL_1 fraction occasionally showed a subdivision into two bands called LDL_{1A} and LDL_{1B}. However, the separation between LDL_{1A} and LDL_{1B} was not as clear as between LDL_{1B} and LDL_2 (Fig. 2). The density of subfraction LDL_{1A} was between 1.025 ± 0.003 and 1.028 ± 0.003 g/ml ($n = 3$); of LDL_{1B} between 1.030 ± 0.002 and 1.033 ± 0.002 g/ml ($n = 10$), and of LDL_2 between 1.036 ± 0.002 and 1.41 ± 0.002 g/ml ($n = 10$). In some pooled sera a faint band was present with density boundaries between 1.044 ± 0.03 and 1.053 ± 0.03 g/ml, presumably representing sinking pre-β lipoproteins. The method appeared to be suitable for the analysis of LDL heterogeneity in hypertriglyceridemic sera. Fig. 2 shows the results obtained in three sera with triglyceride concentratrions of 3.1, 7.5, and 52.2 mmol/l. Even in the last serum the triglyceride-rich lipoproteins did not adhere to the wall and could be recovered in the first 1-ml portion after some experience in performing the aspiration technique. The sera of patients X and Z appeared to contain one single LDL band of relatively decreased intensity; two LDL bands were present in the serum of patient Y. The density of the LDL was related to the triglyceride concentration: the density was lowest in the serum of the subject with the highest triglyceride concentration.

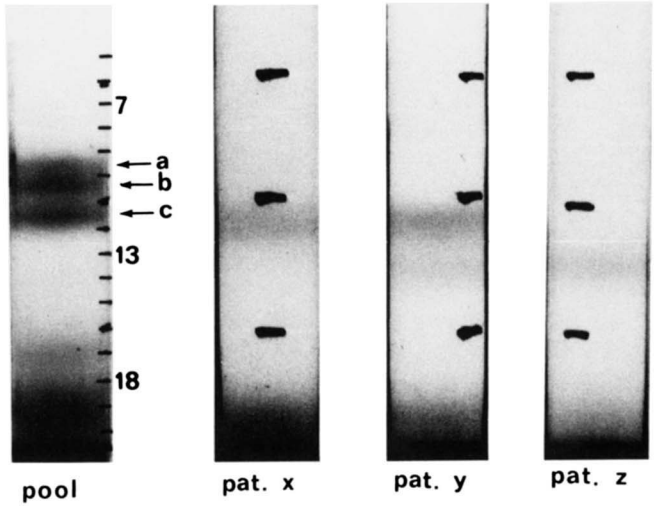

Fig. 2. Banding pattern after density gradient ultracentrifugation of prestained human pooled serum and serum of three hypertriglyceridemic patients. For the pooled serum the arrows indicate the middle position of the visible bands: LDL_{1A} (a), LDL_{1B} (b), and LDL_2 (c). The numbers indicate the fractionation marks as used in Fig. 1. A faint band, presumably sinking pre-β lipoproteins and occasionally observed at fractionation mark no 14, is absent. Sera of the three hypertriglyceridemic patients were obtained after overnight fasting and were ultracentrifuged as described for pooled serum. Plasma triglyceride for patients x, y, and z were 3.1, 7.5, and 52.2 mmol/l, respectively.

Duration of ultracentrifugation

Prolonging the time of ultracentrifugation from 19.5 to 39 hr had no effect on the location of the LDL bands nor on the number of LDL bands (data not shown). In the light of the results of Kuchinskiene and Carlson (11), studies on the effect of shortening the ultracentrifugation time seemed redundant. In addition, considerably shorter ultracentrifugation runs are not attractive because of the increasing risk for contamination with VLDL remnants, HDL, or serum proteins. The length of ultracentrifugation allows one run each day and provides enough time for unloading and loading the rotor.

Recovery, reproducibility, and rebanding pattern

Recovery of cholesterol in the various lipoprotein fractions VLDL, LDL_1, LDL_2, and HDL, together with the serum proteins, amounted to 91.4 ± 8.1% of that in total serum (n = 6). The amount of cholesterol recovered in LDL_1 and LDL_2 was 86.4 ± 2.0% of that in the total LDL fraction isolated sequentially between d 1.019 and 1.063 g/ml (n = 6).

Within-day precision expressed as the CV of the cholesterol content in the LDL_1 and LDL_2 fractions of a serum pool ultracentrifuged in sixfold in the same rotor was dependent on the absolute cholesterol concentration **(Table 1).** Between-day precision determined by ultracentrifugation of a serum pool on 4 successive days was also satisfactory. These results shown in Table 1 were obtained by an experienced technician.

In the rebanding experiment in which LDL_{1A}, LDL_{1B}, and LDL_2 were recentrifuged in the presence of albumin, colored bands were detectable in the tubes with boundaries similar to the bands in the original serum. From LDL_{1A} to LDL_{1B} and LDL_2 a clear stepwise decrease in the density of the colored bands could be seen without any overlap. However, the bands were less sharp and the intermediate layer between the subfractions, clearly present in the first ultracentrifugation, had faded.

TABLE 1. Within- and between-day precision of the density-gradient ultracentrifugation method[a]

	Within-Day Precision[b]		Between-Day Precision[c]	
Fraction	Cholesterol	CV	Cholesterol	CV
	mmol/l	%	*mmol/l*	%
LDL_1	1.69 ± 0.05	3.0	1.69 ± 0.03	1.8
LDL_2	0.77 ± 0.06	7.8	0.76 ± 0.04	2.3

[a] Expressed as the coefficient of variation (%) of the cholesterol content of the LDL_1 and LDL_2 subfractions.
[b] Mean ± SD of six samples of pool A ultracentrifuged in the same rotor.
[c] Mean ± SD of four samples of pool B analyzed on 4 successive days in two different rotors; the pool was stored at 4°C. Isolated subfractions were stored at 4°C and cholesterol was determined on the same day.

TABLE 2. Chemical composition of LDL_1 and LDL_2[a]

Component	LDL_1	LDL_2
Cholesteryl ester	41.3 ± 1.7	37.7 ± 4.0[b]
Free cholesterol	10.7 ± 1.3	10.6 ± 2.5
Triglycerides	3.4 ± 2.0	2.2 ± 1.3[c]
Phospholipids	22.8 ± 0.5	21.3 ± 0.9[b]
Protein	21.6 ± 1.7	28.2 ± 3.0[b]

[a] Relative chemical composition (percent of dry mass, mean ± SD, n = 10).
[b] $P < 0.01$.
[c] $0.05 < P < 0.1$.

Physicochemical characteristics of the LDL subfractions

LDL_1 contained significantly more esterified cholesterol and phospholipids, and less protein than LDL_2 ($P < 0.01$). The percentage of triglycerides in LDL_1 tended to be higher than in LDL_2 **(Table 2)**.

When three subfractions were found, the relative content of cholesteryl esters, free cholesterol, and phospholipids decreased and that of proteins increased from LDL_{1A} to LDL_{1B} to LDL_2 **(Table 3)**.

The mobilities of LDL_1 and LDL_2 on agarose gel were similar **(Fig. 3)**. With SDS-polyacrylamide gel electrophoresis, no differences were detectable in the apoprotein composition. By densitometric scanning, less than 5% of the absorbance was due to staining outside the apoB-100 band **(Fig. 4)**.

On the gradient gel electrophoresis, LDL_2 moved faster than LDL_1 and LDL_{1B} moved faster than LDL_{1A} **(Fig. 5)**. Since smaller particles move the fastest, this indicates that the size is inverse to the hydrated density of the particles within the respective LDL subfractions.

Rocket immunoelectrophoresis of LDL_{1A}, LDL_{1B}, and LDL_2 showed that residual albumin, apoE, and apoA-I contributed less than 5% to the total protein mass of LDL_{1A}, LDL_{1B}, and LDL_2. ApoA-I was the major contaminating protein followed by apoE and albumin **(Table 4)**.

DISCUSSION

By means of a relatively simple method we could confirm heterogeneity of LDL described in previous studies (1-7). The prestaining procedure with Coomassie Brilliant Blue R facilitated the identification of these LDL subfractions as well as of minor fractions present within the density range of LDL, such as sinking pre-β lipoproteins. The staining effect is maximal and the protein-dye complex is most stable at a low pH (19). Staining at a pH between 4.5-5.0, slightly below the isoelectric point of LDL (20), proved to be satisfactory. The LDL bands were

TABLE 3. Chemical composition of LDL_{1A}, LDL_{1B}, and LDL_2[a]

Component	LDL_{1A}	LDL_{1B}	LDL_2
Cholesteryl ester	43.3 ± 2.2	42.2 ± 1.1	39.0 ± 3.2
Free cholesterol	10.3 ± 0.8	9.7 ± 0.8	9.4 ± 2.2
Triglycerides	5.6 ± 1.4	3.8 ± 0.6	4.1 ± 0.5
Phospholipids	20.9 ± 0.3	19.9 ± 0.6	19.4 ± 0.5
Protein	19.8 ± 0.6	24.1 ± 0.8	28.0 ± 0.6

[a]Relative chemical composition (percent of dry mass, mean ± SD, n = 3).

clearly visible and the density ranges of each LDL band could easily be determined by comparing the boundaries of the colored bands in the tubes with the density gradient profile. By comparing the boundaries and the profile of the cholesterol content along the gradient, it could be proved that the staining procedure did not result in a change of the density of the LDL, in agreement with earlier findings for the HDL subfractions (21).

Although the staining procedure is useful for identification, binding of Coomassie Brilliant Blue to the lipoproteins and the change in pH may alter the metabolic behavior. Therefore, for the purpose of in vivo or in vitro experiments it is advisable to use nonstained subfractions, which can be isolated on the basis of the colored bands in a reference tube in which the same serum is ultracentrifuged in the presence of Coomassie Brilliant Blue at pH 4.5–5.0.

Compared to other methods for isolation of LDL subfractions (2, 3, 5, 9) our method is less laborious and very precise. LDL subfractions can be identified as distinct bands in just a single run. In other methods, usually two preceeding ultracentrifuge steps, requiring in total approximately 35 hr, are necessary to isolate pure LDL before LDL subfractions can be isolated in a density gradient (3, 5, 9). The subfractions in these methods are generally isolated at rather arbitrarily defined density limits, whereas the distinct bands we isolated may represent physiological entities.

The sensitivity of our method is at least as good as in the only other single-step ultracentrifuge method performed in a fixed-angle rotor for 26 hr (6). In the latter method a large volume of serum is needed for identification of minor bands. In addition, the method suffers from adherence effects caused by the triglyceride-rich particles present in sera of patients with hypertriglyceridemia,

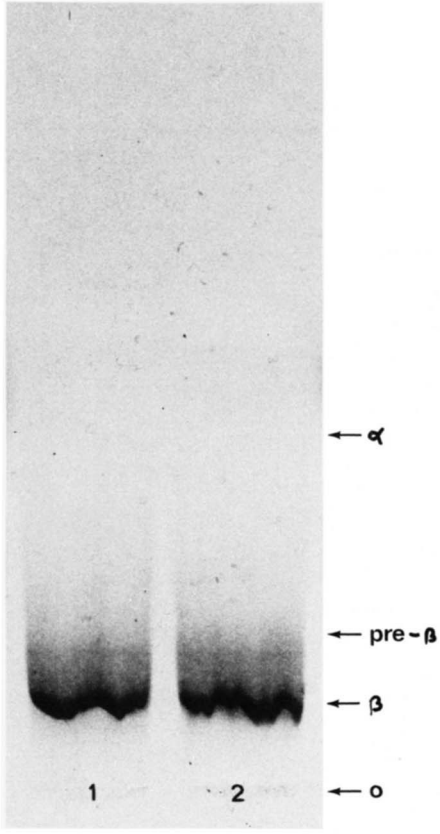

Fig. 3. Agarose gel electrophoresis of LDL_1 and LDL_2; O, origin; 1, LDL_1; 2, LDL_2.

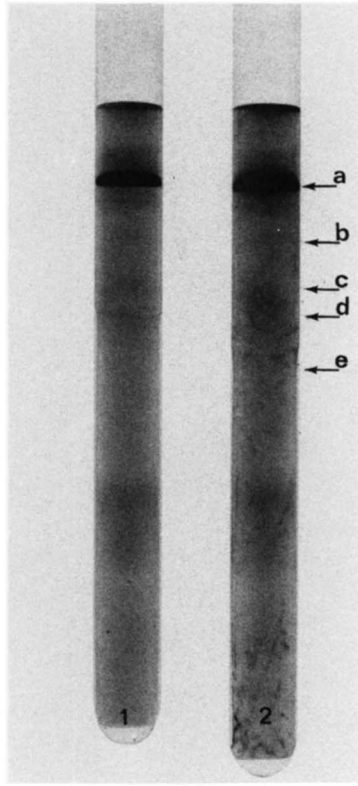

Fig. 4. SDS-polyacrylamide gel electrophoresis of LDL_1 and LDL_2. From top to bottom are shown: apoB-100 band (a), and the potential migration distances of apoB-48 (b), albumin (c), apoE (d), and apoC (e). The numbers indicate LDL_1 (1) and LDL_2 (2), respectively.

whereas our method appears suitable for the analysis of LDL subfractions of sera from patients with various types of hyperlipoproteinemia. For the isolation of LDL of type I, IV, and V, complete removal of the floating particles is important to prevent contamination of the LDL subfractions. In the individual sera of healthy donors, 1 to 3 subfractions could be identified. This excludes the possibility that the number of LDL bands we find in pooled serum is the result of the pooling procedure. On the contrary, the appearance of 2 or 3 LDL subfractions in pooled serum points to the presence in the individual sera of distinct low density lipoproteins with sharp density boundaries. Otherwise, in the case of variation of the density of the LDL bands in the individual sera, pooling would result in a diffuse smear without distinct bands.

The differences in density of the main LDL subfractions, LDL_1 and LDL_2, were related to differences in physicochemical characteristics. The heavy LDL_2 were smaller and contained relatively more protein and less cholesteryl esters and phospholipids than the lighter LDL_1. Chemical composition data of our LDL_1 and LDL_2 subfractions resemble those of fraction 1 or 2 and fraction 4, respectively, obtained by sequential isolation of LDL followed by density gradient ultracentrifugation (5); of layers 2 and 3 obtained by the large scale density gradient in a fixed-angle rotor (6), and also of LDL_{III} and LDL_{IV} obtained by sequential flotation, respectively (2).

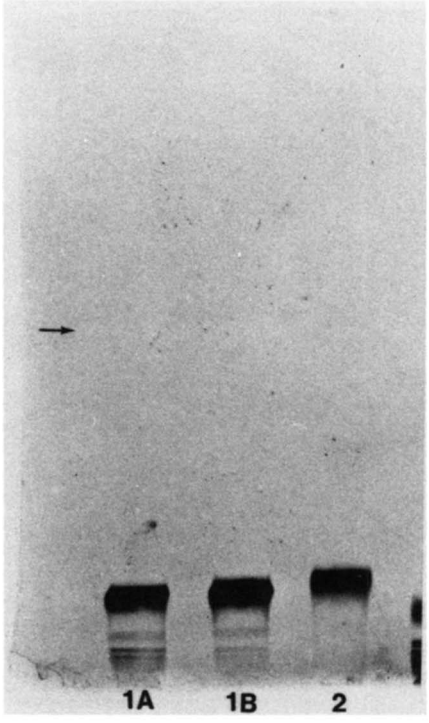

Fig. 5. Polyacrylamide gradient gel electrophoresis of three LDL subfractions, LDL_{1A} (1A), LDL_{1B} (1B), and LDL_2 (2). The arrow indicates the position of HDL.

TABLE 4. Contamination of LDL subfractions by various proteins[a]

Fraction	Albumin (n = 3)	ApoE (n = 3)	ApoA-I (n = 4)
LDL_{1A}	0.2 ± 0.1	0.2 ± 0.1	1.1 ± 0.2
LDL_{1B}	0.2 ± 0.1	0.5 ± 0.2	3.4 ± 0.2
LDL_2	0.6 ± 0.1	1.2 ± 0.1	2.4 ± 0.2

[a]Contamination of undiluted LDL subfractions by apoproteins A-I, E and by albumin was determined by rocket immunoelectrophoresis and was expressed as percent of LDL protein ± SD.

Differences in the chemical composition may be explained by the differences in the sera studied or in methods applied. We used shorter ultracentrifugation runs which may minimize loss of apoproteins during ultracentrifugation (22, 23). The smaller size of the heavy LDL particles we found with gradient gel electrophoresis confirms earlier reports in which size determination of LDL subfractions was performed by means of a similar procedure or by electron microscopy (4, 5, 6). LDL_1 and LDL_2 could not be distinguished on the basis of their electrophoretic mobility, which may be accounted for by the comparable apoprotein composition. More than 95% of the protein consisted of apoB-100; traces of albumin, apoE, and apoA-I were less than 5% of total protein. Similar results of contamination have been reported by Zechner, Moser, and Kostner (24) for isolation of total LDL by sequential ultracentrifugation.

In conclusion, this report presents a simple and quick method for identification and isolation of LDL subfractions from small amounts of serum. The LDL subfractions isolated in this way can be used for physicochemical characterizations and for in vitro or in vivo metabolic studies.

The excellent technical assistance of Pieternel van Heijst and Anneke Hijmans and the skillful preparation of the manuscript by Ans Ruesen-Maandag are gratefully acknowledged. Prof. J. H. Veerkamp and Prof. A. van 't Laar are thanked for comments on the manuscript. This study was supported by a grant (no. G5/84) from the Research Fund of the University of Nijmegen.

Manuscript received 25 November 1986 and in revised form 27 March 1987.

REFERENCES

1. Adams, G. H., and V. N. Schumaker. 1970. Equilibrium banding of low density lipoproteins. III. Studies on normal individuals and the effect of diet and heparin-induced lipase. *Biochim. Biophys. Acta.* **210:** 462–472.
2. Lee, D. M., and P. Alaupovic. 1970. Studies of the composition and structure of plasma lipoproteins. Isolation, composition and immunochemical characterization of low density lipoprotein subfractions of human plasma. *Biochemistry.* **9:** 2244–2252.
3. Hammond, M. G., and W. R. Fisher. 1971. The characterization of a discrete series of low density lipoproteins in

the disease, hyper-pre-β-lipoproteinemia. *J. Biol. Chem.* **246:** 5154–5465.
4. Krauss, R. M., and D. J. Burke. 1982. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *J. Lipid Res.* **23:** 97–104.
5. Shen, M. M. S., R. M. Krauss, F. T. Lindgren, and T. M. Forte. 1981. Heterogeneity of serum low density lipoproteins in normal human subjects. *J. Lipid Res.* **22:** 236–244.
6. Lee, D. M., and D. Downs. 1982. A quick and large-scale density gradient subfractionation method for low density lipoproteins. *J. Lipid Res.* **23:** 14–27.
7. Fisher, W. R. 1983. Heterogeneity of plasma low density lipoproteins manifestations of the physiologic phenomenon in man. *Metabolism.* **32:** 283–291.
8. Patsch, J. R., S. Sailer, G. Kostner, F. Sandhofer, A. Holasek, and H. Braunsteiner. 1974. Separation of the main lipoprotein density classes from human plasma by rate-zonal ultracentrifugation. *J. Lipid Res.* **15:** 356–366.
9. Teng, B., G. R. Thompson, A. D. Sniderman, T. M. Forte, R. M. Krauss, and P. O. Kwiterovich. 1983. Composition and distribution of low density lipoprotein fractions in hyperapobetalipoproteinemia, normolipidemia, and familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **80:** 6662–6666.
10. Demacker, P. N. M., R. W. B. Schade, R. T. P. Jansen, and A. van 't Laar. 1982. Intra-individual variation of serum cholesterol, triglycerides and high density lipoprotein cholesterol in normal humans. *Atherosclerosis.* **45:** 259–266.
11. Kuchinskiene, Z., and L. A. Carlson. 1982. Composition, concentration, and size of low density lipoproteins and of subfractions of very low density lipoproteins from serum of normal men and women. *J. Lipid Res.* **23:** 762–769.
12. Hatch, F. T., and R. S. Lees. 1968. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **6:** 1–68.
13. Terpstra, A. M., C. J. Woodward, and F. J. Sanchez-Muniz. 1981. Improved techniques for the separation of serum lipoproteins by density gradient ultracentrifugation: visualization by prestaining and rapid separation of serum lipoproteins from small volumes of serum. *Anal. Biochem.* **111:** 149–157.
14. Holmquist, L. 1982. Surface modification of Beckman Ultra-Clear® centrifuge tubes for density gradient centrifugation of lipoproteins. *J. Lipid Res.* **23:** 1249–1250.
15. Demacker, P. N., H. E. Vos-Janssen, A. van 't Laar, and A. P. Jansen. 1978. A descriptive study of the different electrophoretic patterns on agarose of human serum very low density lipoproteins. *Clin. Chem.* **24:** 1439–1444.
16. Stalenhoef, A. F. H., M. J. Malloy, J. P. Kane, and R. J. Havel. 1984. Metabolism of apolipoproteins B-48 and B-100 of triglyceride-rich lipoproteins in normal and lipoprotein lipase-deficient humans. *Proc. Natl. Acad. Sci. USA.* **81:** 1829–1843.
17. Stuyt, P. M. J., A. F. H. Stalenhoef, P. N. M. Demacker, and A. van 't Laar. 1985. A comparative study of the effects of acipimox and clofibrate in type III and type IV hyperlipoproteinemia. *Atherosclerosis.* **55:** 51–62.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
20. Sundaram, S. G., K. M. M. Shakir, and S. Margolis. 1978. Preparative isoelectric focusing of human serum VLDL and LDL. *Anal. Biochem.* **88:** 425–433.
21. Demacker, P. N., D. F. van Sommeren-Zondag, A. F. Stalenhoef, P. M. Stuyt, and A. van 't Laar. 1983. Ultracentrifugation in swinging-bucket and fixed-angle rotors evaluated for isolation and determination of high-density lipoprotein subfractions HDL_2 and HDL_3. *Clin. Chem.* **29:** 656–663.
22. Kunitake, S. T., and J. P. Kane. 1982. Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. *J. Lipid Res.* **23:** 936–940.
23. Johnsson, P., R. A. Muirhead, and T. Deegan. 1981. Loss of A-apoprotein immunoreactivity during high-density lipoprotein separation. *Ann. Clin. Biochem.* **18:** 308–313.
24. Zechner, R., R. Moser, and G. M. Kostner. 1986. Isolation of pure LpB from human serum. *J. Lipid Res.* **27:** 681–686.