Sequential microultracentrifugation of lipoproteins in 100 μl of serum

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Abstract A method is described for sequential separation of high density, very low density, and low density lipoproteins (HDL, VLDL, and LDL, respectively) from 100 μl of serum, using an air-driven ultracentrifuge (Airfuge, Beckman). Cesium chloride was used for density adjustment. Precision-within-series (coefficient or variation) depended on the cholesterol concentration in the lipoprotein fractions, > 1 mmol/l, < 2.3%. Recovery within-series was nearly 100%. The results (mmol/l) correlate well with those from an electrophoretic-enzymatic procedure (α-HDL: 1.49 ± 0.34 vs. 1.48 ± 0.33, r = 0.949; pre-β-VLDL: 0.58 ± 0.42 vs. 0.59 ± 0.45, r = 0.975; β-LDL: 3.11 ± 0.93 vs. 3.07 ± 0.88, r = 0.990; n = 48). Recovery of lipoprotein cholesterol from this group was 99.2 ± 4.2%. A combination of ultracentrifugation with high-performance thin-layer chromatography for determination of lipoprotein-lipid profiles was achieved with recoveries of 98–101%, as evaluated from a group of healthy men (n = 31) and women (n = 38). The entire procedure is, therefore, suitable for compositional studies on lipoproteins from small serum samples. In particular, capillary serum from children of all ages, even from premature neonates, is quite adequate.—**Kupke, I. R., and S. Wörz-Zeugner.** Sequential microultracentrifugation of lipoproteins in 100 μl of serum. *J. Lipid Res.* 1986. **27:** 988–995.

Supplementary key words microultracentrifugation • VLDL • LDL • lipoprotein-lipid profiles • HPTLC • young adults

Numerous studies have shown that detailed information on the composition and structure of lipoprotein particles provides a better understanding of lipoprotein metabolism in health and disease.

Preparative ultracentrifugation remains a frequent and useful technique for isolating all serum lipoproteins. However, relatively large serum volumes are required (1, 2), and methods for lipid analysis are time-consuming and laborious.

In 1977, a microprocedure for lipoprotein fractionation using an air-driven ultracentrifuge (Airfuge) was described (3). Inasmuch as only 2 × 175 μl of plasma was required to prepare very low and high density lipoproteins (VLDL and HDL), this procedure seemed to meet the need in pediatrics for analyzing small sample volumes such as capillary blood serum. However, purity and recovery of lipoproteins were poor, as was also recently pointed out by another group (4).

As mentioned above, microultracentrifugation using small sample volumes seems to be especially suited for studies on children. In order to overcome difficulties, and thus, to improve the method, we developed a methodology for the separation of lipoproteins on a microscale (5, 6). However, in plasma samples from hyperlipidemic patients, purity of lipoprotein fractions was not guaranteed in each case. In a further study, separation of HDL was improved, and determination of HDL lipids was performed with high performance thin-layer chromatography (HPTLC) (7), as described previously for serum (8). Purity of HDL fractions was satisfactory from most, but not all, types of sera.

The purpose of the present study is to demonstrate the feasibility of successive separation of lipoproteins starting from 100 μl of serum, using the Airfuge, and to obtain pure HDL, VLDL, and LDL with high precision, good recovery, and reproducibility. For standardization, the method is compared to an electrophoresis-enzymatic procedure (6). Furthermore, the method is combined with HPTLC for subsequent analysis of lipid profiles in all lipoprotein fractions.

In order to test the results of this combined procedure, a group of healthy young men and women was examined. The results suggested that the procedure is highly suitable for lipoprotein analyses and thus can be applied to studies on children, including mature and premature neonates, as well as on small animals.

MATERIALS AND METHODS

Serum samples

Apparently healthy subjects and hyperlipidemic patients of all ages donated venous blood in the fasting state (after a 12–16 hr fast). After centrifugation at 10°C, serum was obtained and stabilized by the addition of ethylenedi-

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; HPTLC, high performance thin-layer chromatography.

aminetetraacetate (EDTA) (1 μl of a 5% EDTA-solution, adjusted to pH 7.4, added to 100 μl of serum). Samples were stored at 4°C.

Instruments and chemicals

The air-driven ultracentrifuge "Airfuge" (No. 350 666, air pressure 103.5 KPa); fixed angle rotor (A-100/30); polyethylene centrifuge tubes, 5 × 20 mm (No. 343622); and tube slicer designed for the small tubes (No. 924116) were from Beckman Instruments, Inc., Palo Alto, CA. The microelectrophoresis system was from IMA/Corning, Giessen, F.R.G. Equipment for lipoprotein electrophoresis on agarose gel followed by enzymatic determination of lipoprotein cholesterol (9) and for lipid quantitation by HPTLC (8) were as previously described.

Cesium chloride solutions (CsCl "Suprapur," Merck, Darmstadt, F.R.G.) were made up as follows: d = 1.019, 1.102, and 1.310 g/ml. Depending on the respective lipid concentrations, the salt solution of d 1.102 g/ml was replaced as follows. A solution of d 1.090 g/ml was used when cholesterol was < 2.59 and triacylglycerols were < 1.13 mmol/l (< 100 mg/dl); a d 1.119 g/ml solution was used when cholesterol was >7.24 and triacylglycerols were > 3.2 mmol/l (280 mg/dl). A solution of d 1.119 g/ml was also used for sera with normal or slightly increased cholesterol but markedly increased triacylglycerols, such as in Type IV or Type V hyperlipidemia. Densities were adjusted by weighing the solutions on an analytical balance. Solutions were tightly sealed and stored in the refrigerator at 4°C.

Microelectrophoresis

For this procedure, 1 μl of serum or lipoprotein fraction corresponding to 1 μl of serum was applied to the agarose gel. After separation of the lipoproteins, the gel was dried in a stream of warm air. After staining with Oil Red O, the gel was dried again. In neither drying procedure was filter paper in contact with the agarose layer. Accordingly, even traces of contaminating lipoproteins could be visualized, making this system highly sensitive.

Checking of serum samples prior to ultracentrifugation

Serum was subjected to microelectrophoresis within 3 hr after the blood was obtained. After longer intervals, the double pre-β-band more or less as a single band. Cholesterol and triacylglycerol concentrations were determined for the purpose of selecting the appropriate CsCl solution

Tube-slicing of lipoprotein fractions separated by the Airfuge

Bottom fractions, as indicated in **Fig. 1**, were specified using a micrometer scale accurate to at least 0.01 mm. Evaporation was negligible as shown by the recovery of lipoprotein lipids (see Tables 2 and 4); thus top fractions very closely approximated the volumes indicated in Fig 1.

Separation of serum lipoproteins by microultracentrifugation

Sera and density fractions were kept in the cold (refrigerator) or kept in ice water during handling (0–5°C). This

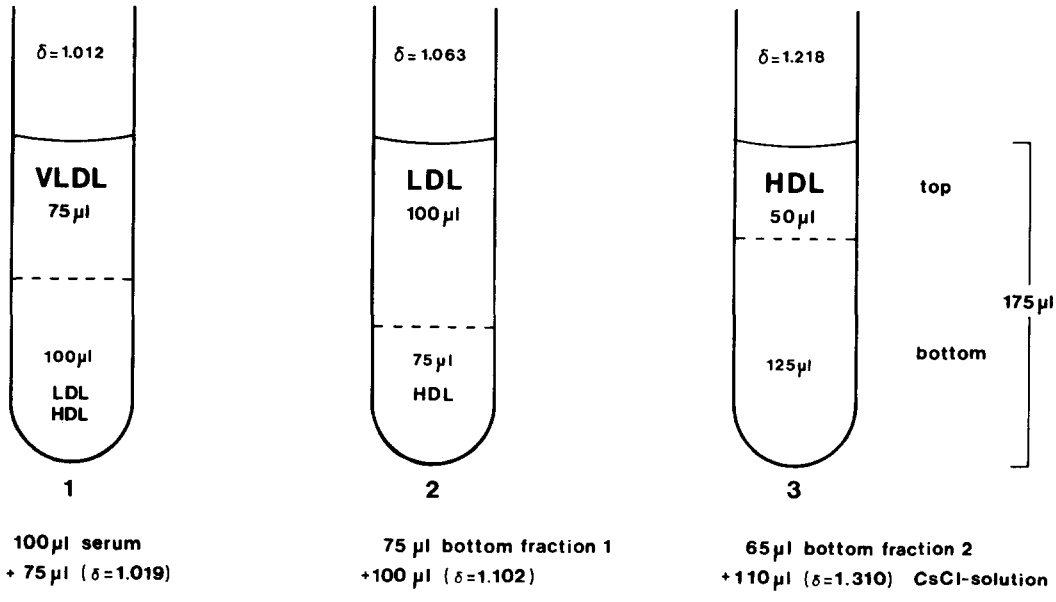

Fig. 1. Separation of lipoproteins from 100 μl of serum by the Airfuge.

was essential to preserve the integrity of lipoprotein structure as well as to prevent evaporation, especially in small sample volumes.

The centrifugation procedure was started on the day that blood was collected or on the following day. Serum samples, bottom fractions, and the precooled CsCl solutions were precisely measured by using Hamilton syringes, and gently mixed. After cutting the centrifuge tubes with the tube slicer, top and bottom fractions were collected with a microliter pipette and transferred to microliter tubes (Eppendorf). Top fractions were stabilized by the addition of human albumin (powder, measured with a tiny spoon). Centrifugation was carried out at 100,000 *g* for several hours (5°C). The entire procedure is summarized in Fig. 1.

Preparation of VLDL

One hundred μl of serum was transferred to a centrifuge tube and mixed gently with 75 μl of CsCl solution (d 1.019 g/ml). Samples were centrifuged for 5 hr. Seventy five μl of top fraction 1 (VLDL) was cut, collected, and stabilized by the addition of approximately 4 mg of human albumin.

Preparation of LDL

Seventy five μl of bottom fraction 1 was mixed gently with 100 μl of CsCl solution (d 1.102 g/ml). Samples were centrifuged overnight for 18 hr. A top fraction of 100 μl was cut and stabilized by the addition of approximately 4 mg of human albumin.

Preparation of HDL

Bottom fraction 2 (65 μl) was mixed gently with 110 μl of CsCl solution (d 1.310 g/ml). Samples were centrifuged overnight (see above). Fifty μl of top fraction 3 was cut and stabilized by the addition of approximately 2 mg of human albumin.

In order to get sharp separation, good recovery, and reproducible results, it was essential to strictly follow this procedure at each step.

Separation of serum lipoproteins by conventional ultracentrifugation

The procedure was carried out with the Beckman L 2-65 B ultracentrifuge, equipped with a Type-60 rotor. Separation started from 9 ml of serum. The appropriate densities were adjusted using potassium bromide solutions. Samples were centrifuged at 10°C, 40,000 rpm: VLDL (d 1.012 g/ml) for 18 hr; LDL (d 1.063 g/ml) for 24 hr; and HDL (d 1.210 g/ml) for 48 hr. This method is essentially that used in the Lipid Research Clinics Program (1).

Control on the purity of the lipoprotein fractions

Density fractions were tested by microelectrophoresis (see above). Furthermore, radial immunodiffusion techniques were used in order to check the lipoprotein fractions for contaminating apolipoproteins, such as apoA and apoB (Behring Institute, Marburg, F.R.G.), as well as apoA-I and apoA-II (Immuno AG, Vienna, Austria).

Separation of serum lipoproteins by electrophoresis on agarose gel followed by enzymatic determination of lipoprotein cholesterol

Ten μl of serum is required for electrophoretic separation of lipoproteins using methods that were described previously (5, 6). After visualization, the lipoprotein fractions were cut with a scalpel, dried, and dissolved in HCl. Addition of a saturated Tris-solution produced a Tris-buffer with pH 7. Cholesterol in these buffered samples was analyzed by a modified enzymatic procedure (catalase method, Boehringer Mannheim, F.R.G.). The procedure was precise and recovery of lipoprotein cholesterol was nearly 100%.

Quantitation of serum and lipoprotein lipids by HPTLC

Serum (0.5 μl) was applied directly on to the silica gel layer, and lipids were separated and quantitated as described previously (8). Precision of determination varied within narrow limits and the recovery of lipids was nearly 100%.

The same procedure was used in this study for the quantitation of lipids in lipoprotein fractions separated by ultracentrifugation.

Serum samples with "sinking pre-β-lipoprotein"

This apoB-containing lipoprotein was precipitated prior to HDL lipid analysis. To 10 μl of top fraction 3 (Fig. 1) was added 4 μl of precipitant (4.8 g/dl phosphotungstic acid, 3.0 mol/l $MgCl_2$ No. 400 971, Boehringer, Mannheim, F.R.G.).

Calculations

Data were examined statistically by linear regression analysis and by two nonparametric tests: a paired test according to Wilcoxon (method comparison) and an unpaired test according to Kruskall-Wallis (study on young adults).

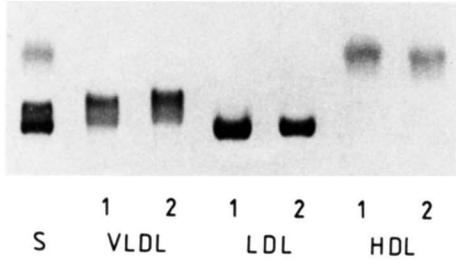

Fig. 2. Microelectrophoresis of lipoprotein fractions separated either by conventional (1) or microultracentrifugation (2).

RESULTS

Sequential separation of serum lipoproteins by ultracentrifugation

Each step of ultracentrifugation was examined by microelectrophoresis. Lipoprotein fractions were visualized by using Oil Red O as lipid stain. As shown in **Fig. 2,** pure lipoprotein fractions were obtained using both the micro and conventional procedures. Sharp separation by the microprocedure was also achieved on all types of sera (**Fig. 3**). The mobility in the electric field of the lipoprotein fractions separated by ultracentrifugation was the same as those of the corresponding fractions in serum, and the bands had the same appearance.

Further test on the purity of the lipoprotein fractions

Table 1 shows data on the contamination of the lipoprotein fractions with apolipoproteins. In both ultracen-

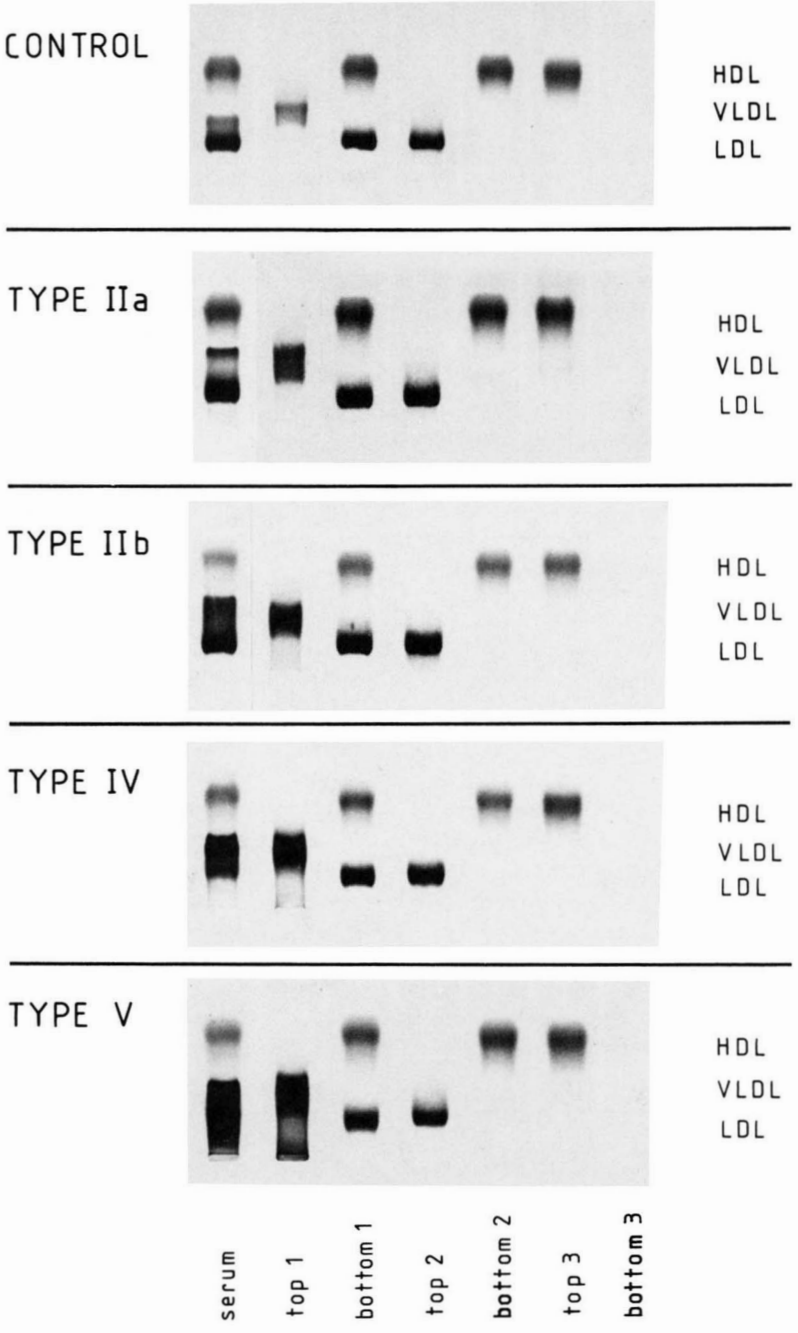

Fig. 3. Lipoprotein density fractions tested for purity and appearance by microelectrophoresis.

TABLE 1. Purity of the lipoprotein fractions

Sample	Total Serum	LDL	VLDL	HDL	Bottom Fraction 3 (see Fig. 1)
	mg/dl		% of total		
Sample 1[a]					
ApoA	245	0.2			1.2
ApoA-I	138	not det.	not det.		
ApoA-II	45	0.1	2.2		
ApoB	160			0.8	
Sample 2[a]					
ApoA	205	0.1			1.1
Apo-I	125	not det.	not det.		
Apo-II	45	<0.1	3.1		
ApoB	150			0.5	
Sample 3[a]					
ApoA	365	0.04			1.2
Apo-I	185	not det.	0.2		
ApoA-II	80	not det.	1.2		
ApoB	182			0.4	
Sample 4[a]					
ApoA	200	0.4			0.8
Apo-I	123	not det.	not det.		
Apo-II	35	not det.	3.6		
ApoB	74			2.0	
Sample 4[b]					
ApoA	200	0.3			0.9
Apo-I	123	0.3	<0.1		
Apo-II	35	0.2	4.3		
ApoB	74			2.7	

Lipoproteins were tested for contamination with apolipoproteins; [a], microultracentrifugation; [b], conventional ultracentrifugation.

trifugation procedures, contamination was either extremely low or undetectable.

Precision and recovery

Table 2 includes data on precision-within-series of lipoprotein separation by the Airfuge and recovery of lipoprotein cholesterol. In lipoprotein fractions with cholesterol concentrations greater than 1 mmol/l, the coefficient of variation was < 2.3%. Recovery was nearly 100%.

Method comparison

For comparative studies, the serum lipoproteins were separated either by ultracentrifugation or by electrophoresis on agarose gel. Lipoprotein cholesterol was determined enzymatically in both cases. Thus, these comparisons indicate the quality and compatibility of separation using these two techniques based on different physicochemical principles. As shown in **Fig. 4,** data were closely correlated and mean values were nearly identical. For this purpose of method comparison, sera were selected that did not exhibit a double preβ-band on microelectrophoresis and had no "sinking pre-β-lipoprotein" in bottom fraction 2 and top fraction 3, as demonstrated in **Fig. 5.**

Combination of ultracentrifugation with high performance thin-layer chromatography

Table 3 includes data on two independent procedures: ultracentrifugation combined with HPTLC for cholesterol determination and electrophoresis combined with enzymatic cholesterol analysis. As shown in this table, data are closely correlated and mean values are nearly identical. Recovery is 98.9 ± 4.5% (n = 48).

Application

In order to test the suitability of this new combined procedure of ultracentrifugatin and HPTLC, a large group of healthy young adults (n = 112) was studied. Twenty four of 55 men (43.6%) and 19 of 57 women (33.3%) exhibited a double-pre-β-band, and "sinking pre-β-lipoprotein" in the HDL-containing fraction (Fig. 5) on microelectrophoresis. A small number of HDL samples exhibiting "sinking-pre-β-lipoprotein" were tested for Lp(a) by Dr. G. Utermann and found to be Lp(a)-positive.

The composition of lipoproteins in the 31 men and 38 women who did not exhibit "sinking pre-β-lipoprotein," are presented in **Table 4**. Recovery of all the lipoprotein lipids measured was nearly 100%. In the subjects with "sinking pre-β-lipoprotein," this lipoprotein was precipitated prior to analysis of HDL lipids. Data obtained on this group are not included in this report.

TABLE 2. Precision-within-series of determination and recovery of cholesterol in lipoproteins separated by the Airfuge

Sample	LDL	VLDL	HDL	Serum	Total	Recovery
			mmol/l			%
Control (n = 5)	3.51 ± 0.07 (2.1)	0.30 ± 0.01 (7.0)	1.12 ± 0.01 (1.1)	4.91 ± 0.12 (2.1)	4.93	100.4
Type IV (n = 6)	2.42 ± 0.06 (2.3)	3.51 ± 0.08 (2.3)	0.79 ± 0.03 (3.8)	6.50 ± 0.05 (0.7)	6.72	103.4

Enzymatic determination of lipoprotein cholesterol. Control, serum of a healthy subject and Type IV, serum from a hyperlipidemic patient (TG = 4.5 mmol/l). Numbers in parentheses represent coefficient of variation (%), n = number of analyses.

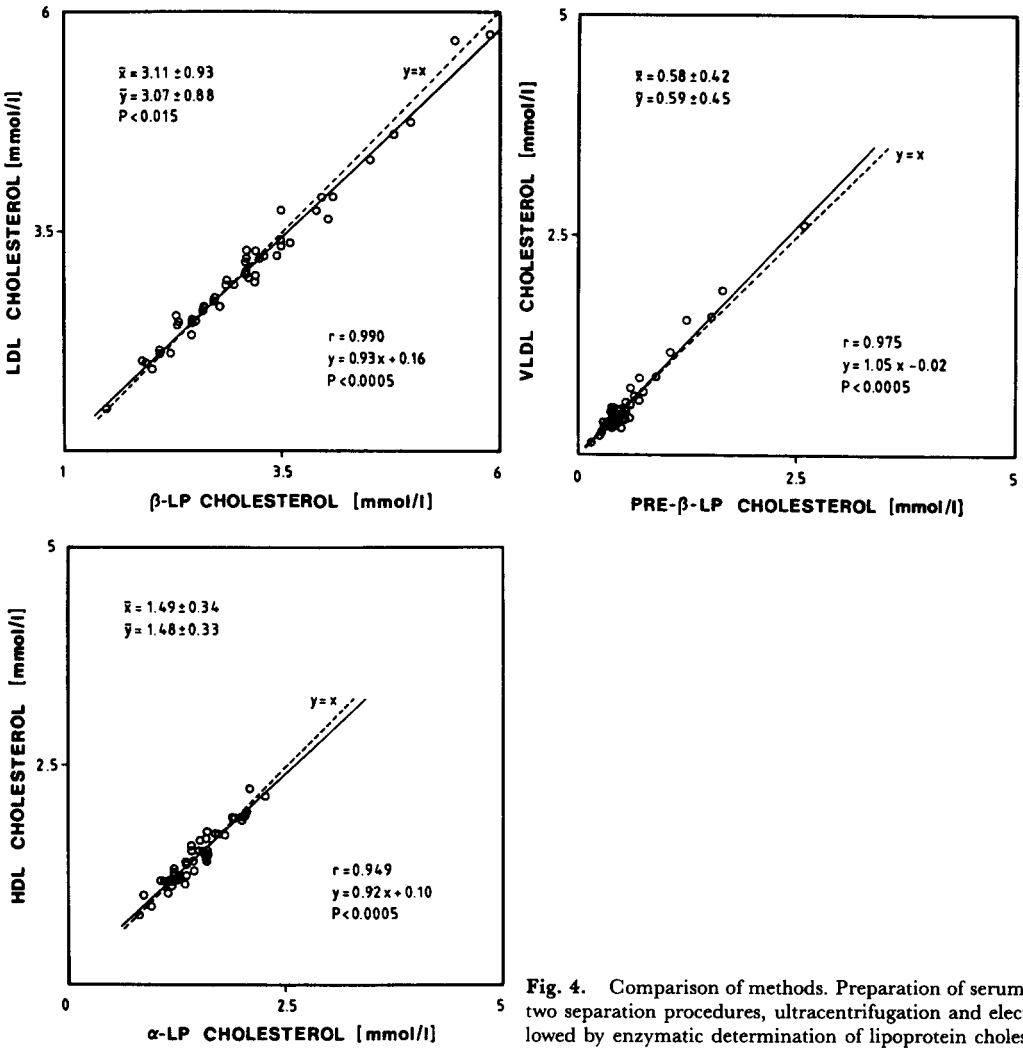

Fig. 4. Comparison of methods. Preparation of serum lipoproteins by two separation procedures, ultracentrifugation and electrophoresis, followed by enzymatic determination of lipoprotein cholesterol.

DISCUSSION

The procedure for sequential separation of lipoproteins on a microscale that was evaluated in this study is precise and reproducible, provided the technical steps are strictly followed. The use of serum instead of plasma gives sharper separation of the fractions. Obviously, fibrinogen interferes with the flotation process. Stabilizing sera with EDTA and keeping sera and density fractions in the cold without interruption had a highly protective effect on the lipoproteins.

CsCl has proved to be useful for ultracentrifugal separation of macromolecules (10). In our studies, using CsCl instead of NaCl or KBr for density adjustment was found to be obligatory. Since the atomic weight of cesium is higher than that of potassium and sodium, the same density of CsCl was achieved at a lower molarity. Apparently, a relatively small number of cesium ions reduces interference with the flotation process due to metal ions. It should also be mentioned that using the appropriate density, based on lipid concentration in serum, is essential for sharp separation of fractions. As an additional advantage, CsCl was shown not to interfere with fluorescence measurement of lipids following HPTLC, compared to KBr.

Thus, using serum instead of plasma and CsCl for density adjustment, as well as keeping all samples strictly in the cold without any interruption, have proved to be prerequisites for quality and reproducibility of this separation procedure on a microscale. As judged by the electrophoretic mobility of the lipoproteins and the appearance of the pherogram spots (Fig. 3), recentrifugation of LDL and HDL under the conditions described above does not seem to influence significantly the composition and structure of these particles (11).

As shown in Figs. 2 and 3, contamination of the lipoprotein fractions by lipid-containing materials was

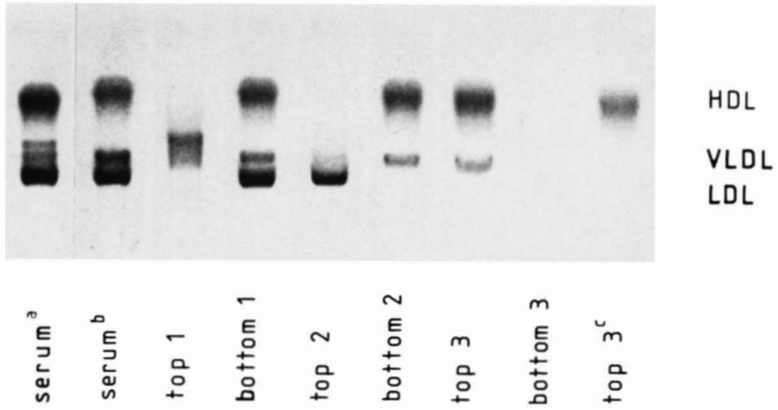

Fig. 5. Serum with a double pre-β-band and "sinking pre-β-lipoprotein;" a, separated by micorelectrophoresis within 3 hr; b, separated on the day after the blood was obtained; c, HDL after precipitation of "sinking pre-β-lipoprotein" by phosphotungstic acid/$MgCl_2$.

either barely visible or undetectable. Contamination of the fractions by apolipoproteins was also either extremely low or undetectable. These statements apply to both the microprocedure and the conventional one. The degree of contamination of the fractions with apolipoproteins is comparable to that reported for conventional ultracentrifugation (12).

Excellent precision and reproducibility of separation as well as recovery of lipoproteins have now been obtained over a period of nearly 3 years, suggesting stability of the entire procedure. Comparison of separation techniques such as ultracentrifugation and electrophoresis revealed that, in sera not exhibiting "sinking pre-β-lipoprotein," α-HDL, pre-β-VLDL and β-LDL correspond closely to each other (Fig. 3). Accordingly, for comparative studies on lipoprotein separation by ultracentrifugation or by electrophoresis, sera without a double pre-β-band and "sinking pre-β-lipoprotein" should be used.

Combination of the Airfuge procedure with HPTLC for detailed lipoprotein analysis was found to be precise and reproducible; recovery of lipoprotein lipids was nearly 100% (Table 3). Therefore, a final test for suitability was carried out with a study on young adults. Again, precision and recovery were nearly 100% (Table 3). Physiological aspects of these results were found to be plausible, but will be discussed elsewhere.

CONCLUSION

Data obtained in this study on detailed analysis of lipoprotein particles on a microscale suggest that the procedure is suited for precise analyses in very small sample volumes (100 μl of capillary serum). This makes the procedure applicable for studies on neonates, even on premature babies. Furthermore, epidemiological studies using capillary blood serum (13–16) as well as experiments on small animals may profit from this procedure.

TABLE 3. Comparison of two procedures for separation of serum lipoproteins, combined with two methods for determination of lipoprotein cholesterol

	α-HDL	Pre-β-VLDL	β-LDL
		mmol/l	
Electrophoresis–enzymatic cholesterol determination	1.49 ± 0.34	0.58 ± 0.42	3.11 ± 0.93
Ultracentrifugation–HPTLC	1.45 ± 0.31	0.56 ± 0.45	3.12 ± 0.93
P	<0.0005	N.S.	N.S.
r	0.959	0.980	0.980

Forty six serum samples from healthy subjects and hyperlipidemic patients of all ages were analyzed. Recovery of lipoprotein cholesterol was 99.2 ± 4.2%.

TABLE 4. Application of the ultracentrifugation-HPTLC procedure for the examination of serum lipoproteins in healthy young subjects

Concentration	Sex	HDL	VLDL	LDL	Serum	Total	Recovery
				mmol/l			%
Phosphatidylcholine (PC)	M	1.17 ± 0.26	0.29 ± 0.13	0.82 ± 0.20	2.29 ± 0.40	2.27 ± 0.40	99.1
	F	1.44 ± 0.44[a]	0.22 ± 0.11[b]	0.84 ± 0.28	2.55 ± 0.63[c]	2.51 ± 0.60	98.4
Phosphatidylethanolamine (PE)	M	0.19 ± 0.04	0.07 ± 0.02	0.14 ± 0.04	0.41 ± 0.06	0.40 ± 0.06	97.6
	F	0.22 ± 0.08	0.06 ± 0.03	0.16 ± 0.08	0.44 ± 0.15	0.43 ± 0.15	97.7
Lyso-PC + sphingomyelin	M	0.23 ± 0.05	0.06 ± 0.03	0.19 ± 0.05	0.49 ± 0.10	0.49 ± 0.10	100
	F	0.27 ± 0.07[d]	0.06 ± 0.03	0.22 ± 0.04[c]	0.55 ± 0.09[d]	0.55 ± 0.09	100
Triacylglycerols	M	0.13 ± 0.04	0.67 ± 0.34	0.25 ± 0.10	1.07 ± 0.37	1.06 ± 0.34	99.1
	F	0.15 ± 0.07	0.54 ± 0.30[c]	0.31 ± 0.14	0.99 ± 0.37	1.00 ± 0.38	101
Free cholesterol	M	0.25 ± 0.07	0.21 ± 0.07	0.70 ± 0.18	1.17 ± 0.22	1.16 ± 0.22	99.1
	F	0.36 ± 0.11[f]	0.17 ± 0.06[b]	0.72 ± 0.17	1.26 ± 0.25	1.25 ± 0.21	99.2
Esterified cholesterol	M	1.06 ± 0.21	0.24 ± 0.08	1.98 ± 0.42	3.30 ± 0.50	3.29 ± 0.48	99.7
	F	1.24 ± 0.24[a]	0.20 ± 0.08[b]	2.01 ± 0.51	3.46 ± 0.56	3.45 ± 0.54	99.7

A group of co-workers and medical students was studied (31 men and 38 women). Levels of significance: [a], $P < 0.005$; [b], $P < 0.05$; [c], $P < 0.07$; [d], $P < 0.01$; [e], $P < 0.03$; [f], $P < 0.0005$.

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