papers and notes on methodology

A simple specific method for precipitation of low density lipoproteins

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Abstract A simple method is described for precipitation of low density lipoproteins. The results correlate well with those from ultracentrifugation in combination with polyanion precipitation (135 ± 35 vs 136 ± 35, r = 0.984, n = 13) or quantitative lipoprotein electrophoresis (r = 0.9287, n = 6394). In addition to lipoprotein analysis, this method may be used for the extracorporeal elimination of plasma low density lipoproteins.—**Wieland, H., and D. Seidel.** A simple specific method for precipitation of low density lipoproteins. *J. Lipid Res.* 1983. **24:** 904–909.

Supplementary key words LDL cholesterol • heparin precipitation • HDL • VLDL

Of the three known major risk factors for coronary heart disease (CHD) hypercholesterolemia, cigarette smoking, and hypertension, hypercholesterolemia is the most prominent. It can, by itself, lead to coronary heart disease at an early age (familial hypercholesterolemia). All clinically significant hypercholesterolemias are due to an increased plasma concentration of low density lipoproteins (d 1.006–1.063 g/ml). Even within normal plasma cholesterol concentrations, an increase in LDL may exist with a concomitant increased risk for coronary heart disease (1). Therefore, prevention and treatment of CHD should focus on reducing the plasma LDL concentration. This has been attempted in the case of homozygous familial hypercholesterolemia by plasma exchange (2), binding of LDL to heparin-Sepharose (3), or surgical intervention (4).

The exact determination of LDL cholesterol by ultracentrifugation is time-consuming and an expensive procedure, whereas the Friedewald formula (5) gives an imprecise value for LDL (6). LDL can also be measured exactly in the form of β-lipoproteins by quantitative lipoprotein electrophoresis (7, 8); however, this technique requires specialized equipment.

In this communication we describe the application of the principle reported by Burstein (9–11) of complete precipitation of apoB-containing lipoproteins from the plasma to the selective precipitation of only low density lipoproteins. Low density lipoproteins can be precipitated with heparin in the absence of additional divalent cations (Mg^{2+}, Ca^{2+}, or Mn^{2+}) by reducing the plasma pH to 5.11. Under these conditions VLDL and HDL remain in solution. For analytical purposes, LDL mass or LDL lipids may then be determined by various techniques, after selective precipitation.

MATERIALS AND METHODS

Precipitation reagents

Sodium citrate buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.04 with 5 N HCl, and containing 50,000 iU/l heparin (Liquemin 25000, Roche).

Precipitation

Samples, control samples, and precipitation reagents were allowed to equilibrate to room temperature. Using a manual pipette or an automatic dispenser, 100 μl of the sample was added to 1 ml of the heparin-citrate buffer. After mixing with a Vortex mixer, 100 μl of the

suspension was removed for cholesterol determination and the remainder was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were then sedimented by centrifugation in an unrefrigerated benchtop centrifuge at 1000 *g* for 10 min. The final pH was 5.11. If higher speeds are used, the temperature should be controlled because of its influence on pH. A 100-μl aliquot of the supernatant was added to 1 ml of cholesterol reagent for cholesterol determination (see below). One hundred μl of the citrate buffer served as a blank.

Calculation of correction factors

The sample was diluted twice during the following procedure. Precipitation: 100 μl sample + 1 ml precipitation reagent. The correction thus involves multiplication by 11. Cholesterol determination: instead of a final volume of 2.02 ml as recommended by most suppliers of enzymatic cholesterol kits, the final volume of the cholesterol assay was 1.1 ml. The correction is multiplication by $\frac{1.1}{2.02}$. However the sample volume was 5-fold that normally used for the cholesterol assay; hence the concentration has to be divided by 5. These corrections can be summarized in a common correction (multiplication) factor: $\frac{11 \times 1.1}{2.02 \times 5} = 1.198$. LDL cholesterol was determined by subtracting the supernatant cholesterol (VLDL + HDL) from the total cholesterol concentration.

Cholesterol determination

Cholesterol was determined in whole plasma or fractions by an enzymatic procedure using a commercially available test kit (CHOD-PAP, Merck Darmstadt, Darmstadt FRG, Boehringer Mannheim, Mannheim FRG).

HDL cholesterol determination

HDL cholesterol was determined by precipitation with heparin and $MgCl_2$ using the test kit supplied by Merck (Darmstadt FRG). Dilutions and calculations were as described for LDL cholesterol.

Immunoelectrophoresis

Immunoelectrophoresis was performed on precast agarose plates (1%) (Lipidophor, Immuno-Diagnostika GmbH, Heidelberg FRG) using antisera to apoA-I, apoB, and apoC (C-I + C-II + C-III) prepared in our laboratory. With this technique one can detect the corresponding apoproteins in serum that has been diluted 1:100 (v/v).

Sample collection and fraction preparation

Serum or plasma (not previously frozen) from fasting or nonfasting subjects can be used for the procedure. In a screening study LDL was measured in serum of 6394 nonfasting male factory workers, age 40–60 years by both the selective precipitation procedure and quantitative lipoprotein electrophoresis. To compare the lipoprotein concentrations obtained by precipitation with those contained by ultracentrifugation, 13 samples were ultracentrifuged in a Beckmann Type 25 rotor for 24 hr at 25,000 rpm at 10°C. Only the peripheral positions were used. Serum (0.8 ml) was layered with 0.2 ml of 0.15 M NaCl and the interphase was marked. After ultracentrifugation, the supernatant (ca 0.4 ml) was aspirated with a syringe and the wall of the tube was blotted with cotton. The volume of the infranatant was adjusted with 0.15 M NaCl to the mark. After complete solubilization and thorough mixing of the infranatants, aliquots were removed for cholesterol determination and precipitation of LDL using either the heparin-citrate method or the commercially available heparin-$MgCl_2$ procedure. In both cases, HDL normally remain in the supernatant and the corresponding LDL concentration can be calculated by difference. VLDL cholesterol concentration is also calculated by difference (whole serum cholesterol minus >1.006 g/ml cholesterol concentration). Since it is possible to determine HDL cholesterol as well as LDL cholesterol from whole serum by precipitation, VLDL cholesterol can also be calculated by difference (whole serum cholesterol minus LDL cholesterol minus HDL cholesterol). For isolation of VLDL, ultracentrifugation was performed on the plasma of healthy donors at serum density following standard procedure (24 hr, 50,000 rpm, 60 TI rotor, 10°C) in a Beckmann L 8 ultracentrifuge. LDL were isolated from the plasma of a hypercholesterolemic subject using sequential ultracentrifugation at the appropriate solution density according to the same procedure. The isolated fractions were then tested for purity by immunoelectrophoresis, dialyzed against 0.15 M NaCl, and subsequently mixed with an equal volume of ten different sera in order to test the specificity of the LDL precipitation. The cholesterol concentration of the VLDL fraction was 100 mg/dl, and that of the LDL fraction was 760 mg/dl.

Evaluation of the precision of the method

The within-assay precision was evaluated by repeated determination of LDL cholesterol (20 times) from a pool of serum, using an automatic dispenser for all pipetting steps. The between-assay precision for a period of 6 months was determined using a lyophilized control

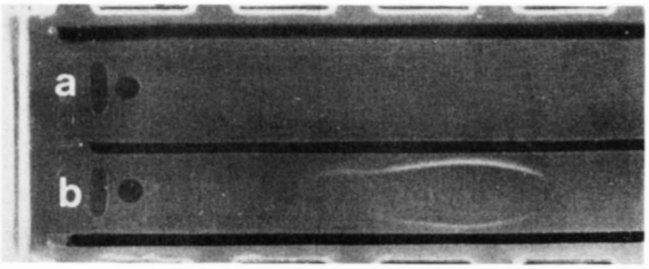

Fig. 1. Immunoelectrophoresis against anti apoA-I (middle trough) and anti apoA-II (upper and lower troughs). In contrast to normal serum (b), no reaction is seen with the dissolved precipitate (a).

serum containing intact human lipoproteins (12, 13) (Lipidophor control, Immuno-Diagnostika GmbH, Heidelberg FRG). To investigate the effects of serum storage on the assay, LDL cholesterol was determined in ten fresh sera and repeated on the same samples after storage for 10 days at 4°C.

RESULTS

Influence of the dilution on cholesterol determination

Cholesterol was determined in 6394 sera using dilutions as indicated by the suppliers of enzymatic cholesterol test kits and compared with the values obtained for the same sera diluted with the precipitation reagent as described in the Methods section. No difference could be observed ($r = 0.982$).

Specificity of the LDL-HDL separation

To investigate whether high density lipoproteins also precipitate under these conditions, the precipitate was dissolved by increasing the pH to 7.2 and submitted to immunoelectrophoresis against anti apoA-I serum. As demonstrated in **Fig. 1,** no precipitin band can be observed in the α position. To test the completeness of the LDL precipitation, VLDL-free sera (d > 1.006 g/ml fractions) were submitted to precipitation by both heparin-citrate and heparin-$MgCl_2$. In both cases the supernatant contained similar amounts of HDL cholesterol (**Table 1,** E and F).

Specificity of the VLDL-LDL separation

To test whether VLDL coprecipitated with LDL under the described experimental conditions, isolated VLDL were added to normal sera; 0.15 M NaCl was used as a control. The increase in total serum cholesterol due to the addition of VLDL was recovered in the supernatant after LDL precipitation (**Table 2**). The difference in the total cholesterol concentration between serum + VLDL and serum and saline (B − A) approx-

TABLE 1. Comparison of the lipoprotein-cholesterol concentration in lipoprotein fractions obtained by ultracentrifugation, polyanion precipitation, and a combination of both

Whole Serum				Ultracentrifugation				
						Precipitation		
A Whole Serum[a]	B HDL(Hep-$MgCl_2$)[b]	C LDL(pH 5.11)[c]	VLDL[d]	D d > 1.006 g/ml (LDL + HDL)	A minus D VLDL	E HDL(Hep-$MgCl_2$)[e]	D minus E LDL	F LDL(pH 5.11) Supernatant
				mg/dl				
285	73	179	33	257	28	75	182	75
203	61	130	12	194	9	64	130	64
213	66	132	15	201	12	60	141	60
210	58	140	12	202	8	56	146	55
218	37	151	30	185	33	38	147	36
186	49	116	21	179	7	63	116	56
258	51	171	36	225	33	61	164	68
219	94	116	9	210	9	82	128	85
162	34	97	31	127	35	30	97	29
187	47	118	22	166	21	45	121	43
197	63	117	17	191	6	67	124	75
116	28	79	9	97	19	25	72	26
295	55	207	33	243	53	42	201	47
$\bar{X}$ 205 + 42	55 ± 17	135 ± 35	21 ± 10	190 ± 43	21 ± 14	54 ± 17	136 ± 35	55 ± 18

[a] A, whole serum cholesterol.
[b] B, HDL cholesterol (heparin-$MgCl_2$ precipitation in whole serum).
[c] C, LDL cholesterol (heparin precipitation, pH 5.11).
[d] VLDL represents cholesterol in heparin-citrate supernatant (VLDL + HDL) minus cholesterol in heparin-Mg^{2+} supernatant (HDL).
[e] E, LDL cholesterol in d > 1.006 g/ml (heparin-$MgCl_2$) fraction.
[f] F, HDL cholesterol in d > 1.006 g/ml fraction after precipitation of LDL at pH 5.11.

TABLE 2. Cholesterol concentrations of different sera mixed 1:1 (v/v) with 0.9% NaCl, VLDL, or LDL and of the corresponding supernatants after LDL precipitation

Total Cholesterol			(VLDL + HDL) Cholesterol[a]			Added VLDL	
A Serum + Saline	B Serum + VLDL	C Serum + LDL	A_1	B_1	C_1	B − A	$B_1 - A_1$
			mg/dl				
151	196	519	37	83	45	45	46
131	186	527	39	92	42	55	53
142	189	541	32	75	36	47	43
139	185	537	55	113	50	46	58
163	218	559	33	76	33	55	43
85	135	465	27	75	23	50	48
107	159	480	71	112	69	52	41
79	126	461	27	66	27	47	39
91	144	467	60	106	64	53	46
83	132	461	32	77	37	49	40
$\bar{X}$ 117 ± 31	167 ± 32	502 ± 41	41 ± 15	87 ± 27	43 ± 15	50 ± 4	46 ± 6

[a] Supernatant after addition of heparin-citrate solution.

imated that found in the respective supernatants ($B_1 - A_1$) after precipitation.

To demonstrate that VLDL does not coprecipitate even in the presence of large amounts of LDL, isolated LDL were added to the same serum samples before precipitation. No marked differences in the cholesterol content of the precipitation supernatant of either serum samples diluted with 0.15 M NaCl or enriched with LDL fractions were apparent (Table 2, A_1, C_1).

Correlation of LDL cholesterol determined after precipitation and ultracentrifugation

The data given in Table 1 demonstrate the excellent agreement of LDL cholesterol determined by precipitation from whole serum (column C) and by precipitation from the corresponding d > 1.006 g/ml fraction by heparin-$MgCl_2$ (D minus E, right side on Table 1): y (heparin-citrate, column C) = 1.01x − 2.8, r = 0.984. Both types of precipitation (the heparin-citrate and heparin-$MgCl_2$) also gave the same HDL cholesterol values when performed on the d > 1.006 g/ml fraction: y (heparin-citrate, column F) = 1.04x − 1.48, r = 0.976.

Correlation of β-lipoprotein cholesterol (quantitative lipoprotein electrophoresis) and LDL cholesterol (precipitation)

There was good correlation between LDL cholesterol and β-lipoprotein cholesterol obtained on 6394 patients: r = 0.9287, y (LDL cholesterol) = 1.021x − 5.62 ($S_{y/x}$ = 13.58). The correlation improved somewhat if only the 5717 individuals with triglycerides not exceeding 400 mg/dl were considered: r = 0.9394, y (LDL cholesterol) = 1.027x − 7.474 ($S_{y/x}$ = 12.2) (**Fig. 2**). For individuals exceeding 400 mg/dl (n = 677), a slight deterioration was observed: r = 0.8779, y (LDL cholesterol) = 0.95x + 13.6 ($S_{y/x}$ = 19.7) (**Fig. 3**). **Fig. 4** shows the cumulative frequency curves of β-lipoprotein-cholesterol and LDL cholesterol of the total population (men, 40–60 years old). Complete congruence of the two curves is demonstrated at values exceeding 130 mg/dl. Below this concentration, the precipitation technique reveals somewhat lower results than the quantitative lipoprotein electrophoresis.

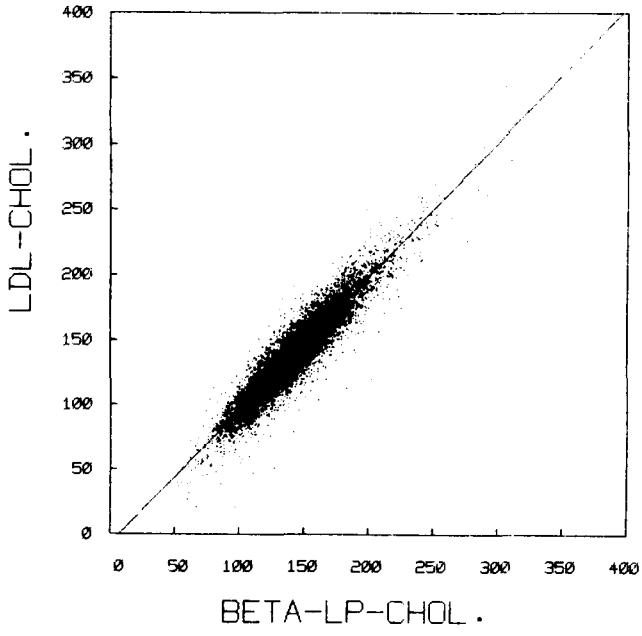

Fig. 2. Correlation of LDL cholesterol determined by the heparin-citrate method, with β-lipoprotein cholesterol determined by quantitative lipoprotein electrophoresis. The data were obtained from a population of 5717 men between the ages of 40 and 60 years, with plasma triglyceride concentrations below 400 mg/dl.

Precision of the method

A coefficient of variation (CV) of 2.8% ($\bar{x}$ = 148 mg/dl, SD = 4.14) was found for the within-assay precision (20 LDL-cholesterol determinations of the same serum) if an automatic dispenser was used. The between-assay precision for a period of 6 months yielded a CV of 3.7% ($\bar{x}$ = 148 mg/dl, SD = 4.14). LDL cholesterol values were essentially the same after storage (10 days at 4°C).

DISCUSSION

The determination of plasma LDL by precipitation using a citrate buffer and heparin at pH 5.11 reveals a strong correlation with results obtained by quantitative lipoprotein electrophoresis and by ultracentrifugation. It does not appear to be greatly influenced by the triglyceride content of the sample and can be performed on sera that have been kept at 4°C for up to 10 days. Sample dilution can be compensated for by use of a larger sample volume without loss of accuracy in the cholesterol determination. The precipitation step has been shown to be specific and complete for low density lipoproteins up to high concentrations. Neither high density lipoproteins nor very low density lipoproteins are coprecipitated. Preliminary results (data not shown) indicate that whereas type III VLDL is not precipitated, LP(a) is partially coprecipitated. If desired, the precipitate can also be analyzed for cholesterol, other lipids, lipoproteins, or apoproteins, since it can be easily dissolved by increasing the pH. The reagents for the procedure are inexpensive and the method can be easily performed with high precision in any laboratory and may also be automated. For this reason and because of the pathophysiological importance of LDL in atherogenesis, this technique may very well be suitable for mass screening.

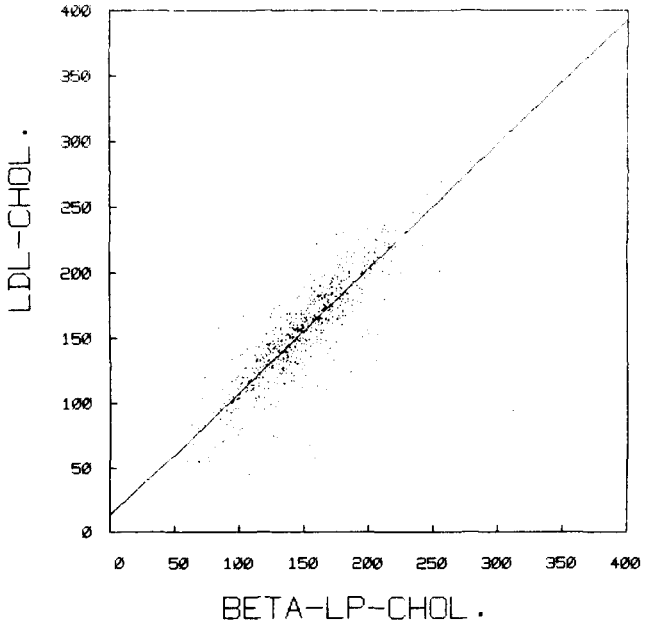

Fig. 3. Correlation of LDL cholesterol and β-lipoprotein cholesterol in 677 individuals with plasma triglyceride concentrations above 400 mg/dl.

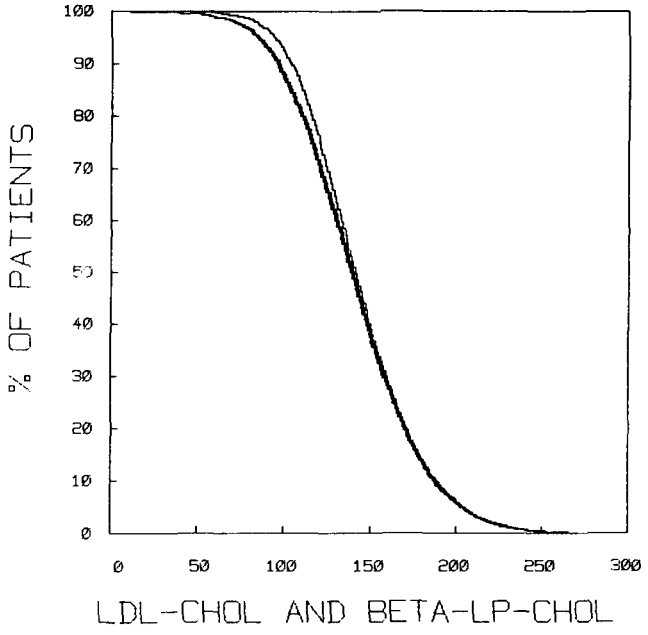

Fig. 4. Cumulative frequency curves of the concentrations of β-lipoprotein cholesterol (thin line) and LDL cholesterol (solid line). At a concentration of 130 mg/dl cholesterol, the curves merge and become congruent.

The principle of the method offers the unique possibility of precipitating lipoproteins in an extracorporeal system using only physiological polyanions such as heparin and no cations. For therapeutical purposes, a continuous flow system is being developed in which plasma is separated by ultrafiltration and then mixed with an appropriate buffer containing heparin. The LDL-heparin precipitate is removed by filtration and the LDL-free plasma is dialyzed to restore physiologic pH and correct volume before reinfusion to the patient. Besides low density lipoproteins, only minor and varying amounts of fibrinogen are precipitated from the plasma at the recommended pH. Preliminary findings of experiments with animals or voluntary humans (members of the research group) indicate that the procedure suffers from no significant or undesired side effects. Details will be reported in due time.

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