

Improved Assessment of Plasma Lipoprotein Patterns. III. Direct Measurement of Lipoproteins after Gel-Electrophoresis

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Based on a previously described technique [*Clin. Chem.* 19, 737 (1973)] of precipitating plasma lipoproteins with polyanions after their electrophoretic separation in gels, a new method is presented for measuring normal plasma lipoproteins densitometrically. The method is fast and easy; the CV for β -, pre- β -, and α -lipoproteins was <5% in one series. Results are linearly related to concentration up to 10 g of total lipoprotein per liter. No unusual equipment is required. Standardization is done with the aid of a commercially available filter. Total plasma cholesterol and cholesterol calculated from quantified lipoprotein fractions were highly ($r = 0.963$) correlated.

Additional Keyphrases: *electrophoresis in agarose · serum lipoprotein pattern · densitometry after precipitation with Ca/dextran sulfate*

In addition to measurements of plasma total cholesterol and plasma triglycerides (triacylglycerols), assessment of plasma lipoprotein patterns has gained significance in the diagnosis of disorders of lipid metabolism. The aim of translating hyperlipidemia into its underlying lipoprotein abnormalities was the basis for the introduction of a system by Fredrickson et al. (1) of classification of hyperlipoproteinemia into five phenotypes, later modified into six phenotypes (2).

Since then, information has accumulated indicating that the various lipoprotein fractions—all of which have cholesterol, triglycerides, and phospholipids as components—behave differently in their interaction with cells in culture and may influence human atherogenesis in different or even opposite ways. For meaningful clinical trials in the future it will therefore be important to have a means of quantifying the plasma lipoprotein fractions in a practical and reproducible way.

A first step towards to fulfilling these requirements was to introduce the use of polyanionic compounds for making visible the lipoprotein bands after electrophoresis in gels (3). Here, we present a technique of quantifying these fractions by densitometry.

Material and Methods

Samples

Blood was sampled from apparently healthy controls and from patients with well-classified hyperlipoproteinemia (IIa, IIb, and Type IV; 3♂, 3♀, 32–56 a) (1, 2) after the subjects had fasted overnight. The serum was promptly separated by low-speed centrifugation. (Plasma should not be used for this technique.)

Isolation of Pure Lipoprotein Fractions

For the isolation of large amounts of pure lipoprotein fractions, two voluntary healthy control donors, two voluntary Type II donor hyperlipoproteinemics, and two voluntary Type IV hyperlipoproteinemic donors underwent plasmapheresis after an overnight fast, and the plasma was collected in bags containing sodium citrate (50 g/liter) as anticoagulant. The isolation scheme is outlined in Figure 1. All ultracentrifugations were done at $150\,000 \times g$ for 22 h at 15 °C in a type 65 TI or type 50 TI rotor of the Omega II ultracentrifuge (Heraeus Christ, Osterode, G.F.R.) and the resulting fractions were separated by tube-slicing. After isolation, the pre- β -lipoprotein band corresponded to lipoproteins of density class $d = 0.95$ – 1.006 g/ml; the β -lipoprotein band to those of the density class $d = 1.02$ – 1.06 g/ml, and the α -lipoprotein band to the filtrate after Na heparin/MgCl₂-precipitation (4) of density class $d < 1.21$ g/ml. The isolated lipoprotein fractions were tested by lipoprotein electrophoresis for freedom from contamination by other lipoproteins and for freedom from contamination with plasma proteins by immunoelectrophoresis vs. an antiserum we produced against human serum devoid of apoproteins. The purified lipoprotein fractions were dialyzed against distilled water at 4 °C for 48 h, and then three 1-ml aliquots were lyophilized and weighed (electronic microbalance) to determine the exact concentration of the lipoproteins in the preparation. Human albumin (Behring-Werke, Marburg/Lahn, G.F.R.) in a final concentration of 50 g/liter was then added to the isolated preparation to preserve the stability of the lipoproteins and make the

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experimental conditions comparable to those of whole serum.

Lipoprotein Electrophoresis

To determine the concentration of serum lipoproteins, we electrophoresed the samples in agarose (8 g/liter; Serva, Heidelberg, G.F.R.) containing human albumin (2 g/liter; Behring-Werke) in barbital buffer (pH 8.6, 50 mmol/liter) on glass plates (75 × 25 × 10 mm) in an LKB electrophoresis system (LKB, Bromma, Sweden). Before electrophoresis each sample was carefully mixed with an equal volume of the agarose solution (30–40 °C) and 10 μl of the mixture was applied to the gel and allowed to migrate for 90 min at 10 V/cm and 40 mA. Immediately after electrophoresis the plates were placed for 4 h at room temperature (for time dependence see *Results*) in a bath consisting of 0.2 mmol of CaCl₂ containing 6 g of Na dextran sulfate, 500 per liter (Pharmacia, Uppsala, Sweden), to completely precipitate all lipoprotein bands.

Quantitation of Lipoproteins

Serum samples, 5 μl, were agarose-electrophoresed with the LKB-system for 90 min and subsequently incubated for 4 h with the polyanionic compounds as described. The plates were then submitted to densitometry. On each sample the total area of all precipitated lipoprotein bands was measured. The conditions used for densitometry were:

Instrument:	Integraph CH (Bender and Hobein, Zürich, Switzerland)
Standardization settings:	Adjustment to 100
wavelength:	500 nm
filter:	E. 0.25 (Type N G 11, Schott, Mainz, G.F.R.)
diaphragm:	2.5
amplification:	1
distance:	4 cm
Measurement settings:	
wavelength:	500 nm
diaphragm:	5
amplification:	6
sensitivity setting:	maximum

From the standard curve prepared separately for each lipoprotein fraction the concentration is calculated as follows:

Pre-β-lipoprotein (VLDL): (Absorbance* × 0.09) – 0.014 = pre-β-LP, g/liter

β-Lipoprotein (LDL): (Absorbance* × 0.047) – 0.007 = β-LP, g/liter

α-Lipoprotein (HDL): (Absorbance* × 0.056) – 0.0025 = α-LP, g/liter

*linear absorbance as percentage of the standard setting as 100.

The densitometer we used (Integraph CH) can automatically scan electrophoretic and other separations. It measures photoelectrically the absorbance of electrophoretic separations and records a linear extinction curve with the aid of a logarithmic amplifier. The area of the fraction is determined automatically and the value indicated in digital terms (linear extinction) as percentage of the standard setting = 100.

Chemical Determinations

We measured total cholesterol concentration of samples and of isolated fractions with an SMA 12/60 AutoAnalyzer (Technicon, Tarrytown, N. Y. 10591), using a double enzymatic method (Boehringer GmbH, Mannheim, G.F.R.).

Results

As indicated in Figure 1 the isolated VLDL, LDL, and HDL fractions migrating as pre-β-, β-, and α-lipoproteins used as reference to establish the standard curves had an electrophoretic mobility and precipitation behavior identical to that earlier described for whole serum (3). Immunochemically, these fractions did not react against antibodies other than those for apoproteins. Each experimental point of the standard curve (Figure 2, a, b, and c) was obtained with lipoprotein preparations of six different subjects (two normal controls, two Type II, and two Type IV hyperlipoproteinemics). The curves showed a linear response from 1 to 16 g/liter for pre-β-lipoproteins and from 1 to 10 g/liter for β- and α-lipoproteins, with no difference between control subjects and hyperlipoproteinemic patients. Lipoprotein concentrations in sera are generally within this range, but in severe cases of Type IV hyperlipoproteinemia in whom triglyceride values exceed 20 g/liter higher lipoprotein concentrations are possible; then dilution of the serum with an equal volume of 50 g/liter albumin solution will improve the quantitation.

The coefficient of variance in one series (n = 20) for this technique was 3.3% for β, 3.4% for pre-β-lipoproteins, and 4% for α-lipoproteins.

To establish the best incubation period with polyanionic compounds, we incubated isolated lipoprotein

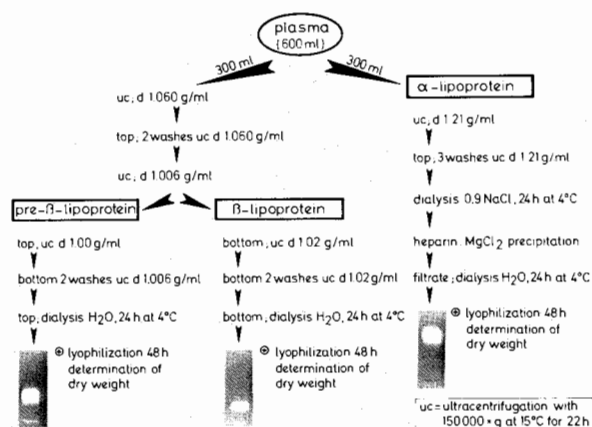


Fig. 1. Scheme for isolation and purification of plasma lipoprotein fractions

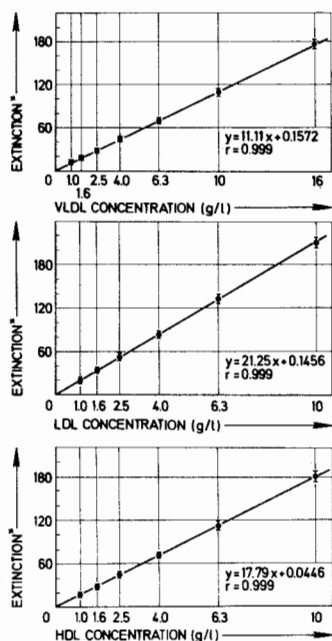


Fig. 2. Standard curves (top to bottom) for pre-β-LP:VLDL (a); β-LP:LDL (b); and α-LP:HDL (c), as determined by densitometry of the agarose electropherograms of lipoprotein preparations obtained from six different subjects (two controls, two Type II, and two Type IV hyperlipoproteinemics)

* Extinction (Absorbance) = absorbance automatically calculated and expressed as percentage of the standard setting (100)

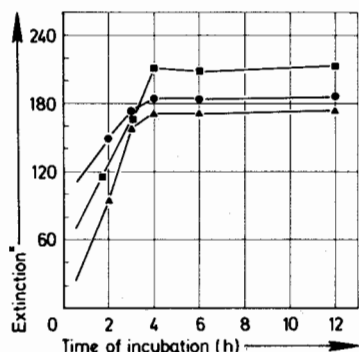


Fig. 3. Time curve for the different lipoprotein bands
Incubation time for polyanionic compounds after agarose electrophoresis
▲ pre-β-lipoproteins; ● β-lipoproteins; ■ α-lipoproteins

fractions in high concentrations (β- and α-lipoproteins 10 g/liter; pre-β-lipoproteins 16 g/liter) for 12 h with the precipitation solution and performed densitometry at hourly. As indicated in Figure 3, precipitation was maximal after 4 h of incubation in all cases. Therefore all other measurements were done after 4 h of incubation. The time needed for complete lipoprotein quantitation by this technique is therefore <6 h. In a series of samples, about 3 min of manual work is required for each sample. The concentration of β-, pre-β-, and α-lipoproteins in various sera can now easily be determined; results for typical samples are shown in Figure 4.

To prove the correlation among total plasma cholesterol, its distribution in lipoprotein fractions, and the amount of cholesterol calculated from the quantitation

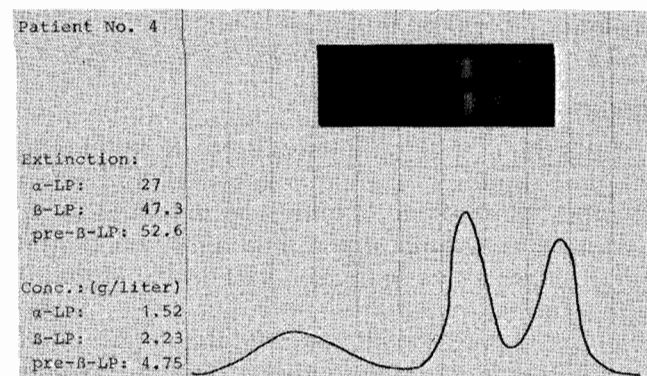
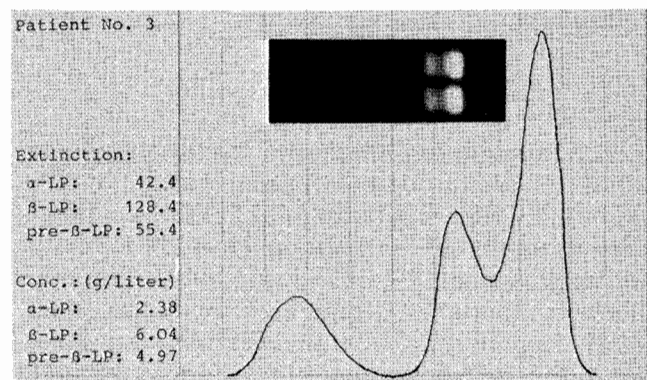
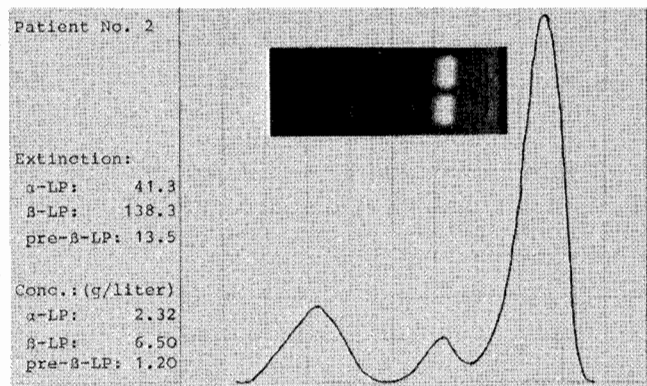
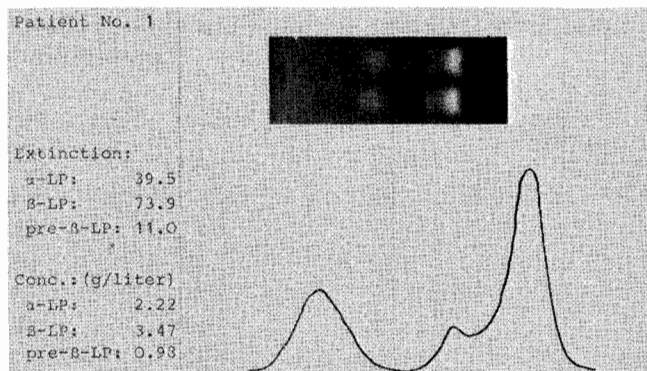


Fig. 4. Quantitation of plasma lipoproteins in different samples

Top to bottom: (a) normal pattern tot. chol.: 2.10 g/liter; TG = 0.96 g/liter; (b) type IIa pattern tot. chol.: 3.55 g/liter; TG = 1.28 g/liter; (c) type IIb pattern tot. chol.: 3.89 g/liter; TG = 3.32 g/liter; (d) type IV pattern tot. chol.: 2.12 g/liter; TC 2.71 = g/liter. Patient 1, ♂, 23 a; patient 2, ♂, 18 a; patient 3, ♀, 22 a; patient 4, ♀, 42 a

Table 1. Comparison of Total Plasma Cholesterol vs. Cholesterol as Calculated from the Lipoprotein Bands after Densitometry.

Fraction sample	β -LP	pre- β -LP	α -LP	Chol., calculated g/liter	Total plasma-cholesterol
1	6.59	1.20	1.72	3.50	3.55
2	6.09	4.62	1.48	3.89	3.60
3	4.75	1.13	3.98	3.07	3.31
4	3.82	3.10	1.35	2.56	2.46
5	3.99	2.24	1.84	2.54	2.52
6	4.17	0.71	2.00	2.38	2.65
7	3.03	1.52	1.20	1.87	1.86
8	4.20	0.74	4.53	2.85	2.63
9	3.30	2.21	2.11	2.28	2.49
10	3.30	1.80	2.89	2.34	1.92
11	3.90	0.63	4.80	2.74	2.55
12	7.99	2.28	0.55	4.13	4.31
13	3.28	0.91	3.47	2.27	2.28
14	5.50	1.37	1.68	3.04	2.97
15	4.71	1.22	1.02	2.53	2.54
16	3.10	1.05	7.32	2.91	2.83
17	3.82	3.11	1.35	2.55	2.11
18	6.04	4.97	2.38	4.09	3.89
19	3.47	0.98	2.22	2.13	2.10
20	6.50	1.20	2.32	3.58	3.55

Percent cholesterol content in β -LP, 45; in pre- β -LP, 19; and in α -LP, 18. $n = 20$; correl. coeff. for total plasma cholesterol vs. calculated cholesterol, $r = 0.963$.

of the corresponding lipoprotein fractions, we determined cholesterol in each isolated fraction and expressed it in per cent of total weight (Table 1). The results obtained (45% for β -lipoproteins, 19% for pre- β -lipoproteins, and 18% for α -lipoproteins) agree with literature reports (5). Comparing total plasma cholesterol values for 20 different samples with total cholesterol calculated from densitometric data on the different lipoprotein fractions and using the indicated key of cholesterol composition (Table 1 and Figure 5), we found a high correlation ($r = 0.9633$; $P < 0.001$).

Discussion

Translation of hyperlipidemia into hyperlipoproteinemia requires quantitation of plasma lipoprotein fractions. Methods so far developed to fulfill this requirement are laborious, time consuming, and depend either on expensive equipment such as the ultracentrifuge or, when based on electrophoresis, involve complicated procedures and standardization (6). It has been known for many years that plasma lipoproteins can be precipitated selectively with polyanions and divalent cations (7, 8). In the first communication of this series (3), we demonstrated that such precipitation is also possible in gels after the lipoproteins have been separated by electrophoresis. On the basis of these findings we herein have described a fast, easy, and inexpensive method for quantifying lipoproteins by densitometry of the electrophoretically separated bands. Because of complete precipitation of all lipo-

proteins (3) it was assumed that the lipoprotein bands make visible by polyanion precipitation reflect the actual concentration of each lipoprotein fraction much better than do the bands obtained by various lipid-staining procedures, which not only are known to depend on the concentration of each fraction but are also known to be affected by dissimilar composition and particular methodological requirements (6). This assumption has been proved in this study when total plasma cholesterol was compared with cholesterol calculated from quantified lipoprotein fractions (Table 1). The additional advantage for routine and experimental work of the method described in the present paper is its

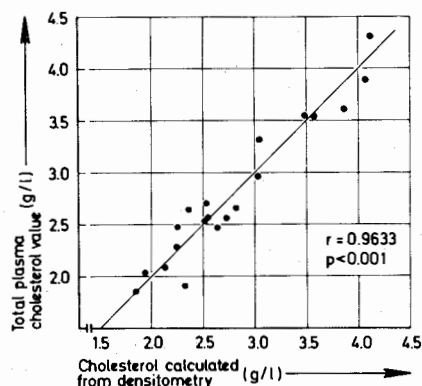


Fig. 5. Comparison of total plasma cholesterol concentrations vs. cholesterol estimated by the described method after densitometry of the lipoprotein bands

simplicity and accuracy (CV <5%) and the wide range of concentration in which the lipoproteins can be assayed. It must, however, be clearly stated, that standardization of the system holds only for plasma-lipoproteins of normal composition and electrophoretic mobility. For abnormal plasma lipoproteins such as lipoprotein-X special equipment and a special standardization system are required (9).

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