Journal of Chromatography, 570 (1991) 382–389
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6012

Short Communication

Comparison of gel permeation chromatography, density gradient ultracentrifugation and precipitation methods for quantitation of very-low-, low- and high-density lipoprotein cholesterol

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(First received January 17th, 1991; revised manuscript received May 21st, 1991)

ABSTRACT

Human VLDL, LDL and HDL (very-low-, low- and high-density lipoproteins) were isolated from plasma by gel permeation chromatography with one pre-ultracentrifugation step. The column effluent was monitored at 280 nm. The cholesterol content of the fractions correlated well with fractions from sequential ultracentrifugation (VLDL, r = 0.839; LDL, r = 0.924; HDL, r = 0.766) or precipitation (LDL, r = 0.975; HDL, r = 0.972) methods. The average triglyceride, phospholipid and protein compositions of the separated lipoprotein fractions were close to those of the ultracentrifugally isolated fractions reported previously. Apolipoproteins A1 and B were determined from fractions to confirm the right distribution between different lipoproteins.

INTRODUCTION

Plasma lipoproteins are classically separated by density-gradient ultracentrifugation [1–5], which fractionates the lipoproteins according to their densities. Sequential density ultracentrifugation has been the principal method used for the isolation and classification of lipoproteins [1]. Currently, methods using gel permeation chromatography have proved to be efficient, rapid and easily automated [6–12]. Gel permeation chromatography separates lipoproteins according to the particle size. Since the cholesterol content of lipoproteins, especially that of LDL (low-density lipoprotein), is a significant risk factor for coronary heart disease, many simpler and faster separation methods for lipoproteins have been presented for clinical purposes. Among these, the precipitation methods for LDL and HDL (high-density lipoprotein) are the most widely used.

We have used gel permeation chromatography for the separation of VLDL (very-low-density lipoprotein), LDL and HDL to obtain lipoprotein fractions

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free from other plasma proteins. Our method is an application of the general scheme [6–9], with one ultracentrifugation step and gel chromatography on modified agarose columns. We compared our method with the classic ultracentifugation method [1] for the quantitative determination of VLDL, LDL and HDL cholesterol in human plasma. Moreover, we determined LDL and HDL cholesterol from the same samples with commercially available precipitation methods.

EXPERIMENTAL

Plasma samples

Blood samples were obtained from seven men and eight women, aged 28–60 years. Total plasma cholesterol varied in the range 4.09–7.37 mmol/l. Blood was collected into K_3EDTA tubes (Venoject, Terumo, Leuven, Belgium). Blood was cooled to 4°C, and plasma was rapidly separated from the cells at 1500 *g* for 10 min. For gel permeation we used 6 ml of plasma, and for sequential gradient ultracentrifugation 3 ml of plasma. Precipitation methods needed 0.2–0.5 ml of plasma.

Ultracentrifugation for gel permeation chromatography

A Beckman XL-90 ultracentrifuge (Palo Alto, CA, USA) with the type 50.4 Ti rotor was used to separate lipoproteins from human plasma. The total lipoprotein fraction was prepared by adding solid potassium bromide to plasma to obtain the requird density, 1.22 g/ml [13], and centrifuging it in Beckman Quick-Seal polyallomer tubes for 23 h at 218 000 *g* and 10°C. The lipoprotein fraction was removed from the top of the tube using a Pasteur pipette. This lipoprotein mixture was concentrated by using Centricon miniconcentrators (Amicon, Danvers, MA, USA), with a cut-off at 10^5 daltons, and filtered through 0.2-μm Acrodisc filters (Gelman Sciences, Ann Arbor, MI, USA) before injection into the gel chromatographic column.

Density gradient ultracentrifugation

Classic sequential density-gradient ultracentrifugation was carried out according to Havel *et al.* [1]. The fractions of VLDL, LDL and HDL were obtained after three different ultracentrifugation steps. We used a Beckman XL-90 ultracentrifuge with the type 50.4 Ti rotor, and polyallomer centrifuge tubes. The VLDL fraction was isolated as the fraction of density < 1.006 g/ml after 18-h centrifugation at 132 000 *g*, LDL with density < 1.063 g/ml after 24-h centrifugation, and HDL with density < 1.21 g/ml after 48-h centrifugation.

Gel permeation chromatography

A fast protein liquid chromatographic (FPLC) system (Pharmacia, Uppsala, Sweden) was used. The system consisted of a P-500 pump, an LCC-500 plus controller, a UV-1 single-path monitor and a REC-482 two channel recorder.

Samples were introduced via a P-1 peristaltic pump and an MV-7 motor valve with 500-μl loop. We used two Superose 6 (Pharmacia) columns in series for the separation of the lipoprotein fractions. The mobile phase consisted of sodium phosphate buffer with 1 m*M* EDTA and 0.02% NaN_3 (pH 7.2). The separated lipoproteins were collected by the Frac-200 fraction collector (Pharmacia) in 0.5-ml fractions. The elution of lipoproteins was monitored at 280 nm.

Precipitation methods and the determination of cholesterol, triglycerides, phospholipids, proteins and apolipoproteins

For the determination of HDL cholesterol, $MgCl_2$–dextran sulphate was used to precipitate VLDL and LDL [14,15]. HDL cholesterol was measured from the supernatant.

LDL was precipitated by poly(vinyl sulphate) (PVS method, Boehringer, Mannheim, Germany) and cholesterol was measured from the supernatant. The content of LDL cholesterol was calculated by subtracting the cholesterol of the supernatant from the total plasma cholesterol.

The cholesterol contents of the plasma and the fractions from density gradient ultracentrifugation, from FPLC and from precipitation methods were determined by an enzymic colorimetric method (Kone Oy, Espoo, Finland).

Triglycerides from FPLC fractions were measured by an enzymic method (Boehringer Mannheim) and choline-containing phospholipids with an colorimetric method (Bio Mérieux, Marcy-l'Etoile, France). The protein contents of the fractions were determined by a sensitive pyrogallol reagent (Labport, Tampere, Finland). The pyrogallol method was standardized against the Bio-Rad protein assay standard (Bio-Rad Labs., Richmond, CA, USA). Apolipoproteins A1 and B were analysed by an immunoturbidimetric method (Kone Oy). All chemical analyses were carried out by an automated analyser Kone Specific (Espoo, Finland).

RESULTS AND DISCUSSION

The separation of at least three lipoprotein fractions by FPLC was good. There was no overlap between VLDL, LDL and HDL in their UV absorption at 280 nm (Fig. 1) In some samples with intermediate-density lipoprotein (IDL) the fourth peak between VLDL and LDL was separated (Fig. 1). HDL eluted as two peaks near each other, probably corresponding to HDL2 and HDL3 in ultracentrifugation. The separation of the lipoprotein fractions took 78 min, but the total run time needed was 105 min because of the small plasma proteins with long retention times in gel permeation.

We determined the coefficients of variation (C.V.) for the FPLC method by analysing the cholesterol content from a plasma sample after having run it ten times through the FPLC system. For VLDL, LDL and HDL cholesterol the means and C.V. (in parentheses) were 0.37 mmol/l (5.4%), 3.41 mmol/l (0.8%) and 1.55 mmol/l (1.4%), respectively.

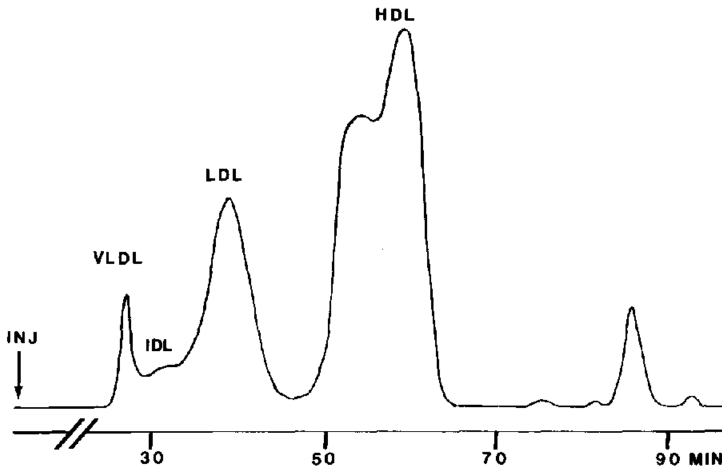

Fig. 1. Typical elution profile of human lipoproteins from the FPLC system after ultracentrifugation for 23 h at a density of 1.22 g/ml. The detection wavelength was 280 nm, and other conditions are described in the text.

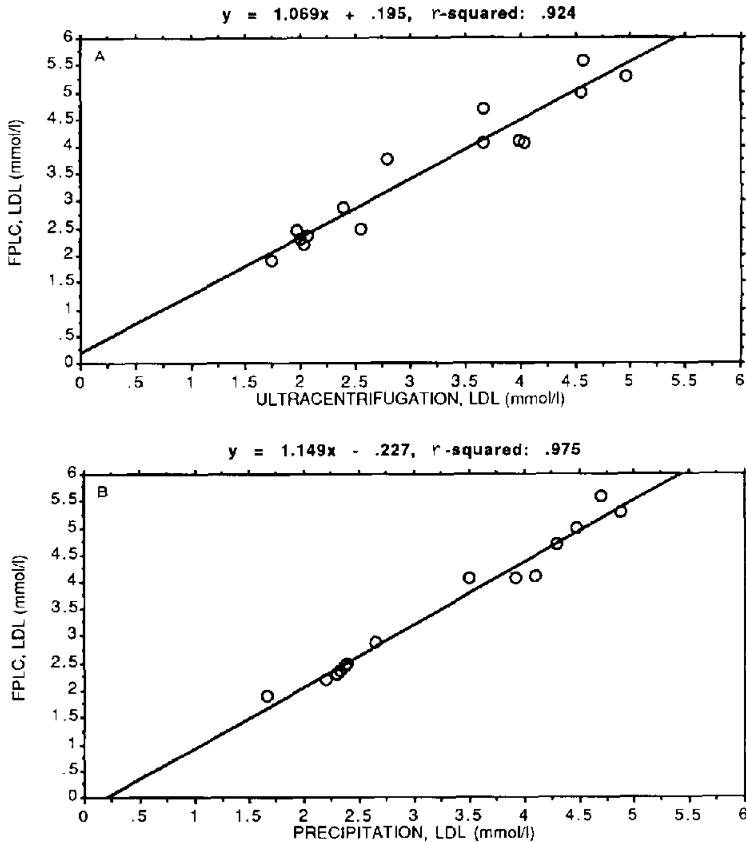

Fig. 2. Correlation of LDL cholesterol values (A) between ultracentrifugation and FPLC methods and (B) between poly(vinyl sulphate) precipitation and FPLC methods.

For LDL cholesterol, FPLC correlated well with both the ultracentrifugation (r = 0.924) and the precipitation (r = 0.975) methods (Fig. 2). In our data, IDL has been included with LDL. For HDL cholesterol there was a slightly weaker correlation (r = 0.766) between FPLC and ultracentrifugation (Fig. 3A). HDL separation with sequential ultracentrifugation demands three different centrifugation steps, which may easily lead to the variation of the results because of the possible mistakes during pipetting. The HDL precipitation method gave a very good correlation (r = 0.972) with FPLC separation (Fig. 3B). In Fig. 3A and B the slopes would be even better without the single point over 2.2 mmol/l; the slope would be 1.15 for FPLC *versus* ultracentrifugation and 1.19 for FPLC *versus* precipitation. For VLDL cholesterol, results from FPLC and ultracentrifugation gave a correlation of 0.839, with no bias in the linear regression equation (Fig. 4).

Our FPLC system for separating lipoproteins from human plasma proved to be time-saving compared with sequential ultracentrifugation. In our system, eight

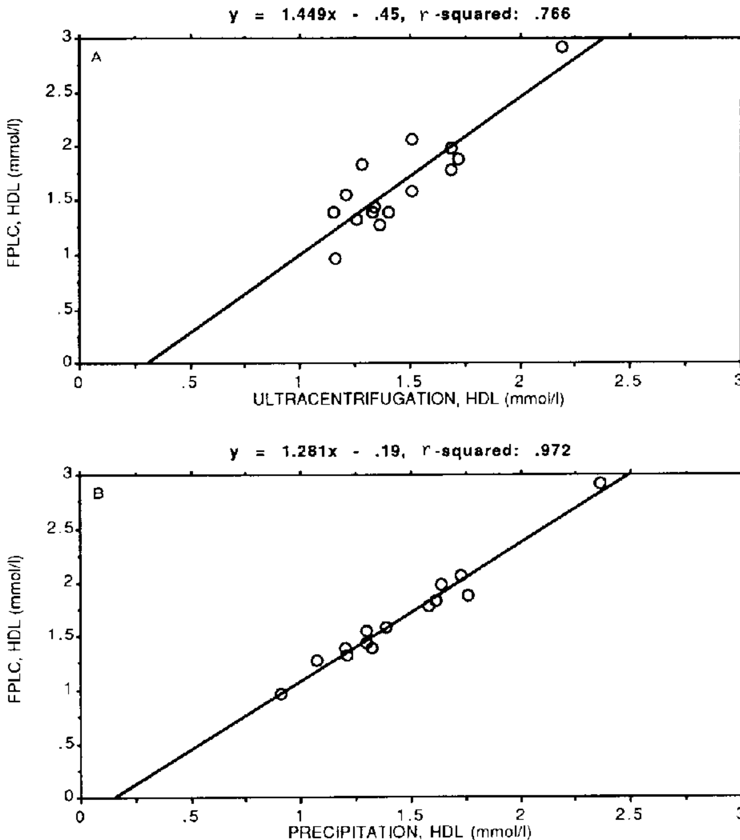

Fig. 3. Correlation of HDL cholesterol values (A) between ultracentrifugation and FPLC methods and (B) between $MgCl_2$–dextran sulphate precipitation and FPLC methods.

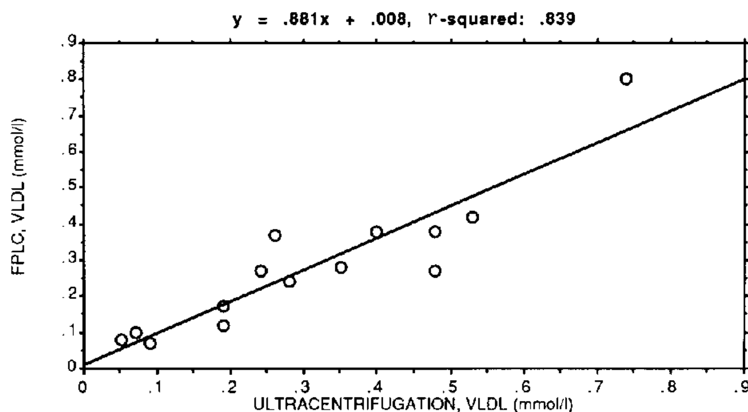

Fig. 4. Correlation of VLDL cholesterol values between ultracentrifugation and FPLC methods.

samples could be injected and fractions collected automatically in the same run. The lipoprotein fractions from FPLC are practically free from other plasma proteins, which is very useful when the content of pure lipoproteins is of interest. We ran some plasma samples by sequential ultracentrifugation to obtain VLDL, LDL and HDL, and then injected these fractions separately into the FPLC system. There were numerous other plasma proteins present in every ultracentrifuged lipoprotein fraction. These other plasma proteins had retention times of 70–100 min and were eluted after HDL in FPLC (Fig. 1). These peaks were collected, and cholesterol was determined. No detectable cholesterol concentration was found in these fractions.

We used ultracentrifugation as a preliminary step before gel permeation in order to separate the total plasma lipoproteins. Gel permeation with a Superose 6 column has been used in lipoprotein analysis without any ultracentrifugation; filtered plasma has been directly injected into a chromatographic system [11]. However, that application produces lipoprotein fractions contaminated with other plasma proteins, *e.g.* immunoglobulin M in LDL and HDL fractions [11]. Moreover, the elution of lipoproteins would be difficult to monitor through UV absorption.

The average lipid and protein compositions of the separated lipoprotein fractions from 68 volunteers are shown in Table I. The values are close to those of the ultracentrifugally separated fractions [16]. We also determined apolipoproteins A1 and B concentrations from the lipoprotein fractions of six healthy volunteers. Apolipoprotein A1 was present only in the HDL fraction, and apolipoprotein B was for the most part found in the LDL (86.9 ± 3.2%) and IDL (12.6,± 3.2%) fractions. VLDL contained 0.4 ± 0.9% of the whole apolipoprotein B found in fractions. Apolipoprotein B was not detectable in HDL fractions. This distribution of apolipoproteins between different lipoprotein fractions separated by gel permeation is similar to the composition of ultracentrifugally isolated lipopro-

TABLE I

COMPONENTS OF PLASMA LIPOPROTEINS ISOLATED WITH GEL PERMEATION CHROMATOGRAPHY

Lipoprotein component	Amount (mean ± S.D., n = 68) (g%)			
	VLDL	IDL	LDL	HDL
Triglycerides	60.8 ± 3.8	25.5 ± 6.1	8.4 ± 2.1	2.8 ± 1.1
Cholesterol	11.1 ± 2.8	31.4 ± 4.2	42.5 ± 1.8	13.8 ± 1.6
Phospholipids	13.3 + 3.0	20.3 ± 3.2	24.6 ± 1.0	23.2 ± 1.6
Procins	14.8 ⊥ 3.0	22.8 ± 3.6	24.5 + 1.2	60.2 ± 2.4

teins [17]. Van Gent and Van Tol [11] reported neither the protein nor apolipoprotein content of the fractions, but the lipid compositions that they found are in close agreement with our values.

Direct detection of cholesterol, triglycerides of phospholipids would be easy to apply to our system. Hara and Okazaki [9] used a post-staining method with enzymic reagents, and lipid components were detected directly from the column effluent. That application is rapid and convenient, if there is no need for many different analyses from the fractions. For our requirements, direct addition of colorimetric reagents is impossible because of later LDL and HDL subfraction analyses.

CONCLUSION

Gel permeation chromatography correlated well with sequential gradient ultracentrifugation and with commonly used precipitation methods for the measurement of cholesterol from VLDL, LDL and HDL fractions. Also, the ratios of triglycerides, phospholipids, proteins and apolipoproteins A1 and B agreed with the distribution obtained from ultracentrifugal methods [16]. This indicates that the separation of lipoproteins, based on either density or size, gives practically the same fractions.

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