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Rapid, Single-Spin Fractionation of Serum Lipoproteins by Density Gradient Ultracentrifugation in Thermo Scientific Vertical Rotors

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Key Words

- Lipoprotein Separation
- Single-spin Isolation
- Discontinuous Salt Gradient
- Ultracentrifugation
- Vertical Rotors

Summary

The rapid, single-spin separation of serum lipoproteins into very low density lipoprotein (LDL), and high density lipoprotein (HDL) fractions by ultracentrifugation in a Thermo Scientific vertical rotor is described. The method uses a discontinuous salt gradient and separation is achieved in under three hours of centrifugation time.

Introduction

Lipids (cholesterol, triglycerides, and phospholipids) circulate in the blood stream as macromolecular lipid-protein complexes called lipoproteins. Lipoproteins can be separated by ultracentrifugation into four main classes differing in their buoyant density, *p*[1,2]. The classes are: chylomicrons (*p*=<0.95 g/mL; very low density lipoproteins, VLDL, (*p*=0.95-1.006 g/mL); low density lipoproteins, LDL, (*p*=1.006-1.063 g/mL; high density lipoproteins, HDL, (*p*=1.063-1.21 g/mL).

The procedure most frequently used to fractionate lipoproteins on a preparative scale involves the use of fixed-angle rotors and multiple centrifugation steps[3, 4, 5]. Separation of chylomicrons, VLDL, LDL, and HDL is accomplished by adjusting the density of the medium between centrifugations to allow sequential floatation of the individual lipoprotein fractions. The disadvantage of the sequential floatation procedure is that it frequently requires relatively long spin times and the complete preparative fractionation of all lipoprotein fractions may require anywhere from 2-5 days of centrifugation time[6].

Sequential floatation by multiple centrifugations in swinging-bucket rotors has also been applied to the fractionation of lipoproteins[7, 8, 9]. In addition, procedures for the isolation of VLDL, LDL and HDL by single-spin ultracentrifugation using discontinuous salt gradients in swinging-bucket rotors has been described; however, these latter procedures still require 24 or more hours of centrifugation time[10, 11, 12].

The use of a vertical rotor for the preparative, single-spin fractionation of serum lipoproteins is described here. The procedure used is a modification of the single-spin procedure reported by Foreman, *et al.*[12] for use with a swinging-bucket rotor. Use of the vertical rotor allows a significant reduction in the time required to achieve the separation (e.g., approximately only 2 hours in the Thermo Scientific StepSaver™ 65V13 vertical rotor).

Materials and Methods

Source of serum:

Pooled human serum from several normolipidemic individuals was obtained from a local clinical laboratory. Samples were stored at 4°C for no more than 6 days prior to use.

Removal of chylomicrons:

If a significant amount of chylomicrons are present in the serum (e.g., serum from lipemic or non-fasted normolipidemic individuals) a lowspeed centrifugation step prior to ultracentrifugation is recommended. This can be accomplished by centrifuging for 30 minutes at 10,000 rpm (9220 x g) in a SS-34 rotor using a Thermo Scientific Sorvall RC-6+™ superspeed centrifuge. Chylomicrons, when present, will "float" to the top of the centrifuge tube and can then be removed by aspiration or pipetting prior to ultracentrifugation in the vertical rotor. This procedure was performed for removal of the chylomicron fraction in the experiments reported here.

Gradient Preparation and Collection of Fractions:

A discontinuous NaCl/sucrose density gradient (17.5 mL total volume) was formed in a 1" x 2" polyallomer tube (Catalog Number 03288). The gradient was formed by layering from bottom to top: 3.5 mL 48% w/w sucrose solution, 5.0 mL of serum (adjusted to 4.0 M in NaCl by adding solid NaCl directly to the serum), and 9.0 mL of a 0.67 M NaCl solution containing 0.5% EDTA, pH 7.0. In order to better visualize the fractionated lipoprotein bands following centrifugation, a parallel gradient was prepared using serum prestained with Fat Red 7B (Sigma Chemical Co., St. Louis, MO. 63178)[13]. 1.0 mL of Fat Red 7B in dimethylformamide (2 mg/mL was activated just prior to use by adding 1.0 mL to 0.1 M NaOH, and one drop of the surfactant Triton X-100. Approximately 0.2 mL of this solution was used to stain 10 mL of serum. Centrifugation was performed at 65,000 rpm (400,000 x g) for 1 hour 45 minutes at 10°C using a StepSaver 65V13 vertical rotor in a Thermo Scientific Sorvall WX Ultracentrifuge. Following centrifugation, the tubes were removed from the rotor, the separations photographed, and each gradient fractionated using bottom puncture and continuous ascending flow. Forty-four fractions, 0.4 mL each, were collected.

Analysis of Fractions:

The distribution of cholesterol, triglyceride, and serum albumin in the gradient following centrifugation was determined by analyzing each fraction using an Automatic Clinical Analyzer (aca). Electrophoretic analysis of the unfractionated whole serum and aliquots of peak fractions obtained by centrifugation was carried out using agarose gel. Electrophoresis was performed according to the manufacturer's instructions with a AC1 Agarose Film/Cassette System

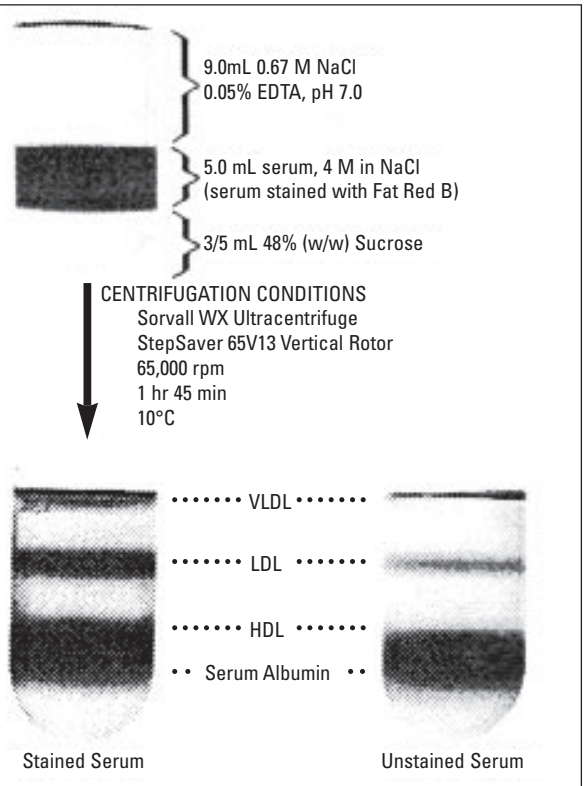

Fig. 1. Photograph of gradient in centrifuge tube after separation of human serum lipoproteins.

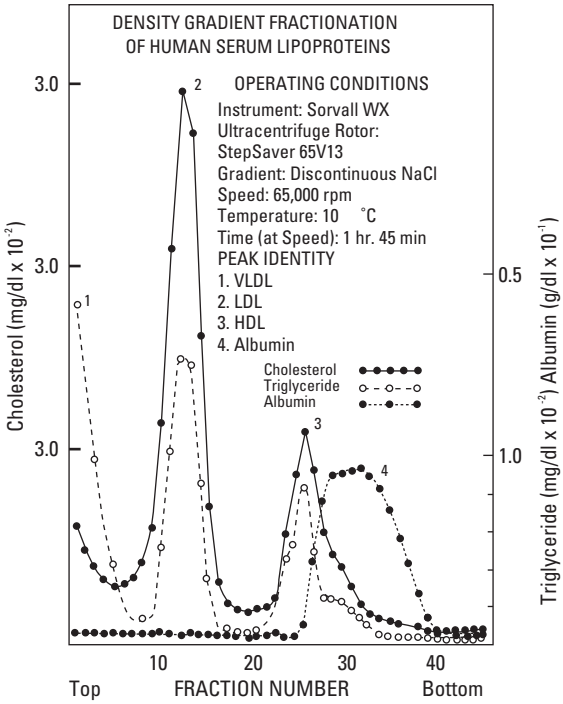

Fig. 2. Density gradient profile showing the distribution of cholesterol, triglyceride, and serum albumin after separation of human serum lipoproteins.

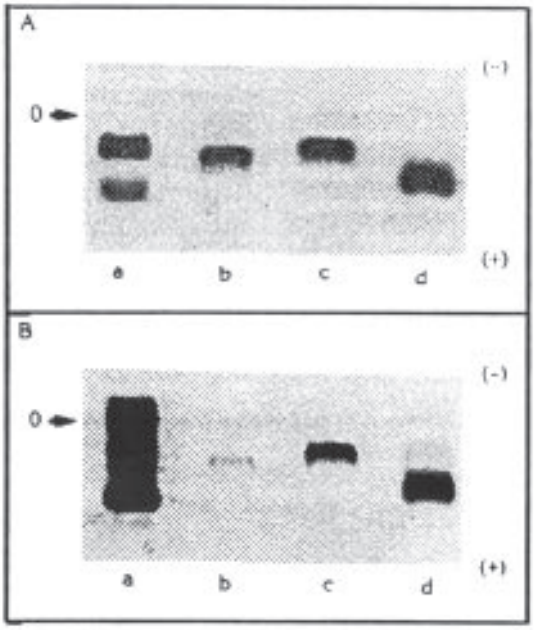

Fig. 3. Electrophoretic patterns of whole serum (a). and fractionated VLDL (b), LDL (c), and HDL (d) on 1% Agarose. A, Serum lipids, fat red B stain; B, serum proteins, amido black 10B stain.

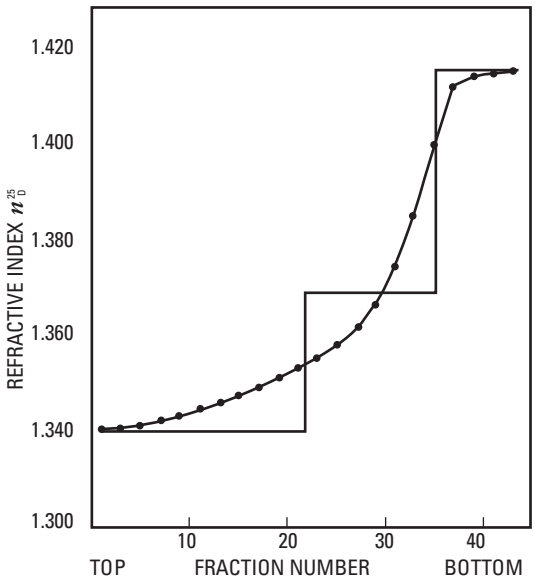

Fig. 4. Shape of the gradient after centrifugation. Refractive index. n_D^{25} determined using a Bausch & Lomb ABBE-3L refractometer. The shape of the discontinuous gradient prior to centrifugation is shown by the dotted line.

(Corning, Pala Alto, CA 94303). Following electrophoresis, the Agarose Films were stained for lipoproteins or serum proteins using Fat Red 7B and Amido Black 10B respectively. Gradient shape following centrifugation was determined from refractive index measurements.

Results

A photograph of a gradient prior to and following centrifugation for 1 hour, 45 minutes at 65,000 rpm (400,000 x g) is shown in Figure 1. The gradient protocol is indicated, as are the run conditions. For comparison the separation obtained using an unstained serum sample is also shown (Figure 1). The distribution of cholesterol, triglyceride and albumin within the fractionated gradient containing unstained serum is shown in Figure 2. Note that use of a sucrose shelf at the bottom of the gradient allows partial separation of serum albumin (Figure 2, peak 4) from HDL (Figure 2, peak 3).

Electrophoretic patterns of whole serum and fractionated VLDL, LDL, and HDL on 1% Agarose gel are shown in Figure 3. Serum lipids (stained with Fat Red 7b) and serum proteins (stained with Amindo black 10B) are shown in Figure 3, A and B respectively. Profile a in each panel is for unfractioned whole serum. Profiles b, c, and d are for fractioned VLDL (fraction 2, Figure 2), LDL (fraction 12, Figure 2), and HDL (fraction 25, Figure 2) respectively.

The relative electrophoretic mobility of the fractionated lipoproteins compares favorably with results reported by other investigators[11].

The shape of the gradient prior to (dotted line) and following centrifugation (solid line) is shown by the plot of refractive index, μ 25/D, versus fraction number (Figure 4).

Conclusion

The centrifugation procedure described here is designed for a rapid, single-spin, preparative fractionation of serum lipoproteins. Suggested protocols for this procedure using various vertical rotors are shown in Table 1.

The procedure described suggests that use of the vertical rotor can provide significant savings in the time and effort required to carry out similar lipoprotein fractionations using either fixed-angle or swinging-bucket rotors. In addition, suitable modification of the discontinuous gradient, and/or gradient materials, should permit further resolution of subcomponents within each lipoprotein class, for example, the resolution of LDL_1 from LDL_2, or HDL_2, from HDL_3.

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Rotor	Run Conditions Speed rpm	Time (at Speed)	Vol. Sucrose 48% w/w, mL	Gradient Protocol Vol. Serum 4M In NaCl, mL	Vol. 0.67M NaCl, ml	Total Serum Capacity 8 tubes /run, mL
StepSaver 70V6	65,000	1 hr 15 min	1.0	1.0	2.4	8.0
StepSaver 65V13	65,000	1 hr 45 min	3.0	5.0	9.0	40.0
TV-860	50,000	2 hr 30 min	3.5	12.0	18.5	96.0

Table 1
Suggested protocols for rapid single-spin density gradient fractionation of serum lipoproteins using Thermo Scientific vertical rotors.

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