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A novel and precise method for simultaneous measurement of serum HDL and LDL subfractions and lipoprotein (a) cholesterol by ultracentrifugation and high-performance liquid chromatography

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ABSTRACT

Background: We developed an ultracentrifugation and high-performance liquid chromatography (HPLC) method for simultaneous measurement of cholesterol in serum high density lipoprotein (HDL) and low density lipoprotein (LDL) subfractions and lipoprotein (a) [Lp(a)].
Methods: Serum aliquots of 0.05 ml were centrifuged at background densities of 1.006, 1.044 kg/l, and in the presence of β-mercaptoethanol (ME) at background densities of 1.044, 1.063 and 1.125 kg/l for the separation of lipoprotein subfractions and Lp(a). Cholesterol levels in the ultracentrifugal bottom fractions were analyzed by HPLC.
Results: ME effectively dissociated Lp(a) into apolipoprotein (a) and Lp(a) remnant [Lp(a-)]. Lp(a-) showed a distinctive density distribution from that of the native Lp(a). Based on these data, a method was developed to separate lipoprotein into subfractions and Lp(a) by ultracentrifugation. The separated HDL and LDL subfractions were not contaminated with Lp(a). This method is highly precise with the total CVs for the measurement of HDL2-C, HDL3-C, LDLa-C, LDLb-C and Lp(a)-C 0.85%–2.66%, 0.87%–3.21%, 0.86%–1.11%, 2.59%–6.35% and 4.42%–12.29%, respectively.
Conclusion: A new method for the separation of HDL and LDL subfractions and Lp(a) and simultaneous measurement of cholesterol by ultracentrifugation and HPLC have been established. It is precise and sensitive and can be used in research or clinical laboratories for lipoprotein profiling.

1. Introduction

Plasma high-density lipoprotein (HDL) and low-density lipoprotein (LDL) populations are composed of heterogeneous subfractions that are different in size, density and protein/lipid content and function. By ultracentrifugation, HDL can be separated into HDL2 and HDL3 subclasses, with hydrated densities of 1.063–1.125 kg/l and 1.125–1.210 kg/l, respectively [1]. Similarly, LDL can also be generally divided into large buoyant LDL (LDLa, 1.006–1.044 kg/l) and small, dense LDL (LDLb, 1.044–1.063 kg/l) [2]. Several lines of evidence suggest that the protective effect of HDL is better reflected in the concentrations of HDL2-C than in those of total-HDL-C or HDL3-C [3,4]. Numerous studies showed that LDLb is more atherogenic than LDLa. Prospective studies have reported the small LDL phenotype to be an important predictor of subsequent cardiovascular diseases (CVD) [2,5]. Therefore, detailed analysis of both major lipoproteins and their subclasses is required for more effective assessment of CVD risk status [6].

A variety of methodologies have been used for determining lipoprotein subfractions, including analytical ultracentrifugation [7], density gradient ultracentrifugation [8], nondenaturing gradient gel electrophoresis [9], nuclear magnetic resonance [10], high performance liquid chromatography (HPLC)[11] and chemical precipitation [12]. However, these methods either require large specimen volume, labor-intensive, technical demanding, or lack of specificity. Variations among currently available methods have made it difficult to compare the test results, interpret the data, and thus hindered their clinical applications [13,14].

Lipoproteins were originally defined by their buoyant densities. Ultracentrifugation still remains the most reliable separation technique for lipoproteins. The major obstacles for the use of ultracentrifugation have been the requirement of large volume of serum samples, and the contamination of recovered HDL and LDL fractions with apolipoprotein (apo) B-containing Lp(a) [8].

Lp(a) has been established as an important risk factor for CVD [15]. Lp(a) is a LDL-like particle, containing apo B100 and apo(a),

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which are covalently linked to each other by at least one disulfide bond [16]. On preparative ultracentrifugation, Lp(a) is found almost completely in the density range of 1.050 to 1.100 kg/l, which overlaps with HDL2 and LDLb. Lp(a) has traditionally been measured by immunoassays directed against apo(a) which vary widely due to the apo(a) genetic polymorphism [17]. Measuring Lp(a) by its cholesterol content will not only avoid the heterogeneity problems but also provide a way to directly compare Lp(a) with the other major lipoproteins that are usually measured by cholesterol [18].

We previously established a novel ultracentrifugation and HPLC (UC/HPLC) method for determination of serum LDL-C and HDL-C with a small amount of serum samples [19]. The method involves using 2-mercaptoethanol (ME) to dissociate serum Lp(a) into apo (a) and apo(a)-depleted Lp(a) [Lp(a-)], thus to effectively eliminate the contamination of Lp (a) in HDL separation.

In this study, we further investigated the density distribution of Lp(a-) and developed an UC/HPLC method for the determination of cholesterol concentrations in 4 lipoprotein subfractions and Lp(a). This method provides a procedure of rapidly profiling HDL2, HDL3, LDLa, LDLb and Lp(a) with a total of 0.25 ml serum samples, allows automatic sampling, involves no bottom fraction reconstitution, is highly precise and sensitive, and can be used in research and clinical laboratories for lipoprotein profiling.

2. Materials and methods

2.1. Serum samples

For methodological studies, fresh serum samples were collected from the leftovers of patient samples in the Department of Laboratory Medicine of Beijing Hospital. Serum aliquots were pooled and re-aliquoted and stored at −80 °C until analysis. For the study of biological variations, twenty healthy volunteers, 10 men and 10 women, were recruited. All subjects maintained a constant weight, their normal diet and lifestyle during the course of study. Blood was collected four times from each individual, after a 12- to 14-hour fast, at 2-week interval between each sampling. For analysis of lipoprotein subfractions, 205 healthy volunteers, 100 male and 105 female, from age 19–81 y, were recruited. Blood samples were taken by venipuncture, into tubes containing clot-activator. Serum was isolated, frozen, and stored in 1-ml aliquots at −80 °C until analyses. This study had been reviewed and approved by the Ethics Committee of Beijing Hospital. All studied individuals had been made aware in writing of the intended use of their sample and provided written consent.

2.2. Lipoprotein and subfraction separation by UC

2.2.1. Separation principles

The UC separation of lipoprotein and subclasses was done by spinning 2 aliquots of 0.05 ml of serum at background densities of 1.006 and 1.044 kg/l without ME, 3 aliquots at densities of 1.044, 1.063 and 1.125 kg/l in the presence of 0.05 mol/l ME, respectively. Cholesterol levels in the ultracentrifugal bottom fractions (BF) were analyzed by HPLC. HDL3-C was represented by the BF cholesterol centrifuged at 1.125 kg/l in the presence of ME ($BF_{1.125ME}C$), and HDL2-C, LDLa-C, LDLb-C, and Lp(a)-C were calculated by subtractions:

$$HDL3-C = BF_{1.125ME}C;$$
$$HDL2-C = BF_{1.063ME}C - BF_{1.125ME}C;$$
$$LDLa-C = BF_{1.006}C - BF_{1.044}C;$$
$$LDLb-C = BF_{1.044ME}C - BF_{1.063ME}C;$$
$$Lp(a)-C = BF_{1.044}C - BF_{1.044ME}C.$$

2.2.2. Density solutions

For LDL plus HDL separation, a solution of 0.098 mol/l NaBr was prepared by dissolving 1.008 g of NaBr in 100 ml of deionized water. For separation of LDLb + Lp(a) + HDL, 0.618 mol/l NaBr solution was prepared by dissolving 6.359 g of NaBr in 100 ml of deionized water. The densities of these two solutions were verified to be 1.006 and 1.0464 kg/l with density meter (DMA 4500 M, Anton Paar, Austria) at 20 °C. For HDL plus LDLb, HDL and HDL3 separation, solutions of 0.615, 0.876, and 1.724 mol/l NaBr containing 0.05 mol/l ME were prepared. These solutions showed a density of 1.0464, 1.0666, and 1.1324 kg/l, respectively. Mixing of 0.8 ml of these above solutions with 0.05 ml of serum would form background densities of 1.006, 1.044, 1.044, 1.063 and 1.125 kg/l respectively, assuming the background density of serum is 1.006 kg/l and serum protein volume 6%.

2.2.3. UC separation

The UC separation was performed on a Beckman Coulter (Brea, CA) XL-90 ultracentrifuge with a Type 25 rotor (Beckman Coulter, 1 ml × 100 in 3 rows) and thick wall polycarbonate UC tubes (1 ml, 8 × 51 mm). The sampling of serum was performed with a MicroLab 500 automatic dilutor (Hamilton, Reno, NV). The dilutor was first primed with the 1.006 kg/l density solution. Aliquots of 0.05 ml of serum samples were delivered with 0.8 ml of the solution to a series of UC tubes. The dilutor was then primed with the next higher density solution and another set of serum aliquots was delivered until each serum sample was delivered with 5 different density solutions. The UC tubes were then loaded onto the rotor and spun at 23,000 rpm for 18.5 h at 20 °C. The g forces for the outmost, middle and innermost rows were 78,196, 68,843 and 59,431 g, respectively. After the centrifugation, the tubes were sliced at the middle of their contents with a CentriTube Slicer (Beckman Coulter) and bottom fractions were obtained for cholesterol analysis [19,20].

2.3. Cholesterol measurement

2.3.1. Calibrators and internal standard

A stock solution of 25.86 mmol/l (1000 mg/dl) was prepared by dissolving a cholesterol reference material SRM 911b (certified value 99.8 ± 0.1%, National Institute of Standards and Technology, Gaithersburg, MD) in ethanol. Calibrators of 0.06, 0.13, 0.26, 0.65, 1.29, 2.59, and 5.17 mmol/l (2.5, 5, 10, 25, 50, 100, and 200 mg/dl) were prepared by diluting the stock solution with ethanol. An internal standard was prepared by dissolving stigmasterol in ethanol (0.65 mmol/l).

2.3.2. Sample preparation for bottom fraction cholesterol measurement

Calibrators were prepared similarly as previously described [19]. After centrifugation, the bottom of the UC tubes containing the centrifugal bottom fractions was gently placed into 10-ml screw-capped glass tubes. 0.05 ml of the internal standard with 0.8 ml of ethanol was added to the tubes containing either calibrators or the bottom fractions. After adding the alcoholic potassium hydroxide, the tubes were then capped, shaken for 15 min and incubated at 50 °C for 2 h for hydrolysis of cholesteryl esters. After incubation, 0.5 ml of the solution was transferred into another set of 2 ml tubes, extracted with hexane, oxidized with chromic acid and analyzed by HPLC as previously described [19,20].

3. Results

3.1. Density distribution of Lp(a) and Lp(a-)

In order to determine the density of Lp(a) and Lp(a-), aliquots of a mixed serum [Lp(a) mass > 500 mg/l] were centrifuged at 33 different background densities in the presence or absence of ME respectively. Cholesterol levels in the bottom fractions were determined