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Rapid, Simple Laser-Light-Scattering Method for HDL Particle Sizing in Whole Plasma, Emerson S. Lima¹ and Raul C. Maranhão²,¹ [¹ Lipid Metabolism Laboratory, Heart Institute (InCor) of the Medical School Hospital, and ² Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo 05403-000, Brazil; * author for correspondence: fax 55-11-3069-5574, e-mail ramarans@usp.br]

HDL particles are composed of an outer layer of phospholipids and free cholesterol surrounding a hydrophobic core that consists primarily of cholesterol esters and small amounts of triglycerides (1). Apolipoprotein A-I (apo A-I) accounts for ~60% of the protein content of HDL. Other apolipoprotein species found in HDL particles include apo A-II, apo A-IV, apo CI, apo CIII, and apo E. Several subfractions of HDL have been identified on the basis of density, electrophoretic mobility, particle size, and apolipoprotein composition (2). Differences in particle size are ascribed mainly to the number of apolipoprotein molecules on the particle surface and the amounts of cholesterol esters in the core (1, 2). Furthermore, there is growing evidence suggesting that most of the cardioprotective properties of HDL are associated with the HDL₂ fraction (larger particles) rather than the HDL₃ fraction (smaller particles) in patients with coronary artery disease (3, 4), in postmenopause women (5), in diabetes (6), and in patients with familial hypercholesterolemia (9).

Several methods, such as sequential ultracentrifugation, chemical precipitation, immunoaffinity chromatography, and nondenaturing polyacrylamide gradient gel electrophoresis, have been used to separate HDL subfractions (8). The sizes of the subfractions have been estimated by nondenaturing polyacrylamide gradient gel electrophoresis (9) and, more recently, by nuclear magnetic resonance spectroscopy (10). In general, these procedures are either laborious or expensive.

Laserscattering (LLS) has been used in the measurement of LDL particle sizes after isolation of this fraction by ultracentrifugation (11). There is great similarity between the data obtained with this approach and those obtained with the nondenaturing polyacrylamide gradient gel electrophoresis method (P <0.0001; r = 0.78).

To date, however, LLS has not been used to perform HDL sizing. HDL sizing by LLS can be performed after chemical precipitation of the apo B-containing lipoprotein (8). This approach is more practical than ultracentrifugation or gel electrophoresis. Here we describe the separation of HDL by chemical precipitation and determine the particles size in the supernatant by the LLS method.

Twenty-nine healthy individuals, 4–66 years of age, participated in the experiments. The plasma concentrations of total cholesterol, HDL-cholesterol, and triglycerides were measured by automated enzymatic methods, and LDL-cholesterol was calculated by the Friedewald formula. Blood samples (15 mL) were collected from an antecubital vein of each participant into three 5-mL glass tubes, one containing EDTA (1.5 g/L), one containing heparin (5 IU/mL), and one containing no anticoagulant. EDTA plasma, heparin plasma, and serum were then obtained by centrifugation at 4 °C for 15 min at 1250 g. Some samples were kept at 4 °C and some at ~70 °C to examine the effect of storage temperature on the assay. For the isolation of HDL for subsequent sizing by LLS, several combinations of polyanions and divalent cations were tested to precipitate apo B-containing lipoproteins (8). The following precipitants were tested: phosphotungstestate-Ca²⁺ (3 g/L and 15 mmol/L, respectively), dextran sulfate-Mg²⁺ (15 g/L and 40 mmol/L, respectively), heparin-Mg²⁺ (40 IU/mL and 30 mmol/L, respectively), and polyethylene glycol (PEG) 8000 (400 g/L) in 0.2 mol/L glycine buffer adjusted to pH 10 with sodium hydroxide. To assess the reliability of the HDL particle size data obtained with the different precipitation techniques used here, we also obtained the HDL fraction for comparison with a standard sequential flotation ultracentrifugation procedure (8) that uses a 90ti rotor in an Optima XL-100K ultracentrifuge (Beckman Instruments Inc.).

Compared with the other tested precipitants, use of PEG gave HDL particle diameter measurements by LLS that were the most reproducible and that most closely resembled those obtained by ultracentrifugation. When LLS analysis was made using samples processed by PEG precipitation, the mean (SD) HDL particle size was 9.1 (0.6) nm, which is consistent with those obtained by the ultracentrifugation procedure [9.3 (0.7) nm; n = 8]. The phosphotungstate-Ca²⁺ and Mg²⁺-based methods yielded HDL diameter results that were not reproducible and with means >14 nm, i.e., inconsistent with the mean values expected for HDL. These results suggest that cations at high concentrations, as required for precipitation of apo B-containing lipoproteins by phosphotungstate-Ca²⁺ or Mg²⁺-based methods, may interfere with LLS measurements of HDL particle size. In this respect, Dias et al. (12) also reported that PEG was the most suitable for HDL isolation by precipitation. Thus, HDL isolation for further LLS analysis was standardized as follows: 0.5 mL of PEG (Merk-Schuchardt) was added to each EDTA-plasma sample (0.5 mL) and stirred in a vortex-mixer for 30 s. Samples were then centrifuged at 1800 g for 10 min at 25 °C in a microcentrifuge (Model 5415 C; Eppendorf). A 0.5-mL portion of the supernatant was added to 1.5 mL of 10 mmol/L NaCl, passed through a
0.22 μm filter (Millipore Products Division) to exclude dust particles, and poured into a disposable 10 \times 10 \times 48 mm cuvette (Sarstedt).

The diameters of HDL particles were determined by use of a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments Corporation). This instrument uses a 29 mW helium-neon laser at 658 nm to excite the samples. Scattered light is collected at an angle of 90° by a photon-counting photomultiplier tube and is then directed to a correlator. The software (BIC particle sizing) derives particle sizes from the correlator function. Results of each sample were expressed as the mean, which is the harmonic intensity-averaged particle diameter. For this study, all LLS measurements were performed at 25 °C, and the results are means (SD) of five runs of 2 min each. To verify the accuracy of the instrument, before conducting the study, we subjected commercially available nanosphere size standards (Duke Scientific) of known diameters [92 (3.7) nm] to size determination under the operating conditions described above. The intraassay CV for the HDL particle diameter measurement (n = 10) obtained with this procedure was 2.1%.

The physical characteristics and laboratory data for the study participants, as grouped by gender, are shown in Table 1. There were no differences between genders with respect to age and body mass index, plasma lipids, and glucose. The HDL particle size measurements are also shown in Table 1, and it is clear that the HDL particles are larger in females than in males. The correlation plots for HDL particle size vs total cholesterol and LDL-cholesterol are shown in Fig. 1. We found negative correlations (Pearson correlation test) between HDL particle size and plasma total cholesterol (r = −0.484; P < 0.05) and between HDL particle size and LDL-cholesterol (r = −0.464; P < 0.05). The correlations between HDL particle size and HDL-cholesterol and triglyceride concentrations were not significant (P = 0.560 and 0.168, respectively). We observed no significant changes in particle diameter size in the frozen EDTA plasma over the first 7 days at −70 °C; thereafter, however, the particle size steadily increased (no data shown). Regarding the EDTA-plasma samples maintained at 4 °C, increases in particle size began to occur as early as after 24 h of storage. Use of serum or heparinized plasma was inadequate for LLS HDL sizing because the diameter values were increased ~12% compared with those obtained from EDTA plasma. The larger diameter values could be attributable to formation of

| Table 1. Age, body mass index, plasma glucose and lipid concentrations, and sizes of the HDL particles for the study participants according to gender. |
|---------------------------------------------------------------|
| Mean (SD) | Men (n = 14) | Women (n = 13) |
| Age, years | 24 (15) | 24 (14) |
| BMI, a kg/m² | 26.4 (7) | 24 (5) |
| Glucose, mg/L | 930 (260) | 800 (130) |
| Cholesterol, mg/L | 1890 (930) | 1660 (800) |
| LDL-cholesterol, mg/L | 1600 (670) | 1100 (780) |
| HDL-cholesterol, mg/L | 480 (210) | 510 (80) |
| Triglycerides, mg/L | 1330 (350) | 1090 (290) |
| HDL particle diameter, nm | 8.4 (1.1) | 9.1 (1.0) b |

a BMI, body mass index.
b Significant difference between men and women (Mann–Whitney test, P < 0.05).

Fig. 1. Plots of HDL particle diameter vs plasma total cholesterol (A) and LDL-cholesterol (B) concentrations.
(A), correlation (Pearson correlation test, r = −0.484; P = 0.0193) between plasma total cholesterol (mg/L) and HDL particle diameter (nm) determined in whole plasma by LLS after chemical precipitation of apo B-containing lipoproteins. (B), correlation (Pearson correlation test, r = −0.461; P = 0.0468) between plasma LDL-cholesterol concentration (mg/L) and HDL particle diameter (nm) determined in whole plasma by LLS after chemical precipitation of apo B-containing lipoproteins.
HDL particle aggregates after precipitation of apo B-containing lipoproteins.

The data on HDL particle size obtained in this study (mean diameter, 8.8 nm) are in agreement with the values described in the literature for other techniques. In healthy normolipidemic individuals, HDL sizing by nuclear magnetic resonance spectroscopy yielded diameters of ~9.2 nm (10), whereas the values obtained from gradient gel electrophoresis ranged from 8.4 to 9.6 nm (9, 13). In this study, similar to other published results obtained by gradient gel electrophoresis (13), the HDL particle diameter (nm) was greater in premenopausal women than in men (9.1 vs 8.4 nm; Mann–Whitney test, \( P = 0.013 \)).

Negative correlations between HDL particle size and total cholesterol and LDL-cholesterol were also reported by Pascot et al. (13), thus strengthening the link between HDL particle size and coronary artery disease. In the present study, the correlations between HDL particle size and HDL-cholesterol and triglyceride concentrations were not statistically significant, probably because of the small number of samples. Pascot et al. (13) may have found significant correlations for these relationships because they studied >400 individuals. Therefore, LLS analysis after chemical precipitation of apo B-containing lipoproteins gave results for HDL diameter in the same range as those obtained by established techniques for lipoprotein sizing (9, 10, 13). Furthermore, the differences between genders and the correlations reported here are in agreement with those described in the literature (13).

Because LLS analysis after chemical precipitation is a practical and less time-consuming approach for HDL sizing, it could be used in large trials and in routine clinical laboratory analysis.

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Quantitative Assessment of PML–RARα and BCR–ABL by Two Real-Time PCR Instruments: Multiinstitutional Laboratory Trial, Pascal Bonufer, Dolores Colomer, Mary T. Gomez, Joaquín Martínez, Silvia M. González, Marcos González, José Nomdedeu, Beatriz Bellosillo, Eva Barra-gañ,1 Francesco Lo-Coco,8 Daniela Diverio,9 Lourdes Hermosín,10 José García-Marco,11 Maria D. de Juan,12 Francisco Barros,13 Rafael Romero,13 and Miguel A. Sanz,15 for the Group of Molecular Biology in Hematology (1 Molecular Biology, Department of Medical Biopathology, Hospital Universitario La Fe, Avda Campanar 21, 46019 Valencia, Spain; 2 Hematopathology Unit, Hospital Clinic, Barcelona, Spain; 3 Molecular Biology, Hematology, Hospital Gran Canaria Dr. Negrín, Las Palmas de GC, Spain; 4 Molecular Biology, Hematology, Hospital 12 de Octubre, Madrid, Spain; 5 Immunopathology and Molecular Biology, Hematology, Hospital Clínico Universitario, Salamanca, Spain; 6 Laboratory of Hematology, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; 7 Laboratory of Cyto-genetics and Molecular Biology, Service of Pathology, Hospital del Mar, Barcelona, Spain; 8 Laboratory of Integrated Diagnosis of Oncohematologic Diseases, University Tor Vergata, Rome, Italy; 9 Laboratorio di Diagnostica Molocolare Oncoematologica, Dipartimento di Biotec-nologie Cellulari ed Ematologia, Università degli Studi “La Sapienza”, Rome, Italy; 10 Biology, Hematology, Hospital de Jerez, Jerez de la Frontera, Cádiz, Spain; 11 Molecular Cytogenetic Unit, Servicio de Hematología, Hospital Universitario Puerta de Hierro, Madrid, Spain; 12 Unified Laboratory, Immunology, Hospital Donostia, San Sebastian, Gipuzkoa, Spain; 13 Molecular Medicine Unit-INGO (Sergas), University of Santiago de Compostela, Hospital Clínico Universitario de Santiago, Santiago de Compostela, Spain; 14 Departamento de Estadística e Investigación Operativa, Universidad Politécnica de Va-lencia, Valencia, Spain; 15 Clinical Hematology, Service of Hematology, Hospital Universitario La Fe, Hospital Universitario La Fe, Valencia, Spain; * author for correspondence: fax 34961973030, e-mail bolufer_pas@gva.es)

The recent introduction on the market of instruments for real-time PCR has prompted the development of quanti-