Lipoprotein separation in a novel iodixanol density gradient, for composition, density, and phenotype analysis

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Abstract Separation of lipoproteins by traditional sequential salt density floatation is a prolonged process ($\sim$72 h) with variable recovery, whereas iodixanol-based, self-generating density gradients provide a rapid ($\sim$4 h) alternative. A novel, three-layered iodixanol gradient was evaluated for its ability to separate lipoprotein fractions in 63 subjects with varying degrees of dyslipidemia. Lipoprotein cholesterol, triglycerides, and apolipoproteins were measured in 21 successive iodixanol density fractions. Iodixanol fractionation was compared with sequential floatation ultracentrifugation. Iodixanol gradient formation showed a coefficient of variation of 0.29% and total lipid recovery from the gradient of 95.4% for cholesterol and 84.7% for triglyceride. Recoveries for VLDL-, LDL-, and HDL-cholesterol, triglycerides, and apolipoproteins were approximately 10% higher with iodixanol compared with sequential floatation. The iodixanol gradient effectively discriminated classic lipoproteins and their subfractions, and there was evidence for improved resolution of lipoproteins with the iodixanol gradient. LDL particles subfractionated by the gradient showed good correlation between density and particle size with small, dense LDL ($<$25.5 nm) separated in fractions with density $>$1.028 g/dl. The new iodixanol density gradient enabled rapid separation with improved resolution and recovery of all lipoproteins and their subfractions, providing important information with regard to LDL phenotype from a single centrifugation step with minimal in-vitro modification of lipoproteins.—Yee, M. S., D. V. Pavitt, T. Tan, S. Venkatesan, I. F. Godsland, W. Richmond, and D. G. Johnston. **Lipoprotein separation in a novel iodixanol density gradient, for composition, density, and phenotype analysis.** *J. Lipid Res.* 2008. **49:** 1364–1371.

Supplementary key words ultracentrifugation • apolipoprotein B • very low density lipoprotein • low density lipoprotein • high density lipoprotein • lipoprotein (a)

The use of iodixanol for density gradient ultracentrifugation for isolation of lipoproteins was first described by Dr. John Graham (1–3). Iodixanol density gradient

ultracentrifugation (IDGU) is a rapid method of separating lipoproteins and, unlike conventional sequential floatation ultracentrifugation (SFU) (4) or salt gradient systems (5), does not require prolonged ultracentrifugation time, salt density adjustment, or dialysis.

We have developed a new three-layered gradient using iodixanol, which can be used for preparative and analytical work and, when compared with published two-layered iodixanol gradients, has the advantages of improved resolution of lipoproteins and their separation from plasma proteins as well as ability to subfractionate individual lipoprotein density classes. We describe the separation of plasma using both the IDGU and SFU methods and demonstrate the ability of the iodixanol gradient technique to provide additional useful information about lipoprotein distribution and composition and hence phenotype from a single ultracentrifugation step.

MATERIALS AND METHODS

Materials

Sixty percent iodixanol solution (Optiprep™) was supplied by Axis-Shield (Oslo, Kimbolton, UK). The 13 g × 89 mm Luer lock syringe-filling cannula was from Holborn Surgical and Medical Instruments, Ltd (Margate, UK). Cholesterol H (L-type), triglyceride (L-type), phospholipid B, free cholesterol C, apolipoprotein A1-HA, and apolipoprotein B-HA assays, and apolipoprotein calibrator, triglyceride, and free cholesterol and phospholipids standards were supplied by Wako (Alpha Laboratories, Neuss, Germany). Lp(a), albumin, and total protein were measured routinely by the Chemical Pathology Department of St Mary's Hospital on the Beckman Immage system (High Wycombe, UK), using the respective assay kits. Optiseal polyallomer tubes (362181), 11.2 ml capacity; (361621), 4.7 ml capacity; and Thinwall Ultra-Clear (344088), 6.5 ml capacity ultracentrifuge tubes were supplied by Beckman Coulter (High Wycombe, UK).

Abbreviations: ApoA-I, apolipoprotein A-I; HMW, high molecular weight; IDGU, iodixanol density gradient ultracentrifugation; SFU, sequential floatation ultracentrifugation; TBE, Tris borate EDTA.
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Spectra/Por membranes (MWCO 6-8000) were supplied by Spectrum Laboratories (Breda, Netherlands). Fluorinert FC-40 was supplied by Sigma-Aldrich (Poole, UK). Precast nondenaturing polyacrylamide gels (LPE-Gel 2/16) were supplied by CBS Scientific (Magna Park, UK). Tris borate EDTA (TBE) electrophoresis buffer (T-3913) was from Sigma-Aldrich. High-molecular-weight (HMW) protein standards were from Amersham Bioscience (Little Chalfont, UK). Polystyrene carboxylate (0.04 μm) modified (4% solid) beads (W004CA) were from Duke Scientific (Hatton, UK). Magnesium chloride hexahydrate and dextran sulfate (Dextralip 50) were from Sigma-Aldrich.

Blood samples

Sixty-three plasma samples from normal, healthy controls (n = 6, total cholesterol 4.2 ± 1.0 mmol/l and triglyceride 0.9 ± 0.3 mmol/l) and type 2 diabetic volunteers (n = 57, total cholesterol 4.4 ± 1.2 mmol/l and triglyceride 1.8 ± 0.9 mmol/l) with various lipoprotein profiles were studied, with both centrifugation methods. All subjects gave informed, written consent, and the study was approved by the local ethics committee (EC 02.95). The gradient was developed and optimized using plasma from normal controls. Twelve milliliters of blood were drawn after an overnight fast into a tube with 120 μl of 10% EDTA via venepuncture and placed on ice. Red cells were pelleted by centrifugation at 3,000 rpm (1,600 g) in an AccuSpin 3R centrifuge (Fisher Scientific, Loughborough, UK) for 15 min at 4°C. Chylomicrons were routinely removed in diabetic subjects by centrifugation of the plasma at 14,000 g for 30 min at 4°C in an Optiseal polyallomer tube (4.7 ml), using a 50.3 Ti rotor (Beckman Coulter). Chylomicron-free plasma (4.5 ml) was then carefully extracted with a syringe fitted with a metal cannula, and 25 μl of 5% sodium azide and 25 μl of 5% gentamycin were added.

Preparation of iodixanol gradients and separation of plasma lipoprotein subfractions

Samples from each individual were analyzed in duplicate, as described below. A 20% iodixanol plasma solution was prepared by diluting 2.25 ml Optiprep with 4.5 ml chylomicron-free plasma. Iodixanol solutions (9% and 12%) were prepared by diluting 9 ml of 60% iodixanol (Optiprep) with 51 ml of 0.1 M HEPES-buffered saline (0.85 g NaCl in 90 ml distilled water, with 10 ml of 1 M HEPES added, adjusted to pH 7.4) and 12 ml Optiprep with 48 ml HEPES-buffered saline, respectively. The density of the solution was then checked against the expected densities in the product information leaflet.

The initial three-step gradient was prepared by careful under-layering using a 10 ml disposable syringe with a long, wide, bore metal cannula. Three milliliters of the 9% solution was placed in the Optiseal polyallomer centrifugation tube (11.2 ml); this was then under-layered with 3 ml of the 12% solution and then with a final 3 ml of the 20% iodixanol/plasma solution. The tube was then carefully filled to the top with HEPES-buffered saline. To distribute the plasma lipoproteins in a continuous iodixanol gradient, the tube was capped and placed in an NVTi 65 rotor (Beckman Coulter) and centrifuged at 65,000 rpm (342,000 g) for 4 h at 4°C in an Optima XL-100K ultracentrifuge (Beckman Coulter) set at slow acceleration and deceleration. Samples were fractionated within 1 h of centrifugation.

Fraction collection

Fractions were collected from each gradient in a cold room (5–8°C), by upward displacement using a Beckman gradient unloader that pierced the bottom of the tube and pumped Fluorinert into the bottom of the tube via a syringe driver set at 90 ml/h. The fractions were collected into a LKB Bromma 2212 Helirac (Bromma, Sweden) fraction collector set at 20 s per fraction, with resultant 0.5 ml vol per fraction. The fractions were stored in cryotubes (Sarstedt, Leicester, UK) at −70°C until further analysis.

Fraction analysis

A minimum volume of 50 μl from each fraction was pipetted into a sample cup and assayed for total cholesterol, triglyceride, phospholipid, free cholesterol, apoA-I, and apoB-100 on a Cobas Mira S analyser (Roche, Burgess Hill, UK) and albumin, total protein, and apo[a] on an Immage analyser (Beckman).

The density gradient profile and its reproducibility

To define the density profile of the gradient, a blank centrifugation tube was set up with 3 ml each of 9%, 12%, and 20% iodixanol diluted with HEPES-buffered saline and centrifuged as described. The density of each fraction was measured using a hand-held oscillating U tube DMA 35N densitometer (Anton Paar, Hertford, UK).

SFU

Ultracentrifugation was performed according to the modified method of Havel, Eder, and Bragdon (4) by density adjustment with potassium bromide that has been previously established in our laboratory (6). Plasma VLDL was separated by ultracentrifugation of chylomicron-free plasma (4 ml) in a Thinwall Ultra-Clear tube (Beckman Coulter, High Wycombe, UK) in a 50.3 Ti rotor at 48,000 rpm (166,000 g) for 18 h at 4°C. The VLDL was collected using a Beckman tube slicer set to cut the top 1 cm of the tube. The cap was washed twice with saline, and the final volume was recorded. The density of the remainder of the tube was then adjusted by the addition of 0.55 g potassium bromide for LDL, and the process was repeated. These steps were repeated with the addition of 0.63 g potassium bromide for HDL2 and 0.92 g for HDL3.

Dialysis of lipoprotein fractions

LDL, HDL2, and HDL3 lipoprotein fractions that underwent density adjustment with high concentrations of potassium bromide were dialyzed prior to assays. The samples were dialyzed in a 10 cm strip of Spectra/Por membrane in a large tank of dialysis buffer (10 liters distilled water, 100 mg sodium azide, and 0.84 g ammonium hydrogen carbonate, adjusted to pH 7.8–8.4) for 1–2 h. The buffer was then changed, and the samples were left overnight at 4°C. The following morning, the buffer solution was changed again, and the samples were left for a further 1–2 h before being transferred to cryotubes. Final sample volume was recorded and used to calculate the recovery from the concentrations of each lipid and apolipoprotein. Samples were then stored at −70°C.

LDL particle-sizing gradient gel electrophoresis

A modified approach to that of Krauss and Burke (7) was used for LDL particle sizing. Forty microliters of sample was mixed with 20 μl of 31% sucrose in TBE buffer. Twenty microliters was loaded onto a 2–16% precast nondenaturing polyacrylamide gel, following a 15 min prerun at 125 V. Electrophoresis was carried out using a stepwise run at 25 V for 15 min; 70 V for 20 min; and 125 V for 24 h. The gel was stained with a solution of 0.3 g Brillant Blue R, 50 ml acetic acid, 125 ml methanol, and 125 ml deionized water and destained with several washes in a solution of 100 ml acetic acid, 300 ml methanol, and 600 ml

deionized water. The gel was calibrated with the high molecular weight protein standard and 0.04 μm polystyrene beads. The polystyrene beads were loaded onto the standard lanes 2 h into the electrophoresis. The gel was scanned and analyzed with Kodak 1D Image Analysis software (Hemel Hempstead, UK).

HDL-cholesterol measurement

The Centers for Disease Control and Prevention designated comparison method for HDL-cholesterol measurement (8) was used as follows. A working solution of magnesium dextran sulfate was prepared [0.5 g dextran sulfate (M_r 50,000 Dalton), 3.56 g magnesium chloride, and 0.025 g sodium azide in deionized water to a final volume of 50 ml]. Fifty microliters of magnesium dextran sulfate solution was added to 0.5 ml of EDTA plasma, mixed thoroughly, and incubated at room temperature for 10 min. The samples were then centrifuged at 4°C and 1,500 g for 30 min. HDL-cholesterol was determined in the supernatant on a Cobas Miras S analyser.

Reproducibility and recovery

The reproducibility of the formation of the iodixanol density gradient was assessed by measuring the density in each fraction in eight separate runs. The coefficient of variation was calculated in each fraction.

Recovery data for the samples were calculated from the sum of the mass of each lipid and apolipoprotein in each fraction expressed as percentage of the mass in the original samples. The overall recovery of plasma lipids in the IDGU method was calculated from eight tubes and for the SFU method from six tubes. The lipid and apolipoprotein mass of the supranatant and the infranatant was used to estimate the recovery for each ultracentrifugation step of the SFU method. The lipoprotein recovery in the SFU method was calculated from whole plasma and each subsequent ultracentrifugation step, whereas in the IDGU method, recovery was calculated from each isolated lipoprotein formed in the gradient. Recovery for VLDL, LDL, HDL2, and HDL3 in the IDGU method was run in duplicate by substituting the lipoprotein samples for plasma in the iodixanol density gradient preparation.

Statistical analysis

Paired t-tests were used to compare lipid and protein composition of the lipoprotein fractions by the two methodologies. Correlations were assessed with Pearson's correlation coefficient. The Bland-Altman plot was used to compare bias between methodologies.

RESULTS

Iodixanol density gradient

The density profile generated by the iodixanol gradient is shown in **Fig. 1**. The gradient from fractions 1–16 (density 0.997–1.063 g/ml) was linear, giving good resolution of the lipoprotein particles, and from fractions 17–21 (density 1.076–1.217 g/ml) of the tube, the density increased sharply in a curvilinear fashion, separating the plasma proteins from all lipoproteins except HDL. The coefficient of variation in each fraction ranged from 0.09–1.13%, with a mean of 0.29%. Using the distribution of plasma albumin as a marker, plasma proteins were seen to be predominantly confined within the bot-
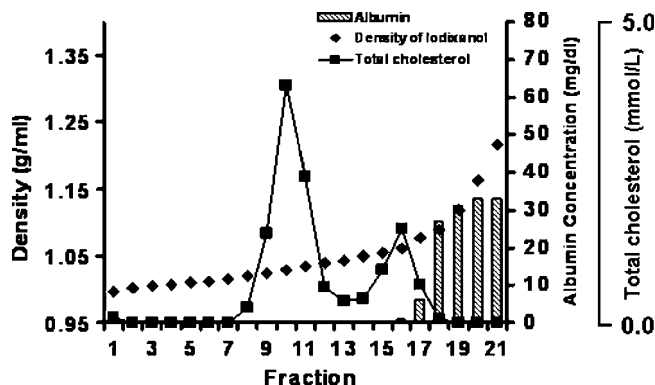

Fig. 1. Density profile of the iodixanol gradient and distribution of plasma lipoproteins and albumin following ultracentrifugation. Lipid data shown are from an individual; density and albumin profile is from four experiments in duplicate.

tom four fractions of the tube. The total cholesterol profile of plasma is shown, illustrating the distribution of lipoproteins in the iodixanol density gradient and their separation from plasma proteins.

Separation of lipoprotein fractions in iodixanol

We determined the location and distribution of the conventional VLDL, LDL, HDL2, and HDL3 isolated by SFU in the iodixanol density gradient in duplicate (**Fig. 2A–D**). The lipoproteins show distinct distributions in the gradient. VLDL was recovered in fractions 1–4 (density <1.007); LDL in fractions 7–13 (density 1.016–1.043); HDL2 in fractions 13–17 (density 1.043–1.076), and HDL3 in fractions 14–19 (density1.049–1.119). For the purposes of analysis, HDL in the iodixanol density gradient was taken as fractions 14–19. **Figure 3A** shows the plasma lipid and apolipoprotein profile of a normal subject from which the conventional lipoproteins in Fig. 2 were derived. In comparison, Fig. 3B shows the plasma profile of a dyslipidemic type 2 diabetic subject. Figure 3C shows a subject with a significant amount of apo[a], which forms a distinct shouldering or peak between LDL and HDL, as illustrated.

The density ranges for the lipoproteins in iodixanol were typically lower than those derived from salt gradients, as previously described by others (1, 3). This phenomenon is believed to be to the result of the dehydration of the lipoprotein particles by the hyperosmolar salt solutions used in the salt gradients (3). To investigate this further, we compared the peak distribution of the lipoprotein fractions of the same plasma separated by the two different methods. We isolated lipoproteins using the conventional SFU method and then separated the harvested fractions by the IDGU method and compared them to the lipoproteins in whole plasma separated by the IDGU method alone. A shift in the peak concentration of LDL apoB-100 toward increased density, from 1.029 to 1.034 g/ml, was seen in samples that had undergone SFU with high salt concentrations, compared with the lipoprotein distribution of the same plasma that had not
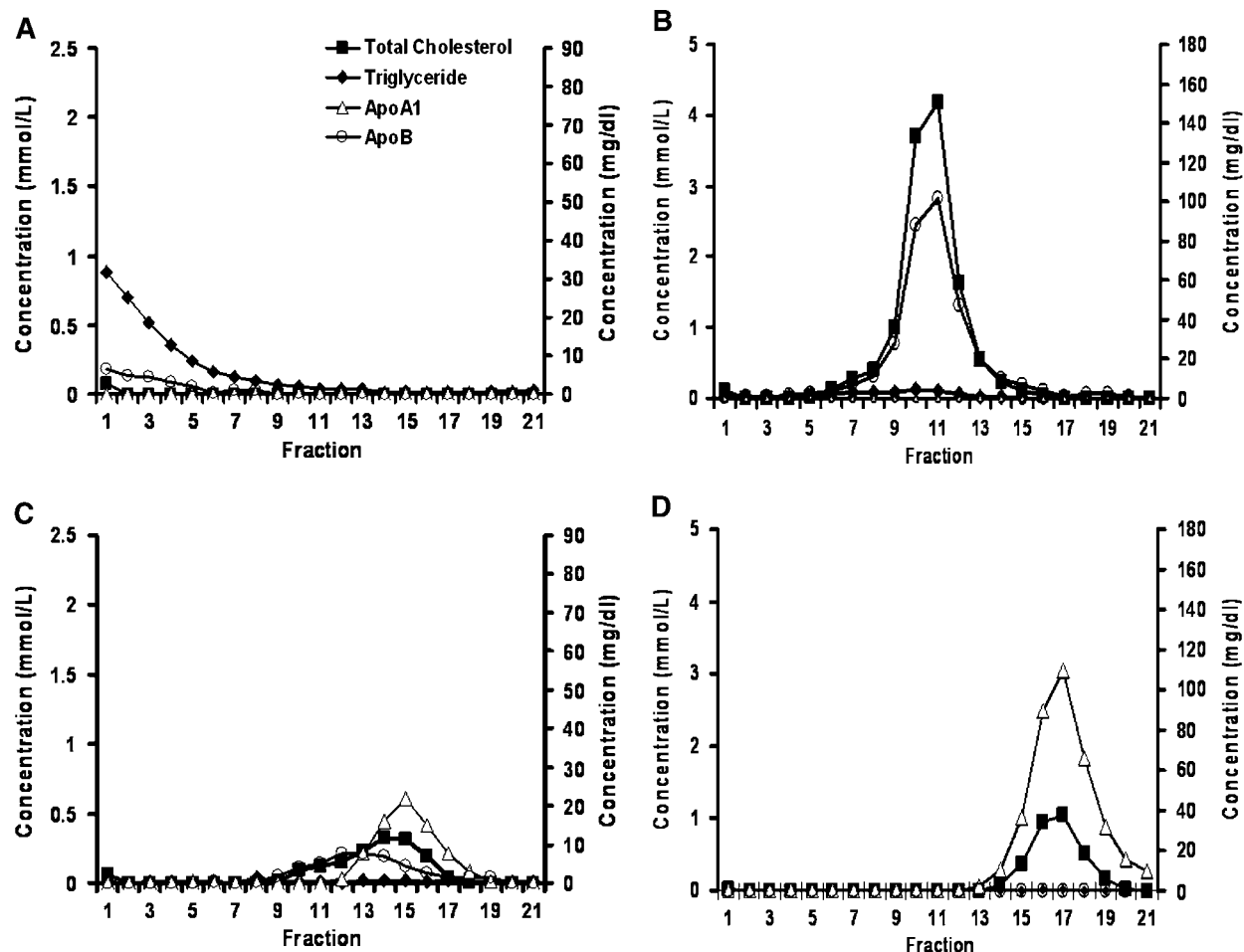
Fig. 2. Lipid and apolipoprotein profile of (A) VLDL, (B) LDL, (C) HDL2, and (D) HDL3 (isolated by sequential floatation ultracentrifugation) in the iodixanol gradient following ultracentrifugation.

undergone SFU. A similar shift in the distribution of the peak concentration of apoA-I, from 1.063 to 1.076 g/ml, was noted in the HDL3 from SFU, compared with apoA-I in HDL that had not undergone SFU. The contribution of HDL2 to the total pool of apoA-I in HDL, in this sample, was less than 20%.

Recoveries from the IDGU

The recoveries for the various lipoproteins are shown in **Table 1**. The recovery for the IDGU method was higher compared with a single step in the SFU method. Overall, the total recovery for the IDGU method was 95.4 ± 2.7% for total cholesterol and 84.7 ± 2.0% for triglyceride, and for the SFU method, 56.0 ± 6.4% for total cholesterol and 59.3 ± 12.2% for triglyceride. The difference was significant ($P < 0.001$). The coefficient of variation was 2.8%, 2.4%, 11.5%, and 20.6%, respectively.

Comparison of HDL-cholesterol measurements

We compared the HDL-cholesterol measurements from the IDGU method with those from the CDC HDL-cholesterol reference method. **Figure 4A** shows a scatterplot comparing the results. Analyzing the results with Pearson's correlation gave $r = 0.91$ with a $P < 0.0001$.

A bias plot comparing the results in Fig. 4B showed a bias of −0.032 mmol/l, and the 95% limits of agreement were −0.327 and 0.263 mmol/l.

LDL subtypes and LDL particle size

The high resolving power of this iodixanol density gradient can be exploited to analyze LDL subfractions and LDL particle size. **Figure 5** shows the apoB-100 distribution and cumulative percentage in LDL2 and LDL3 in successive iodixanol density fractions (reference samples kindly provided by Dr. Muriel Caslake). Unfortunately, the LDL1 sample was not suitable for analysis, because the total cholesterol concentration was 0.3 mmol/l and the apoB-100 concentration was 9 mg/dl, too low for accurate measurement after fractionation. The smaller and denser LDL subfractions showed a shift in the apoB-100 distribution toward the right (higher density), which was also apparent when total cholesterol was measured (data not shown). Therefore, depending on the position and shape of the LDL distribution in the lipid profile of the plasma separation, the predominant LDL subfraction and proportion in the plasma can be readily identified.

In addition to information regarding the density distribution of the lipoprotein subclasses, their particle
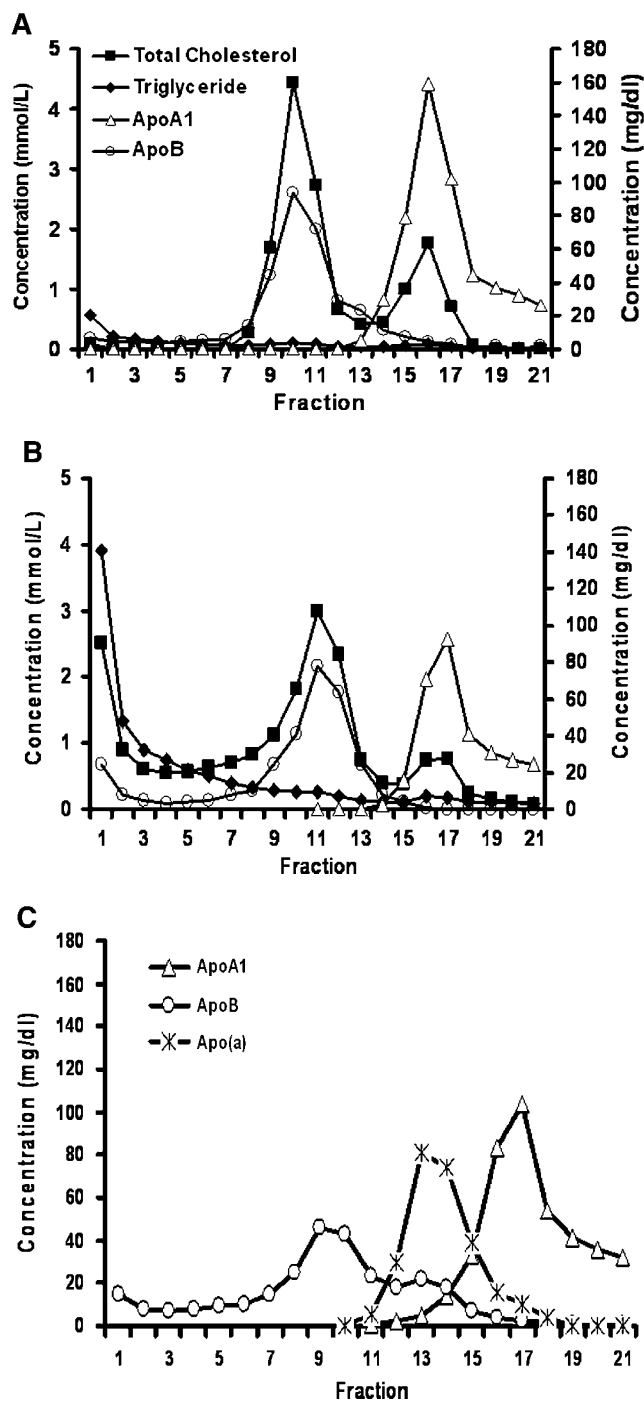
Fig. 3. Lipoprotein distribution and composition in (A) normal plasma (total cholesterol 4.4 mmol/l and triglyceride 0.8 mmol/l); (B) dyslipidemic plasma (total cholesterol 5.6 mmol/l and triglyceride 3.5 mmol/l); and (C) elevated levels of apolipoprotein[a] (apo[a]) (total cholesterol 3.7 mmol/l and triglyceride 1.4 mmol/l); apoA-I, 120 mg/dl; apoB-100, 71 mg/dl; and apo[a], 68 mg/dl using iodixanol density gradient.

size profile can also be readily determined in the iodixanol density fractions. Figure 5 shows the size of the LDL particles in each density fraction as assessed on polyacrylamide gradient gel electrophoresis. As illustrated, LDL particles from successive increasing-density fractions show

a decrease in size corresponding to smaller, denser LDL particles. Taking the LDL particle size of 25.5 nm as the cutoff between LDL phenotypes A and B (9), LDL particles in fractions 7–9 are classified as LDL1 and LDL2, and those from fractions 10–13 are LDL3. This is illustrated in Fig. 5, which shows the apoB-100 profile of LDL2, with 27.5% of the particles having a density >1.028 g/ml and a particle size of <25.5 nm, in contrast to LDL3 with 72.1%.

Comparison of the lipoprotein fractions from patients using IDGU and SFU

We compared the results from the plasma of 57 subjects with type 2 diabetes separated using both the IDGU and SFU methods (Table 1). The results showed significant differences between the two methods, in particular with regard to LDL and HDL, although there was a strong correlation between the methods, with Pearson's coefficient of correlation of 0.875 for total cholesterol, 0.806 for triglyceride, 0.921 for apoA-I, and 0.888 for apoB-100, significant at $P < 0.001$.

DISCUSSION

Our objectives in developing this new three-layered iso-osmotic density gradient procedure with iodixanol were to achieve rapid separation and preparative subfractionation of all three major lipoprotein density classes in a single run. Compared with SFU, the standard preparative method of lipoproteins, we have demonstrated improved fractionation and recovery of lipoproteins, although there was contamination of some subfractions of HDL3 with albumin, which is a common drawback of gradient ultracentrifugation methods. To completely remove albumin, a further ultracentrifugation step, use of albumin binding columns or gel electrophoresis, is required. Compared with the reported iodixanol density gradient from Davies, Graham, and Griffin (1), which effectively discriminates LDL from HDL but with poor separation of LDL from VLDL, or from Sawle et al. (3), which discriminates LDL from VLDL but with poor separation from HDL, our new gradient was able to give better fractionation of all three lipoproteins and separation from plasma proteins and was able to subfractionate LDL, enabling phenotyping of individuals in terms of cardiovascular risk.

The study was performed using a three-layered self-generating continuous density gradient. This gradient was linear over the range of densities corresponding to the lipoprotein particles and was highly reproducible (coefficient of variation, 0.29%). Consequently, a complete lipoprotein profile was obtained from a single 4 h ultracentrifugation step. Assuming that the lipoprotein particles come close to their isopycnic density following ultracentrifugation in the density gradient, the ability of the system to resolve and subfractionate lipoprotein density classes will be determined by the slope of the density gradient over the requisite range and the number/volume of fractions collected. The steeper the slope of the density gra-

TABLE 1. Table of lipoprotein composition, density, and recovery from iodixanol density gradient and sequential floatation ultracentrifugation.

		Iodixanol Density Gradient Ultracentrifugation	Sequential Flotation Ultracentrifugation	P
VLDL	Density	<1.007	<1.006	
	Total cholesterol	0.24 ± 0.30	0.18 ± 0.21	0.033
	Triglyceride	0.52 ± 0.41	0.55 ± 0.49	0.432
	Phospholipid	0.26 ± 0.18	0.20 ± 0.17	<0.001
	Free cholesterol	0.20 ± 0.14	0.13 ± 0.12	<0.001
	ApoB-100	6.8 ± 4.5	3.1 ± 2.3	<0.001
	Triglyceride recovery	90.9 ± 2.4%	73.3 ± 13.0%	0.121
	ApoB-100 recovery	81.7 ± 2.4%	86.1 ± 2.8%	0.148
LDL	Density	1.016–1.043	1.019–1.063	
	Total cholesterol	2.02 ± 0.66	1.13 ± 0.48	<0.001
	Triglyceride	0.27 ± 0.12	0.20 ± 0.09	<0.001
	Phospholipid	0.79 ± 0.24	0.44 ± 0.16	<0.001
	Free cholesterol	0.71 ± 0.20	0.36 ± 0.14	<0.001
	ApoB-100	52.1 ± 13.9	26.0 ± 10.8	<0.001
	Total cholesterol recovery	82.3 ± 3.0%	73.7 ± 7.9%	0.199
	ApoB-100 recovery	95.9 ± 0.6%	81.3 ± 10.4%	0.109
HDL HDL	Total cholesterol	0.96 ± 0.34	0.56 ± 0.24	<0.001
	Triglyceride	0.15 ± 0.07	0.07 ± 0.04	<0.001
	Phospholipid	1.03 ± 0.26	0.44 ± 0.18	<0.001
	Free cholesterol	0.32 ± 0.10	016 ± 0.06	<0.001
	ApoA-I	82.8 ± 20.6	36.5 ± 15.2	<0.001
HDL2	Density	l043–1.076	1.063–1.125	
	Total cholesterol recovery	71.5 ± 4.9%	83.2 ± 1.8%	0.071
	ApoA-I recovery	92.9 ± 2.7%	96.7 ± 1.6%	0.043
HDL3	Density	1.049–1.119	1.125–1.210	
	Total cholesterol recovery	84.4 ± 5.2%	73.0 ± 3.4%	0.010
	ApoA-I recovery	96.7 ± 3.1%	85.3 ± 3.6%	0.008

Composition data from 57 diabetic subjects (mean ± standard deviation). Total cholesterol, triglyceride, phospholipids, free cholesterol results in mmol/l and apolipoprotein AI (apoA-I) and apoB in mg/dl.

dient, the smaller will be the subfraction volumes required to achieve adequate resolution in a given density range. This will compromise the preparative capacity of the procedure and limit the volumes available for compositional analysis. Our iodixanol gradient achieves a low linear density gradient slope over a wide density range to enable good resolution of adjacent density classes and their preparative subfractionation in 0.5 ml fraction volumes. Although our gradient has slightly less power to resolve LDL subfractions, compared with the method of Davies, Graham, and Griffin (1), we are able to resolve all major lipoprotein species in one centrifugation step.

The HDL-cholesterol results from IDGU were comparable to the CDC reference method for measuring
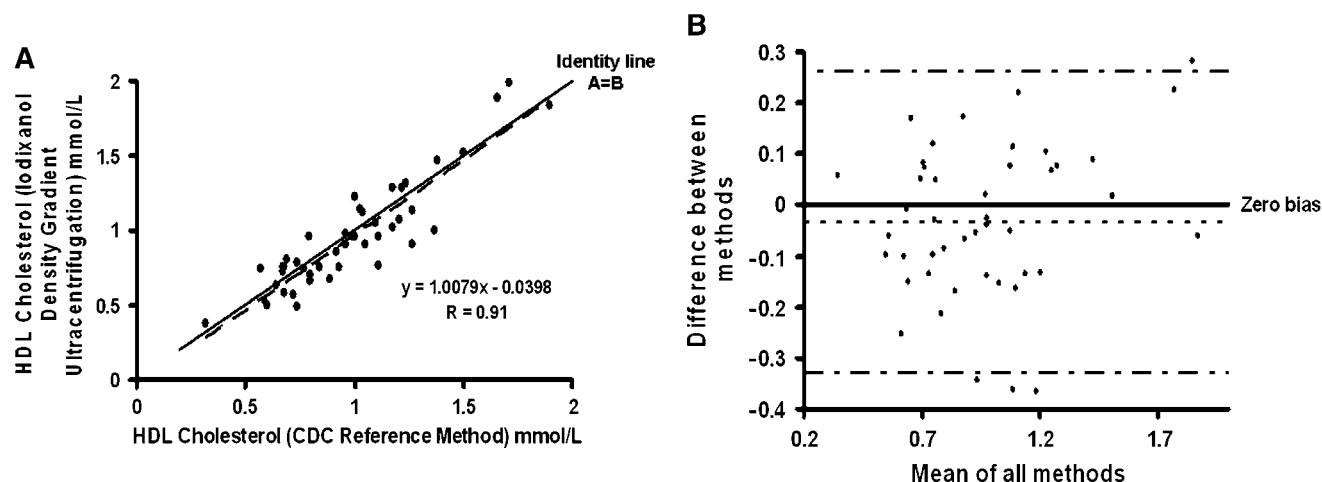

Fig. 4. Comparison of HDL-cholesterol results between the CDC reference method and iodixanol density gradient ultracentrifugation. A: Correlation between methods. B: Bland-Altman plot.
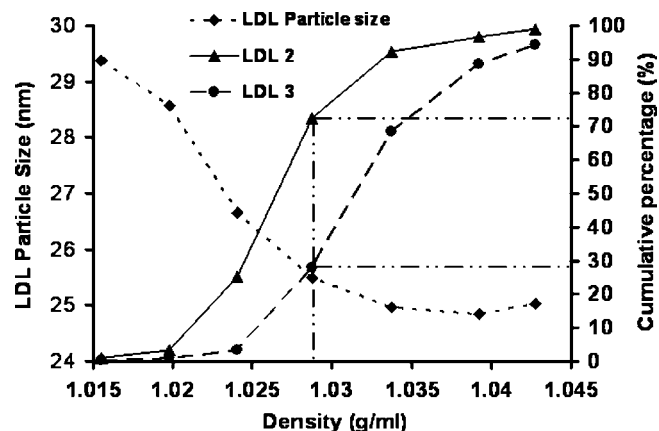
Fig. 5. The cumulative percentage of apoB-100 in LDL2 and LDL3 according to its density and a plot of the size of the LDL particles in each density fraction. The dotted line shows the density of 1.028 g/ml and the cumulative percentage of apoB-100 up to that point in LDL2 and LDL3 (LDL subfractions from three individuals performed in duplicate).

HDL-cholesterol used to standardize routine laboratory measurements, and the VLDL-cholesterol and triglyceride results were comparable to the traditional SFU method. Differences in LDL and HDL between IDGU and SFU methods were largely due to the difference in recoveries between the two methods, because correlations between the methods were strong, although recoveries were significantly different. However, lipoprotein recoveries from the SFU method are known to be highly variable, with reported recoveries from 88% in a single step to 36% involving multiple steps. Our recoveries are consistent with those from other published groups (2, 10). Recovery of VLDL was comparable between the methods, but recoveries for LDL and HDL were significantly better using the IDGU method due to minimal handling of samples and a methodology that is less likely to cause alteration to the lipoprotein particle. Recovery of HDL2 was lower in IDGU due to low concentrations, with cholesterol of 0.4 mmol/l and triglyceride of 0.01 mmol/l; when fractionated, the limits of the enzymatic assay were reached. Resolution of the lipoproteins isolated by IDGU was similar if not better than that of SFU when assessed by apolipoprotein content. IDGU gave a result comparable to traditional single-step SFU but was superior when multiple steps were involved.

Lipoproteins isolated using IDGU were more representative of lipoproteins in vivo and unaltered physiologically, because the ultracentrifugation time was reduced from 72 h to 4 h, which reduced the risk of oxidation and enzymatic alteration of the lipoprotein particles, as well as potential changes associated with prolonged exposure to high *g* forces (11–14). In addition, because iodixanol is iso-osmotic to plasma up to solutions containing 40% of iodixanol (data from manufacturer's information leaflet), lipoproteins are less likely to be affected by osmotic shifts associated with methods requiring the high salt concentrations and high osmotic pressures needed to achieve the densities required to separate lipoproteins and the subsequent need for dialysis. We have demonstrated increased density of lipoproteins following exposure to the high salt concentrations with SFU that are not corrected following dialysis of the lipoprotein particles.

In addition to the advantages stated, the IDGU also gave important information with regard to the density and particle size of the lipoproteins. The previous methodology for the subfractionation of LDL was performed using salt gradients (15). Lipid profiles derived from salt gradients require long centrifugation times and dialysis of samples that lead to the changes discussed earlier; in addition, salt gradients involved complicated procedures for preparation, often requiring the use of gradient-making equipment. This is avoided when using iodixanol, because the gradient formed is self-generating during centrifugation.

In all patients studied, three separate lipoprotein fractions were obtained, corresponding to VLDL, LDL, and HDL. Additional information was also obtained with regard to the subfraction distribution of the lipoproteins, because the position and shape of the lipoprotein profile gives information concerning the relative proportions of small, dense particles and larger, less-dense forms. Differences in the profile of normal and dyslipidemic plasma (as defined by the International Diabetes Federation, with plasma triglyceride $\geq$1.7 mmol/l, and HDL-cholesterol <0.9 mmol/l in men or <1.29 mmol/l in women) are clearly shown in Fig. 3, with elevated levels of VLDL and reduced levels of HDL and an increased proportion of small, dense LDL present in the dyslipidemic plasma. Because the fractions were correlated with the size of LDL particles, as assessed by gel electrophoresis, one can determine the LDL phenotype by comparing the proportion of LDL cholesterol in fractions 7–9 with that of fractions 10–13 [LDL particles >25.5 nm and density <1.028 g/dl compared with LDL particles (<25.5 nm and density >1.028 g/dl)]. Therefore, using the total cholesterol or apolB-100 profile of plasma, one can determine each individual's lipoprotein phenotype, which can be used in cardiovascular risk assessment. The density of the small, dense LDL particles in iodixanol is lower than that described in traditional salt gradients, with a described density for small, dense LDL >1.044 g/dl (15, 16); however, the densities we derived in iodixanol were reported by Davies, Graham, and Griffin (1) using a specific iodixanol density gradient designed for separation of LDL subclasses only. Because there is strong association between small, dense LDL and increased cardiovascular risk, such additional information is valuable in assessing an individual's lipid profile and cardiovascular risk (17).

To summarize, we have presented a new iodixanol density gradient with improved resolution and recovery of all lipoproteins and able to subfractionate lipoprotein particles, giving important information with regard to LDL phenotype from a single centrifugation step. Lipoprotein samples recovered are more representative of the physiological state, because of the reduced time and handling of samples.

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