methods

A rapid single-step centrifugation method for determination of HDL, LDL, and VLDL cholesterol, and TG, and identification of predominant LDL subclass

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Abstract **Determination of the circulating levels of plasma lipoproteins HDL, LDL, and VLDL is critical in the assessment of risk of coronary heart disease. More recently it has become apparent that the LDL subclass pattern is a further important diagnostic parameter. The reference method for separation of plasma lipoproteins is ultracentrifugation. However, current methods often involve prolonged centrifugation steps and use high salt concentrations, which can modify the lipoprotein structure and must be removed before further analysis. To overcome these problems we have now investigated the use of rapid self-generating gradients of iodixanol for separation and analysis of plasma lipoproteins. A protocol is presented in which HDL, LDL, and VLDL, characterized by electron microscopy and agarose gel electophoresis, separate in three bands in a 2.5 h centrifugation step. Recoveries of cholesterol and TG from the gradients were close to 100%. The distribution profiles of cholesterol and TG in the gradient were used to calculate the concentrations of individual lipoprotein classes. The values correlated with those obtained using commercial kits for HDL and LDL cholesterol. The position of the LDL peak in the gradient and its shape varied between plasma samples and was indicative of the density of the predominant LDL class. The novel protocol offers a rapid, reproducible and accurate single-step centrifugation method for the determination of HDL, LDL, and VLDL cholesterol, and TG, and identification of LDL subclass pattern.**—Sawle, A., M. K. Higgins, M. P. Olivant, and J. A. Higgins. **A rapid single-step centrifugation method for determination of HDL, LDL, and VLDL cholesterol, and TG, and identification of predominant LDL subclass.** ***J. Lipid Res.*** **2002.** 43: **335–343.**

Supplementary key words self-generating gradients • iodixanol • agarose gel electrophoresis • electron microscopy • reproducibility • comparison with kits • sample storage • high density lipoprotein • low density lipoprotein • triglyceride • very low density lipoprotein

Determination of the circulating levels of plasma lipoproteins is important in the diagnosis of primary and secondary disorders of lipid transport and in risk assessment for atherosclerosis and coronary artery disease (1, 2). In the fasting state, three main lipoprotein classes have been identified: VLDL, LDL, and HDL, each of which differs in size and density, and in lipid and apolipoprotein composition. It is well established that there is a positive correlation between risk of premature coronary heart disease and total plasma cholesterol and plasma LDL-cholesterol (LDL-C) (1, 2). There is also a correlation between decreased HDL-cholesterol (HDL-C) and increased plasma TG (1). Heterogeneity in the size and density of LDL is well documented (3, 4) and has also been shown to have clinical relevance (5–9). Small dense LDL (Pattern B) has an increased relative risk compared with large light LDL (Pattern A) (6–9). One of the most prevalent lipid/lipoprotein patterns associated with risk of coronary artery disease is the atherogenic lipoprotein phenotype (ALP), which is characterized by moderately raised plasma TG, low levels of HDL-C, elevated total and LDL-C, and small, dense LDL particles (10, 11). Although methods are available in the clinical laboratory for measurement of HDL, LDL, and VLDL, methods for the identification of the predominant LDL subclass are technically difficult and time-consuming.

The main methods for separation and analysis of the plasma lipoprotein levels, based on differences in physical properties, include ultracentrifugation, electrophoresis, and differential precipitation. Of these, ultracentrifugation is the "gold-standard" for analysis of plasma lipoproteins and potentially provides the greatest amount of information as the lipid and apolipoprotein compositions of the separated lipoproteins can be analyzed. Methods for density gradient centrifugation have generally been based on salt solutions, and include sequential flotation with adjustment of the density of the plasma and infranatants after each centrifugation step, or centrifugation on discontinu-

Abbreviations: ALP, atherogenic lipoprotein phenotype; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

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ous or continuous gradients (12, 13). The use of salt gradients has a number of disadvantages. These are technically difficult to prepare and relatively unstable, and reproducibility is difficult to achieve. In addition, prolonged centrifugation is often necessary to float the lipoproteins into the gradients and the high salt concentrations can modify the protein structure and lead to loss of apolipoproteins from the lipoprotein fractions. For further analysis of the lipoprotein fractions, it is usually necessary to remove the salt (dialysis being one means of salt removal). This results in loss of material and poor recoveries. Iodixanol is a nonionic isoosmotic medium that forms self generating gradients on centrifugation that overcome many of the disadvantages of salt gradients (14). We have previously reported a novel method for separation of plasma lipoproteins using self-generating iodixanol gradients (15). The method is technically simple, involves short centrifugation steps (1–3 h), the gradients produced are extremely stable and reproducible, and recoveries of lipoprotein lipids from plasma are close to 100%. The values obtained for the determination of VLDL-, LDL-, and HDL-C were consistent with those found using beta-quantitation (15). We have now modified the gradient conditions to improve the separation of lipoprotein classes, especially in individuals with dyslipidaemias, to reduce the amount of plasma needed for assay, and to resolve LDL subclasses. This procedure provides a plasma lipoprotein cholesterol and TG profile in which total HDL, LDL, and VLDL can be directly compared in the same sample and in which the predominate LDL subclass can be determined.

MATERIALS AND METHODS

Materials

Iodixanol solution formulated for lipoprotein separation (Liposep) was obtained from Lipotek, Ltd. (Merseyside, UK). Preformed agarose gels for lipoprotein identification (Hydragel-lp-a), which resolve lipoprotein [a] (Lp[a]), LDL, HDL, and VLDL were purchased from YSL (Clandon, UK). These are supplied with all materials for electrophoresis and staining of the gels. Cholesterol analysis kits were from Boerhinger Ingelheim Pharmaceutical, Inc. (Germany) and TG analysis kits from Alpha labs (Bio-stat Diagnostics Stockport, UK). Kits for assay of LDL-C and HDL-C were from Sigma (St. Louis, MO) and Genzyme (Cambridge, MA), respectively.

Blood samples

A total of 377 plasma samples from a range of individuals expected to exhibit different lipoprotein profiles were used in these studies. They included normal volunteers in the University of Sheffield (39 volunteers), non-insulin-dependent diabetic patients attending the Diabetes Clinic, University College Hospital of Wales, Cardiff (64 volunteers), patients attending the Obesity clinic of Leeds General Infirmary (74 volunteers), and subjects screened prior to and after dietary intervention studies carried out in the Department of Food Sciences, Reading University (200 volunteers). Blood was drawn into tubes containing EDTA by venipuncture. Red cells were pelleted by centrifugation at 1,000 g for 20 min and the plasma collected. In some cases, to compare results from plasma with serum, EDTA was not used and bloods were allowed to clot for 1–2 h before centrifugation. Blood samples were drawn after overnight fasting. However, fasting plasma samples were not always chylomicron-free, especially in samples from diabetic patients. Any chylomicrons present were removed routinely by centrifugation of plasma at 13,000 g for 10 min before separation of the lipoproteins. Chylomicron-free plasma was carefully removed from the bottom of the tube using a syringe fitted with a metal cannula. Aliquots of the original plasma and the chylomicron-free plasma were retained for analysis.

Separation of plasma lipoproteins in self-generating gradients

Four parts of plasma were mixed with one part of Liposep in a plastic conical tube (lower phase). The tube was mixed by tilting, taking care to avoid bubble formation. A second iodixanol solution was made up of 0.75 parts of Liposep mixed with 4.25 parts of 0.14 M sodium chloride buffered to pH 7.4 with HEPES (HBS) (upper phase). From the upper phase, 1.4 ml were transferred to Beckman Optiseal tubes for the TLN100 rotor (nominal volume 2.9 ml); 1.4 ml of the lower phase were transferred to the bottom of the tube below the upper phase using a syringe with a long metal cannula. HBS (~0.1 ml) was carefully layered on top of the upper phase to fill the tube. The tubes were capped and centrifuged at 100,000 rpm, (350,000 gav) for 2 h 30 min at 16°C in the TLX100 or the Beckman Optima bench top ultracentrifuge, set at slow deceleration. Similar gradients were prepared without plasma and the density of the gradient fractions determined by measurement of the refractive index.

Collection of gradients

Gradients were collected using a Beckman Gradient Unloader, which pierces the tube bottom, and a Gilson fraction collector. Different arrangements can be used, including unloading from the top by upward displacement with Maxidens (a water-immiscible dense liquid fluorinated hydrocarbon) pumped into the bottom of the tube, or from the bottom by gravity, or from the bottom using a pump fitted with narrow gauge tubing linked directly to the fraction collector. Routinely we used the latter method, and in the present studies we collected gradients dropwise in 20 fractions (7 drop), or 44 fractions (3 drop) into 96 well microtitre plates.

Analysis of fractions

Using a multipipette, 10 μl aliquots of each fraction were transferred from the wells of the microtitre plates used for collecting the gradients into new microtitre plates in duplicate. Triplicate aliquots (10 μl) of plasma and chylomicron free plasma, cholesterol standards, and blanks were also pipetted into wells in each plate. The same plate layout was always used to facilitate analysis. Separate microtitre plates were used for assay of cholesterol and TG. Two hundred microliters of cholesterol assay reagents or TG assay reagents were added to each well and the plates incubated according to the manufacturer's instructions. Absorbance was read at 450 nm for cholesterol and 570 nm for TG in a Multiscan ascent plate reader.

To analyze the data, a Microsoft Excel template was set up to calculate the cholesterol and TG (μmol) in each gradient fraction. The fractions from the bottom of the gradient are more viscous than those from the rest of the gradient, so that the volumes are smaller for the first two or three samples. In initial experiments the volumes of fractions were measured. However, under the same collection conditions, the fraction volume was found to be extremely reproducible. This volume was entered into the template. The absorbances from the plate reader were transferred directly into the template, which calculated the amount (μmol) of cholesterol or TG in each gradient fraction using the fraction volume, the absorbance for the sample, and

the absorbance for the standard cholesterol or TG sample. Cut-off points for HDL, LDL, and VLDL were selected for individual plasmas after examining the cholesterol profile and the agarose gel. Cholesterol (mM) and TG (mM) concentrations in total plasma, chylomicron free plasma, HDL, LDL, and VLDL were calculated. The recoveries of cholesterol and TG were also calculated from the lipids in the appropriate gradient fractions taking into account the volume of chylomicron free plasma used. The gradient distribution profiles for cholesterol, TG, and histograms showing the lipid content of total HDL, LDL, and VLDL were plotted.

Agarose gel electrophoresis

To identify the lipoproteins in each fraction, aliquots (2 μl) were separated by flat-bed electrophoresis using Hydragel (Lp[a]) agarose gels according to the manufacturer's instructions.

Electron microscopy

Lipoprotein gradient fractions were negatively stained with 1% uranyl acetate applied to coated grids and examined in a Phillips CM12 electron microscope.

RESULTS

Separation of plasma lipoproteins in self generating gradients of iodixanol

After centrifugation, three bands were visible in the centrifuge tube (**Fig. 1**). The lipoprotein class in each band was identified by agarose gel electrophoresis (see below). An opalescent white band of VLDL formed at the top of the tube. A red band of LDL was visible in the middle of the tube. At the bottom of the tube was a viscous red layer of plasma proteins. Immediately above this was a reddish band of HDL. In the electron microscope, the peak fraction from each band was seen to consist of particles of different diameter. Band one (VLDL) contained particles with diameters 30–80 nm; band two (LDL) contained particles of diameters 20–25 nm; and band three (HDL) consisted of small particles, diameters 6–15 nm, that tended to aggregate in chains. This may be an artifact of electron microscopy. The diameters are consistent with those reported for the main lipoprotein classes (13).

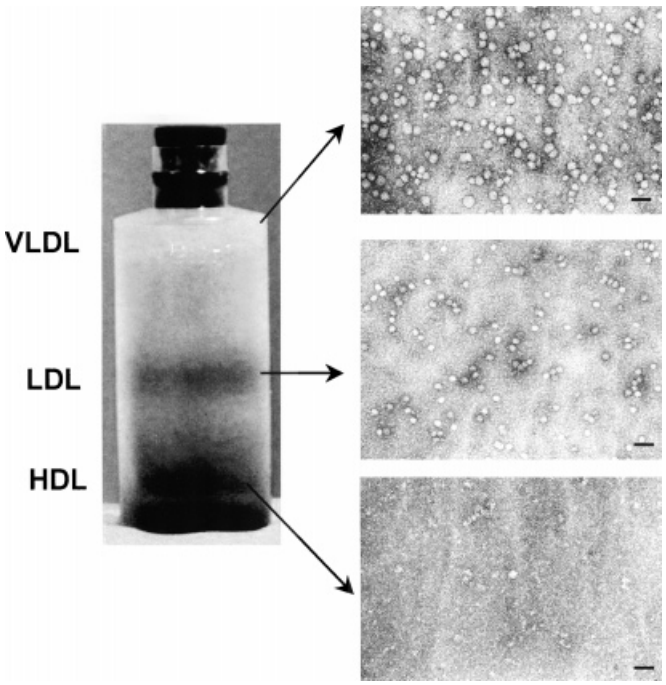


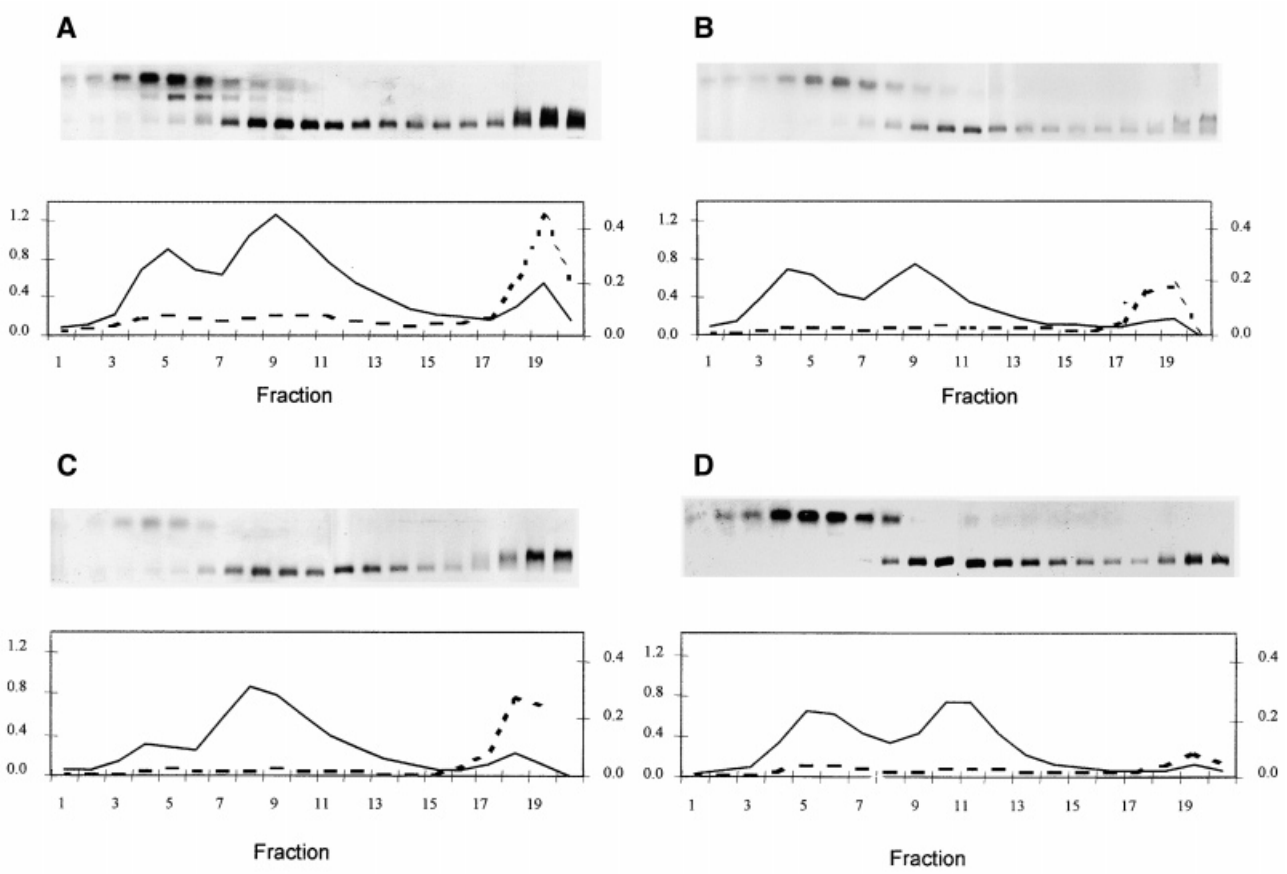
Fig. 1. Separation of plasma lipoproteins in self-generated gradients of iodixanol. Plasma was separated in a self-generated gradient of iodixanol as described in Materials and Methods. The gradients were collected and a sample from the fraction containing the peak VLDL, LDL, and HDL was examined in the electron microscope after staining with uranyl acetate. The centrifuge tube illustrating the separation is shown on the left and the morphology of the gradient fraction on the right (×40,00, the bar indicates 100 nm).

Cholesterol distributed in three peaks coincident with the peaks visible in the centrifuge tube. TG was concentrated in the top VLDL band, but was also detectable in the LDL and HDL bands. There was an overlap between HDL and LDL, usually of one or two fraction. For calculation of the cholesterol of each lipoprotein class, the cholesterol content in the overlap region was divided into two, with half of the value assigned to HDL and half to LDL. In practice, the overlap fraction contained a very small proportion of the total cholesterol and its inclusion in HDL, LDL, or both had very little effect (<2%) on the values for calculated HDL and LDL cholesterol. Although we have used agarose gels to identify the lipoproteins in gradient fractions, this is not necessary in routine analysis as the cut-off points for HDL/LDL and LDL/VLDL can be ascertained from the lipid profiles. Examples of separations of plasma with different lipoprotein patterns are illustrated in **Fig. 2**. Plasma A has high cholesterol, high LDL-C, and high Lp[a] visible in the agarose gels between LDL and HDL. In the gradient, Lp[a] distributes at the junction of HDL and LDL, and in the analysis contributes cholesterol to both of these lipoprotein classes. However, even in this sample, in which Lp[a] is unusually high, it is possible to assign a cut-off point between LDL and HDL. Plasma B, C, and D have total cholesterol levels that are normal or only slightly elevated. However, there are significant differences in the lipoprotein cholesterol profiles obtained. Plasma B and D have relatively high HDL and low LDL, whereas plasma C has low HDL and high LDL. The position of the peak of LDL is in fraction 8, 10, and 12 in plasma samples C, B, and D, respectively, indicating that the density of the predominant LDL is different in the individuals.

Values obtained for LDL-C and HDL-C correlated with those obtained using commercially available kits for assay of LDL (Sigma) and HDL (Genzyme) (**Fig. 3**). The LDL kit has been evaluated by the manufacturers and in other laboratories by comparison with beta quantitation and calculation using the Friedwald formula (16, 17). The HDL-C kit has been evaluated by the manufacturers against the Designated Comparison Method (precipitation with dextran sulfate-Mg^{++}).

Reproducibility and effect of sample storage

Iodixanol gradients are extremely reproducible. Almost identical cholesterol profiles and VLDL, LDL, and HDL



	A		B		C		D	
	Chol mM	TAG mM	Chol mM	TAG mM	Chol mM	TAG mM	Chol mM	TAG mM
plasma	7.66	1.27	4.78	0.58	5.10	0.92	5.38	0.58
HDL	2.41	0.30	2.08	0.14	0.75	0.07	2.15	0.16
LDL	5.68	0.61	3.00	0.28	3.69	0.19	2.65	0.19
VLDL	1.08	0.86	0.36	0.40	0.40	0.55	0.28	0.19
recovery	101%	99%	104%	92%	97%	93%	98%	94%

Fig. 2. Cholesterol profiles and agarose gels of plasma samples separated in self-generating gradients Plasma samples (A–D) were separated in self-generated gradients of iodixanol as described in Materials and Methods. Each gradient was collected in 20 fractions. Fraction 1 is the bottom of the tube. Cholesterol and TG were determined in each fraction, and aliquots of each fraction were separated on agarose gels as described in Materials and Methods. The upper photograph in each case illustrates the agarose gel. The top band is HDL (fractions 1–7, approximately), the bottom band LDL (fractions 7–15, approximately) and the middle band VLDL (fractions 18–20). A: Lipoprotein [a] is visible immediately below HDL (fractions 5–7). The lower graph shows the distribution of cholesterol (left-hand scale; solid line) and TG (right-hand scale; broken line). The values plotted are μmol of each lipid in the fraction. The cholesterol and TG concentrations (mM) in total plasma, the contribution of HDL, LDL, and VLDL calculated from the distribution profile, and the recoveries of lipids from the gradient are shown in the table at the bottom of the figure. The cut-off between HDL and LDL, and LDL and VLDL, were determined from the profile in each case (see text).

concentrations were produced when four gradients were prepared at the same time (**Fig. 4**). The calculated values from the gradient for the cholesterol were also close with variation coefficients of 2.8%, 4.0%, and 4.7% for HDL, LDL, and VLDL cholesterol, respectively (Fig. 4). There were slight differences in the shape of the VLDL-TG peak at the top of the gradient. However, the calculated values for TG were also close with variation of 4.1%, 4.5%, and

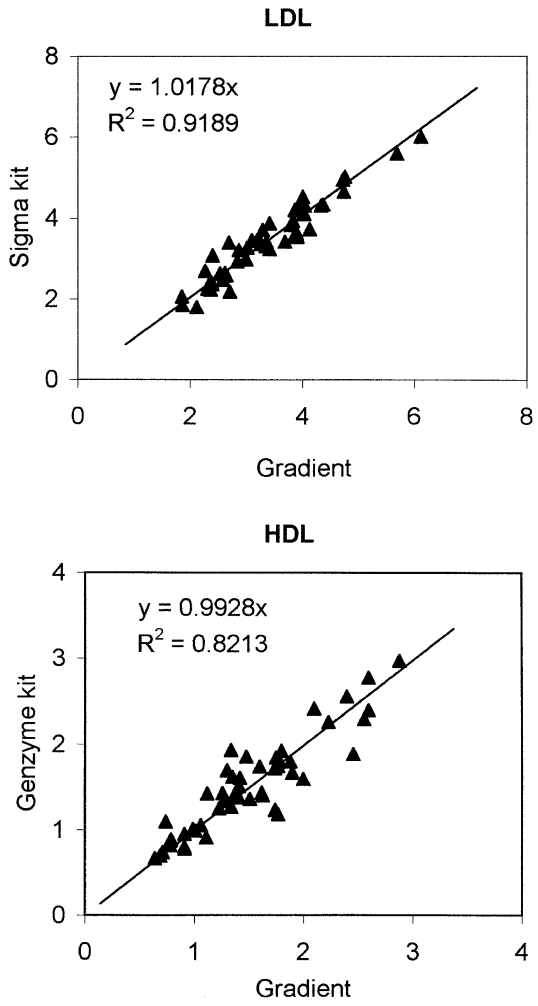


Fig. 3. Comparison of results obtained for LDL and HDL cholesterol from iodixanol gradients with those from commercial kits. A: Plasma samples from 50 individuals were analyzed in duplicate using the Sigma LDL-cholesterol (LDL-C) kit and further aliquots were separated on idoxanol gradients and the LDL-C determined in duplicate as described in Materials and Methods. B: Plasma samples from 50 individuals were analyzed in duplicate using the Genzyme HDL cholesterol (HDL-C) kit and were separated in iodixanol gradients, and the HDL-C determined in duplicate as described in Materials and Methods. Average values of the duplicates are plotted.

1.3% for HDL, LDL, and VLDL, respectively. Similar profiles and values for lipoprotein cholesterol and TG were obtained when either plasma or serum was used (**Table 1**). The results from cholesterol assay were not affected by storage of plasma either at 4°C or at −30°C for several weeks (Table 1). However, storage under either condition resulted in considerable variation in the values obtained for TG. In the case of VLDL, this is probably because freezing or prolonged storage denatured the particles resulting in variable recovery of the TG. VLDL also tended to form more diffuse smeared bands on agarose gel after freezing.

Separation of LDL subfractions

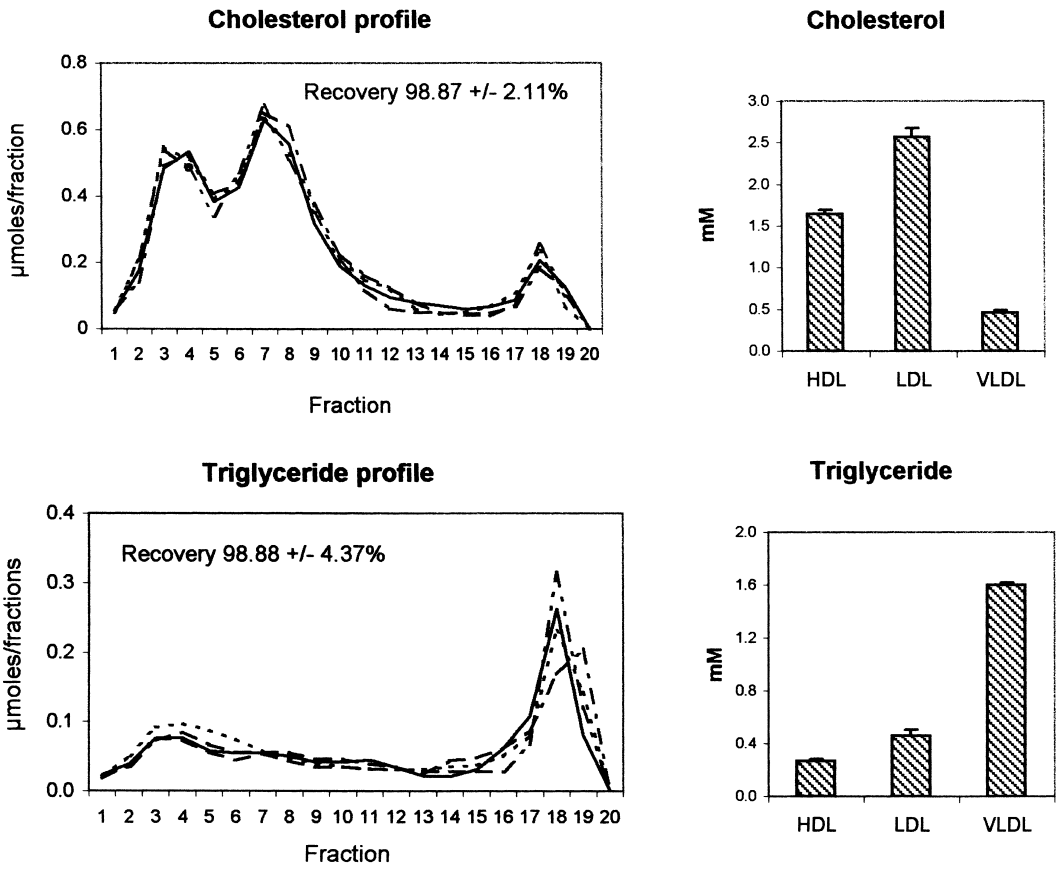
The position of the LDL cholesterol peak varied on the iodixanol gradient between different plasma samples (Fig. 2). The diameter of the LDL particles in the gradient fractions, determined by gradient gel electrophoresis, exhibited a linear relationship with the density of the gradient fraction indicating that LDL particles are resolved on the basis of size and density (**Fig. 5**). To improve resolution of the iodixanol gradients, a larger number of smaller fractions (44 instead of 20) were collected. When 272 samples were compared, the peak position of the LDL varied between fractions 19 and 29. In many samples, secondary peaks, shoulders, or inflections could be detected in the LDL-C curve consistent with three major groups of LDL density peaks between fractions 15–22, fractions 23–26, and fractions 27–33, corresponding to apparent densities of 1.038–1.060, 1.019–1.038, and 1.011–1.019 g/ml iodixanol, respectively. Examples of the lightest, densest, and intermediate LDL-C profiles observed are illustrated in **Fig. 6**.

Comparison of different rotor/centrifuge combinations

To test the transfer of the basic protocol to other rotors using a floor-mounted ultracentrifuge, the same plasma sample was separated and analyzed simultaneously using the TLN100 rotor (tube capacity 2.9 ml) in the Optima bench top ultracentrifuge, the NVT65.1 (tube capacity 5.9 ml), and the Vti65.1 (tube capacity 11.2 ml). The same number of fractions was collected in each case. Although the position of the peaks varied between rotors, essentially similar separations were achieved and the values for lipoprotein cholesterol were close (**Fig. 7**).

DISCUSSION

Methods for the analysis of plasma lipoproteins are based on differences in physical characteristics, usually density and charge. Ultracentrifugation is generally considered the reference method for lipoprotein analysis. For routine analysis, however, ultracentrifugation has the disadvantages that the protocols are often time-consuming, sequential flotation can result in considerable loss of material, and salt gradients are unstable and difficult to prepare. Moreover, it is necessary to remove the high salt before further analysis can be carried out. This frequently results in loss of material. Self-generating gradients of iodixanol have a number of advantages for the separation of plasma lipoproteins. Gradients form in a relatively short time, so that separation and analysis of plasma lipoproteins can be achieved within 4 h of removing blood. The gradients are reproducible and stable and can be collected in a varied number of fractions depending on the resolution required. Because iodixanol does not interfere with analysis of lipids, enzymes, or apolipoproteins, gradient fractions can be analyzed without further treatment, and recoveries of cholesterol and TG from the gradient are close to 100%. The procedures used are technically easy and gradient collection of cholesterol and TG can be automated. For example, gradients can be collected in tubes and analyzed using an autoanalyser or, when gradients are collected in microtitre plates, robotic handling systems



Cholesterol mM	average	sd	CV%
HDL	1.64	0.046	2.8
LDL	2.57	0.099	4.04
VLDL	0.46	0.022	4.74

Triglyceride mM	average	sd	CV%
HDL	0.27	0.011	4.09
LDL	0.46	0.021	4.47
VLDL	1.6	0.021	1.32

Fig. 4. Reproducibility of separation. Plasma from the same individual was separated in four separate gradients, which were collected in 20 fractions. The cholesterol and TG profiles (left-hand graph) and the HDL-, LDL-, and VLDL-C and TG concentrations (right-hand histogram) were determined as described in Materials and Methods. The recoveries as a percentage of the total plasma separated ± SD are indicated on the graphs. The lower table shows the average values for HDL-, LDL-, and VLDL-C and TG ± SD and the variation.

can be used to carry out lipid analyses. The values obtained for HDL-C and LDL-C correlate with those from differential precipitation using commercial kits. However, in self-generating gradients, lipoprotein lipids are measured in the same sample and recoveries are routinely checked, thus providing an internal control not possible with precipitation methods that are carried out separately.

Lp[a] occurs in a small proportion of plasma samples. This is concentrated in iodixanol gradients into 2–4 fractions (concentration factor ~3 fold), so that levels of Lp[a] that are low or undetectable in agarose gels of whole plasma become apparent. This is an additional diagnostic feature of the iodixanol gradients combined with agarose gel electrophoresis. However, as Lp[a] overlaps the HDL/LDL boundary, this will contribute to the cholesterol content of both of these lipoproteins. In precipitation methods, Lp[a] cholesterol is recovered in the LDL; for accurate measurement in plasma with high Lp[a], this should be measured separately and taken into account in calculations of HDL and LDL cholesterol.

TABLE 1. Effect of sample preparation and storage on lipoprotein analysis

	A					B							
	Plasma Average	SD	Serum Average	SD	Variation	Plasma Average	SD	Stored at 4°C Average	SD	Variation	Stored at −30°C Average	SD	Variation
		mM			%		*mM*			%		*mM*	%
Cholesterol													
HDL	1.23	0.012	1.21	0.009	1.63	1.67	0.11	1.62	0.09	3.09	1.69	0.08	1.19
LDL	4.13	0.023	4.09	0.021	0.96	2.52	0.12	2.49	0.11	1.19	2.53	0.15	3.95
VLDL	0.29	0.011	0.30	0.012	1.72	0.22	0.03	0.21	0.04	4.54	0.23	0.27	4.34
Triglyceride													
HDL	0.09	0.003	0.091	0.002	1.11	0.07	0.001	0.08	0.001	14.28	0.08	0.002	14.28
LDL	0.26	0.014	0.25	0.012	4.00	0.22	0.21	0.23	0.019	4.34	0.21	0.018	4.54
VLDL	0.51	0.011	0.49	0.009	3.92	0.41	0.32	0.36	0.006	12.19	0.35	0.051	14.6

In (A) blood was drawn and divided into two parts. EDTA was added to one part (plasma) and the other part was allowed to clot (serum). Red cells were pelleted by centrifugation and four aliquots of the plasma or serum were separated in iodixanol gradients and the HDL, LDL, and VLDL cholesterol and TG were determined as described in Materials and Methods. In (B) plasma from one individual was used. Aliquots were separated immediately in iodixanol gradients and stored in the refrigerator at 4°C or frozen at −30°C for two weeks before separation of iodixanol gradients. In each case, four plasma aliquots were separated in iodixanol gradients and the HDL, LDL, and VLDL cholesterol and TG were determined as described in Materials and Methods. The results are given as average ± SD for the four separate analyses.

There is firm evidence for the structural heterogeneity of LDL particles (4) and for the clinical importance of the different subclasses in assessing risk of coronary heart disease (5–9). However, the methods for analysis of LDL subclass are frequently technically difficult, time-consuming, and not appropriate for routine use. Two methods have been used generally: non-denaturing gradient-gel electrophoresis combined with densitometry (8, 9, 18), and density gradient ultracentrifugation on salt-gradients (4, 7, 19, 21). The former method provides particle size measurement rather than composition. In the later case, in some protocols LDL is pre-isolated by differential centrifugation, and in others whole plasma is used. The density distribution of LDL on the salt-gradient is generally measured by densitometry using prestained lipoproteins, or by collection of the gradient and measurement of absorption at 280 nm, or in some cases by determination of apolipoprotein-B (apo-B). A consensus model has emerged from studies using different analytical methods, that LDL consists of populations of particles including large buoyant particles: Pattern A or LDL I (LDL density 1.025–1.034), Pattern I or LDL II (LDL density 1.034–1.040), and Pattern B or LDL III (LDL density 1.040–1.045) (7). Recently a method for determination of lipoprotein subclass was reported in which lipoproteins are stained with a fluorescent ceramide and separated by capillary isatachophoresis (22). Although this method overcomes many of the problems with the use of gradient gels and salt gradients, it cannot be used to measure lipoprotein cholesterol or TG, and further analysis of the subfractions (determination of apo-B, for example) is not possible. In the current study, LDL-C density profiles were obtained as part of the overall analysis of plasma lipoproteins. Thus, total plasma, HDL-, LDL-, and VLDL-C, TG, and the shape and position of the LDL-C peak are determined simultaneously in the same sample without loss of material. This allows accurate analysis of different parameters (for example, of the relationship

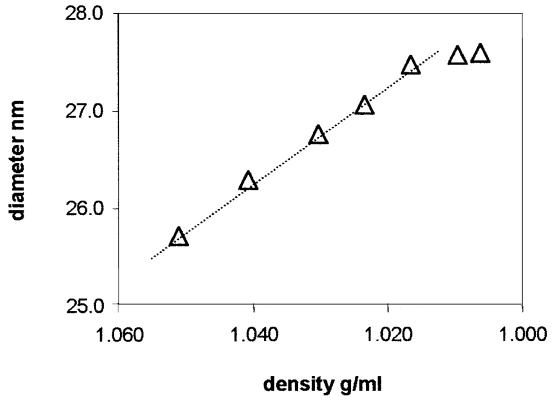


Fig. 5. Relationship of LDL particle diameter and density in iodixanol gradients. Plasma was separated in iodixanol gradients as described in Materials and Methods. Samples of the gradient fractions containing the LDL were analyzed by gradient gel electrophoresis. The diameter of the LDL particles is plotted against the apparent density on the iodixanol gradient. We are grateful to Dr. Muriel Caslake, University of Glasgow, for carrying out the gradient gel electrophoresis.

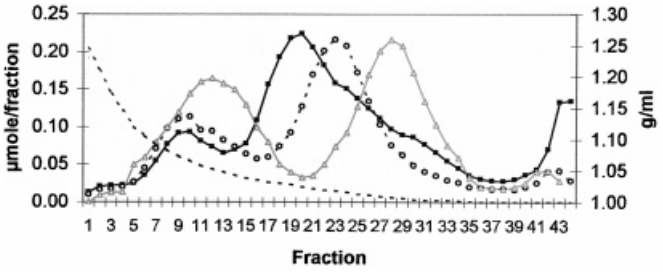


Fig. 6. Examples of cholesterol profiles exhibiting a range of LDL densities. Plasma samples were separated in iodixanol gradients that were collected in 44 fractions. The cholesterol (µmol/fraction) is plotted against fraction number (left-hand scale). A parallel iodixanol gradient without plasma was also prepared, and the density determined by measurement of the refractive index of the fractions (15) (right-hand scale). Gradients containing the most dense, least dense, and intermediate-density lipoproteins are illustrated.

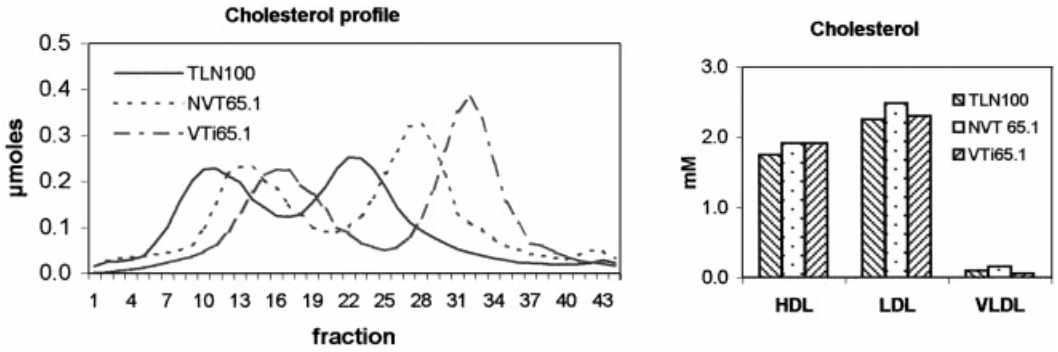


Fig. 7. Comparison of different rotors for separation of the same plasma sample. Plasma from the same individual was mixed with Liposep (1:4). Three rotor/centrifuge combinations were used to separate the lipoproteins. TLN100 rotor in the Optima bench top ultracentrifuge, as described in Materials and Methods; NVT65.1 rotor in the Beckman floor mounted L80 ultracentrifuge, as described in Materials and Methods except that 2.8 ml of each layer was used in 5.9 ml Optiseal tubes; Vti65.1 rotor in the Beckman floor mounted L80 ultracentrifuge, as described in Materials and Methods except that 4.8 ml rotors were used in 11.2 ml Optiseal tubes. The tubes were centrifuged in the L80 ultracentrifuge for 2.5 h at 650,000 rpm. The same number of fractions were collected from each gradients; three drops from the TLN100 tubes; six drops from the NVT65.1 tubes and 14 drops from Vti65.1 tubes. Cholesterol profiles were prepared (A) and the cholesterol concentration of the VLDL, LDL, and HDL were determined (B) as described in Materials and Methods. The results are the average of two gradients assayed in duplicate.

of plasma or VLDL TG to predominant LDL subfraction). In the protocols we used routinely for LDL analysis, 44 gradient fractions were taken, and overall, the cholesterol profiles obtained exhibited three shoulders consistent with three LDL subclasses. If finer resolution is required, a greater number of gradient fractions can be collected. The apparent densities of the LDL peaks were 1.011–1.019, 1.019–1.038, and 1.038–1.060 g/ml and are thus lower than the apparent densities of LDL subfractions prepared in salt gradients. However, density is an operational parameter, as this is measured from a blank gradient without plasma, which interferes with refractive index measurement. Although the plasma proteins move to the bottom of the gradient during centrifugation, their presence alters the density profile of the gradient. In addition, comparison with salt gradients is complicated by the fact that, in high salt concentrations, water is removed from the lipoproteins and this alters the native density of the particles.

In summary, the use of self-generating gradients of iodixanol to separate and analyze plasma lipoproteins offers a rapid, reproducible, and accurate method that is technically simple. HDL-, LDL-, and VLDL-C and TG are measured and the predominant LDL subclass can be identified in a single step. All of the information for comparative analysis of total and lipoprotein lipids and LDL density is provided in the same analysis.

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