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Comparison of lipoprotein fractionation by sequential density gradient ultracentrifugation with precipitation or fast phase liquid chromatography☆

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Abstract

Lipoprotein fractions, i.e. very low density (VLDL, $\rho = 0.93-1.019$), low density (LDL, $\rho = 1.019-1.063$), and high density lipoprotein (HDL, $\rho = 1.063-1.21$) were separated by sequential gradient density ultracentrifugation (SDGU) and their cholesterol values were determined and compared with values determined by fast phase liquid chromatography (FPLC) or HDL cholesterol by phosphotungstic acid-$MgCl_2$ (PTMg) precipitation in hamsters fed diets containing various levels of cholesterol. The correlation coefficient (r) for LDL between the SDGU and FPLC methods in plasma from hamsters fed 0–3% cholesterol diets was 0.21–0.51, $n = 45$. The FPLC method over estimated (+45%) LDL and the HDL values were under estimated (−18%). The agreement between FPLC and SDGU methods was also evaluated by plotting mean values against the differences between the values obtained by the two methods. FPLC method overestimated LDL 22–49% (mean 36%) and HDL was underestimated 14–27% (mean 20%). This was significant systematic bias with the FPLC method in VLDL, LDL and HDL values with the level of cholesterol in the diet. As FPLC is a fast method, it could be used in intervention type experiments to monitor the progress, however final results may need validation for research studies with the SDGU method. HDL determined by the SDGU method and phosphotungstic acid $MgCl_2$ precipitation in hamsters ($n = 26$) fed 0.25% cholesterol diets was represented by two significantly different ($P \leqslant 0.05$) divergent lines when a regression fitted model and a one-to-one relationship model by the two methods were plotted. The data suggest that in hamsters fed either no added cholesterol or cholesterol-containing diets, lipoprotein fractions determined by the precipitation method or by FPLC need to be validated against a SDGU for critical samples. Published by Elsevier Science Ltd.

Keywords: Cholesterol; HDL; LDL; Lipoproteins; Methods; Plasma; Ultracentrifugation; VLDL

1. Introduction

Elevated levels of circulating low density lipoprotein (LDL) cholesterol are associated with an increased risk of premature coronary artery disease, atherosclerosis and stroke. Lipoprotein metabolism studies require the quantitative separation of very low density lipoprotein (VLDL, $\rho = 0.93-1.019$), low density lipoprotein (LDL, $\rho = 1.019-1.063$) and high density lipoprotein (HDL, $\rho = 1.063-1.21$) fractions. Lipoprotein fractions are defined by their hydrated densities rather than biochemical composition, size or charge. VLDL, LDL and HDL were fractioned by the sequential density gradient ultracentrifugation (SDGU) method (Havel, Eder, & Bragdon, 1955). This is an accurate and time-consuming method as the lipoprotein subclasses are fractionated by their density, other methods are compared and validated against the SDGU. Fast phase liquid chromatography (FPLC) has been described for separation and analysis of lipoproteins (Rudel, Marzetta, & Johnson, 1986). The chromatographic separation occurs primarily due to differences in size, however, the size–density

☆ Names are necessary to report factually on available data; however, the US Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of a name implies no approval of the product to the exclusion of others that may also be suitable.

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relationships of lipoprotein molecules are not completely understood, separation by FPLC often requires further characterization (Rudel et al., 1986). High-performance liquid chromatography (HPLC) has been described for the separation of plasma lipoprotein fractions in humans and nonhuman primates (Hara & Okazaki, 1986). HPLC method is simple, takes less than 1 h, there is no need for sample pretreatment, and requires a small sample (less than 20 μl of intact serum). However, a quantization of lipoproteins is not practical by HPLC method, as the amounts of lipoproteins are small compared with those of albumin or immunoglobulins, which elute overlapping with LDL and HDL fractions. HPLC method can be applied for changes in HDL subfractions during drug therapy. Although HPLC method cannot be used for the preparation of plasma lipoproteins, this method can offer additional information after the lipoprotein fractionation by ultracentrifugation (Hara & Okazaki, 1986). A low temperature flotation method to isolate lipoproteins was described using 2 ml samples in a benchtop ultracentrifuge, the method required 18 h, there is a recovery problem as the lipoproteins stick to the sides of a narrow tube, and the method results in 9–12% higher values for HDL (Tong, Knapp, & VanRollins, 1998). HDL cholesterol in human serum is also determined after precipitation of apo-B-containing lipoprotein by heparin–$MnCl_2$ (Warnick & Albers, 1978), dextran sulfate-$MgCl_2$ (Warnick, Benderson, & Albers, 1982), phosphotungstic acid-$MgCl_2$ (Assman, Schriewer, Schmitz, & Hagele, 1983) or the Beta-Quant method [National Heart and Lung Institute, DHEW Publication No. (NIH) 75-628, 1974]. The Beta-Quant method removes VLDL and chylomicrons (density range of 0.93–1.006) by a single short time ultracentrifugation spin, HDL is determined in the supernatant after dextran-sulfate precipitation, and LDL is determined by difference. Good correlations were reported between the phosphotungstic acid-$MgCl_2$ and ultracentrifugation methods in humans (Assman et al., 1983). Bland and Altman (1986) have shown that high correlation between the methods compared may lead to the wrong conclusion that the new method can replace the old one, a plot of difference in values between the methods against their mean may be more informative. Precipitation methods are currently used for routine clinical determinations of HDL. An excellent correlation was established between the FPLC and Beta-Quant methods for lipoprotein fractionation in humans and several animal species, including the hamsters fed 0.06% cholesterol diet with total plasma cholesterol (TC) values of 6.93 mmol/l (Kieft, Bocan, & Krause, 1991). However, the FPLC method has not been evaluated when hamsters were fed higher levels of dietary cholesterol and this method has not been validated against lipoprotein fractionation by SDGU.

In this paper, the validity of plasma lipoprotein (VLDL, LDL and HDL) cholesterol fractionation with FPLC and HDL by precipitation methods were investigated and compared against SDGU method in hamsters fed semipurified diets containing various levels of cholesterol.

2. Experimental procedures

2.1. Experimental animals

Male, 23-day-old weanling Syrian golden hamsters (Sasco, Inc., Omaha, NE) were fed individually in wire bottom cages. One hundred eighty hamsters were fed diets containing various levels of cholesterol in nutritional studies. For HDL by precipitation vs. SDGU method, 26 pooled plasma samples (pools of 3 or 4) from 90 hamsters fed 0.25% cholesterol diets were compared. For comparison of VLDL, LDL and HDL cholesterol by FPLC vs. SDGU method, 90 hamsters were fed diets containing 0–3% cholesterol diets. Pooled samples of fresh plasma were prepared (two animals per pool) using an equal volume of plasma from each animal. Feeding and experimental conditions are described in detail (Kahlon, Chow, Irving, & Sayre, 1996).

2.2. SDGU

Hamsters were fasted for 16 h and anesthetized with CO_2 for tissue sample collection. All the procedures described were approved by the Animal Care and Use Committee of the Western Regional Research Center, USDA, Albany, CA, and conformed to the principles in "*Guide for the Care and Use of Laboratory Animals*" (National Research Council, National Institutes of Health. Publ. No. 85-23 rev., 1985). Blood was drawn by cardiac puncture into plastic tubes containing anticoagulant (ethylene diamine tetra acetic acid, dipotassium salt, 0.8 mg/ml of blood) and centrifuged at $1500 \times g$ for 30 min at 4 °C to obtain plasma. Fresh plasma pooled samples were prepared (two animals per pool) using an equal volume of plasma from each animal because lipoprotein fractionation by SDGU requires 1 ml of plasma for each sample, which is more than half that obtained from each animal by heart puncture. A protease inhibitor (ϵ-amino caproic acid, ICN Biomedicals, Inc., Costa Mesa, CA, 1.3 mg/ml plasma) and an antimicrobial agent (garamycin 50 mg/ml, Schering Corp. Kenilworth, NJ, 10 μl/ml of plasma) was added to stabilize the plasma. Lipoproteins were fractionated using density gradient ultracentrifugation (Havel et al., 1955). After adjusting the background density of 1 ml plasma to 1.019 g/ml with 5 ml of NaCl solution (1.0214 g/ml), plasma was centrifuged in an ultracentrifuge (model L8, Beckman Inc., Palo Alto, CA)

at 40 K for 18 h at 17 °C in a fixed-angle rotor (model 50.3, Beckman, Inc.). The top 1 ml was removed as the VLDL fraction, and another 1 ml was removed as background. The subnatant density was adjusted to 1.063 g/ml and centrifuged similarly for 24 h. The top 1 ml (1.019–1.063 g/ml) was removed as the LDL fraction, and another 1 ml was removed as background. The subnatant contained the HDL fraction. Lipoprotein fractions and plasma were analyzed in triplicate for cholesterol by an enzymatic colorimetric procedure (diagnostic kit no. 352, Sigma Chemicals, St. Louis, MO). Cholesterol values were determined using standard curves obtained by running several concentrations of standards provided with the kit. HDL values were corrected for a reagent dilution factor. With each ultracentrifugation, two salt solution tubes with similar density were run and the densities of their fractions were monitored with a density meter (model DMA-48, Anton Paar, Inc., Richmond, VA). Some reports have used a density cutoff of 1.055 for hamster LDL (Gallaher, Hassel, Lee, & Gallaher, 1993; Spady & Dietschy, 1988); others have reported that hamster LDL density extends up to 1.072 (Goulinet & Chapman, 1993); however, our own examination of hamster lipoprotein by agarose gel electrophoresis suggests that LDL density up to 1.063 is appropriate.

2.3. HDL-C by phosphotungstic acid–$MgCl_2$ precipitation (PTMg)

VLDL and LDL fractions in 0.5 ml plasma were precipitated with 0.1 ml phosphotungstic acid (30.3 mmol/l) and $MgCl_2$ (100 mmol/l) reagent mixture. After 5 min, supernatant was obtained by centrifugation at $1500\times \boldsymbol{g}$. Cholesterol concentration in the supernatant was determined by the same procedure as that used for the ultracentrifugation method. HDL values were corrected with an appropriated reagent dilution factor.

2.4. FPLC

This method has been described in detail by Kieft et al. (1991) and Marz, Siekmeier, Scharnagl, Seiffert, and Gross (1993). The FPLC system used was a Waters 710 Wisp (auto sample injection system), Waters 510 pump (Waters–Millipore, Milford, MA), and a Superose 6HR FPLC column, 1×30 cm No. 17-0537-01 (Pharmacia, Piscataway, NJ). A 20 μl aliquot of plasma was injected and lipoprotein fractions eluted at a flow rate of 1.0 ml/min, with 0.15 M NaCl, pH 7.0 buffer containing 0.2% sodium azide. VLDL, LDL and HDL were determined by post-column reaction with cholesterol assay reagents (Boehringer–Mannheim Diagnostica, No. 236691) using a mixing coil (Bodman No. 1615-50) in a temperature control jacket (Bodman CJB-75) and isocratic pump model 79851A (Hewlett-Packard, Wilmington, DE) to deliver cholesterol reagents at a rate of 0.2 ml/min. The cholesterol reaction chromogen absorbs at 550 nm. The separation of lipoprotein is primarily by size; however, peaks were designated as VLDL, LDL and HDL with peak retention times of 10.0, 13.0 and 18.0 min, respectively. The areas under the peak for Cholesterol Calibrator (bovine) 200 mg/dl, No. C0284 (Sigma, St. Louis, MO) was for VLDL, 28%; LDL, 27% and HDL, 45%. There was $\leqslant$5% variability in the peaks areas with the repeat injections of the same sample. In case a hamster plasma sample with very high cholesterol levels there was peak broadening. Those samples were diluted before injection and appropriate dilution factors were used to correct the final values. Peak areas were integrated using Hewlett-Packard Chem Station software. VLDL, LDL and HDL values were calculated from pooled (two animals/pool) TC values using percent area under respective designated peaks.

All analyses were conducted in triplicate. Data were statistically analyzed using SAS software (SAS Institute, NC). Data were normally distributed and tested for homogeneity of variances. Analysis of variance, correlation coefficients and regression analysis were conducted. Data were also analyzed by the method described by Bland and Altman (1986) for assessing agreement between two methods of clinical measurements. A value of $P \leqslant 0.05$ was considered the criterion of significance.

3. Results and discussion

3.1. HDL-C by PTMg vs. SDGU

Since precipitation by phosphotungstic acid gave a clearer supernatant and a lower standard error of the means (S.E.M.) than other precipitation methods, a comparison of HDL analysis by PTMg was made against the SDGU method in 26 pooled plasma samples (pools of 3 or 4) from 90 hamsters fed 0.25% cholesterol diets. Total plasma cholesterol values ranged from 6.67 to 7.81 mmol/l. The mean values for HDL by PTMg and SDGU methods were 3.64 and 3.61 mmol/l, respectively. Although the mean values for HDL by the two methods were identical, the correlation coefficient (r) between PTMg and SDGU methods for HDL-C was only 0.60, and the coefficient of determination (r^2) was 36%. The coefficient of non-determination ($1-r^2=k^2$), the unexplained portion of the sum of squares of HDL quantization by the precipitation method, was 64%. When the HDL mean values of each of 10 treatments determined by both SDGU and PTMg were plotted against each other in a structural regression model (Fig. 1, dotted line) and compared against a hypothetical one-to-one relationship between the two methods (Fig. 1, solid line), the slopes of the two lines were

significantly ($P \leqslant 0.05$) different. The data reveals that in hamsters fed 0.25% cholesterol diets the precipitation method gives higher HDL values than the SDGU method at the high end and lower values at the low end. This suggests that determination of HDL by the phosphotungstic-$MgCl_2$ precipitation method is not an accurate predictor of HDL values in plasma from hamsters fed hypercholesterolemic diets when compared against the SDGU method. Precipitation methods are routinely used for clinical determination of HDL. LDL is determined using Friedewald equation, LDL = TC−HDL−triglycerides/5 (Friedewald, Levy, & Fredrickson, 1972). Calculated LDL values are not reliable with very low or very high triglyceride values. Data reported herein suggests that HDL values determined by precipitation were low at the low end and high at the high end compared with those determined by the SDGU method. Thus, HDL by precipitation may need to be validated by the density gradient method for critical samples.

3.2. Lipoprotein cholesterol by FPLC vs. SDGU

In 42 hamsters (21 pooled plasma samples) fed diets containing 15–20% saturated fat with no added cholesterol or low (0.05%) cholesterol, in which the mean TC values were 7.65 mmol/l, LDL values were similar by both the FPLC and SDGU methods. However, by FPLC there were significantly ($P \leqslant 0.05$) higher VLDL and significantly lower HDL values compared with those obtained by SDGU (Table 1). The percent of TC distributions for VLDL and HDL with SDGU vs. FPLC methods were 10 and 73 vs. 15 and 66%, respectively. The correlation coefficients between the two methods for VLDL and HDL values were 0.70 and 0.49, respectively. The correlation coefficient for LDL between SDGU and FPLC was only 0.21. The coefficient of determination (r^2) for plasma LDL and HDL between FPLC and SDGU methods in hamsters fed no or low added cholesterol diets ranged from 4–25%. The coefficient of non determination (the unexplained portion of the sum of squares for LDL and HDL mean values) by the FPLC method ranged from 75 to 96%. LDL-C is the most atherogenic lipoprotein and its accurate determination is critical in all evaluations and recommendations for the amelioration and prevention of atherosclerosis.

In 32 hamsters fed 0.5% added dietary cholesterol (16 pooled samples), where mean TC values were 15.18 mmol/l. Similar VLDL values were observed by FPLC and SDGU methods. LDL values were significantly higher and HDL values significantly lower by FPLC compared with those obtained by SDGU. Percent distribution of LDL and HDL by the SDGU vs. FPLC methods were 26 and 46 vs. 43 and 30%, respectively. The correlation coefficient between the LDL values

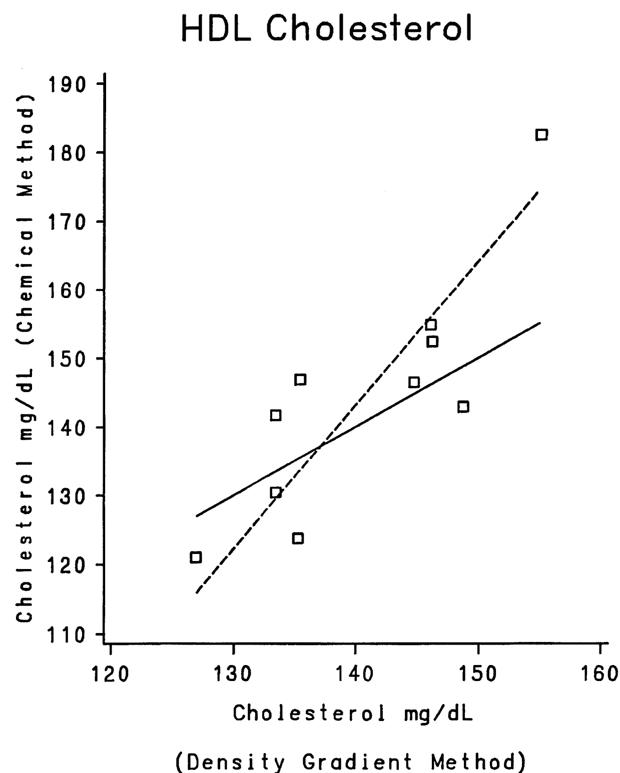

Fig. 1. Structural regression plot using the mean plasma high density lipoprotein cholesterol (HDL-C) values in 10 groups of hamsters fed 0.25% cholesterol diets. The dotted line is the fitted model for HDL-C by the sequential density gradient ultracentrifugation method (SDGU) vs. the phosphotungstic acid $MgCI_2$ (PTMg) precipitation chemical method. The solid line represents a hypothetical one-to-one relationship between the two methods. HDL-C values by the precipitation chemical method are high on the high end and low on the low end.

determined by these two methods was 0.51. In hamsters fed 0.5% cholesterol, r^2 for LDL by the FPLC method was only 25%. Although the correlation coefficient for HDL values between the two methods is extremely good (0.97), the mean HDL values by the FPLC method were 34% lower than those by the SDGU method. In these hamsters, LDL was overestimated by 59% with the FPLC method compared with the SDGU method.

In 16 hamsters (eight pooled samples) fed very high (3.0%) cholesterol diets, TC values ranged from 13.8 to 20.4 mmol/l (mean 18.6 mmol/l). Mean VLDL, LDL and HDL values were similar by SDGU and FPLC methods. However, relative difference in values by FPLC compared with SDGU method were VLDL, −22%; LDL, +53%; and HDL, −18%; these differences were not significant due to a small number of samples and high variability among samples from animals fed the very high cholesterol diet. Relative distribution by SDGU and FPLC for VLDL, LDL and HDL was (36, 27 and 38 vs. 28, 42 and 30% of TC, respectively). In hamsters fed high (3%) cholesterol diets correlation coefficients between the two methods for VLDL, LDL and HDL were 0.70, 0.22 and 0.60,

Table 1
Total Plasma Cholesterol (TC), Very Low Density Lipoprotein Cholesterol (VLDL), Low Density Lipoprotein Cholesterol (LDL) and High Density Lipoprotein Cholesterol (HDL) by Sequential Density Gradient Ultracentrifugation (SDGU) vs. Fast Phase Liquid Chromatography (FPLC) in plasma from hamsters fed diets with various levels of cholesterol[a]

Lipoprotein	n	Dietary cholesterol (%)	Pooled (mmol/l)	SDGU (mmol/l)	FPLC (mmol/l)	SDGU (% of TC)	FPLC (% of TC)	r[b]
VDLC	21	0.00–0.05		0.78±0.06 b	1.13±0.08 a	10.2±0.6 b	14.8±1.0 a	0.70
LDL	21	0.00–0.05		1.34±0.06 a	1.48±0.09 a	17.1±0.6 a	19.4±1.1 a	0.21
HDL	21	0.00–0.05		5.52±0.14 a	5.04±0.19 b	72.7±0.8 a	65.8±1.7 b	0.49
TC	21	0.00–0.05	7.65±0.20					
VLDL	16	0.5		4.75±0.73 a	4.42±0.70 a	28.8±2.6 a	27.1±2.5 a	0.80
LDL	16	0.5		4.14±0.54 b	6.58±0.63 a	25.7±1.6 b	43.0±1.5 a	0.51
HDL	16	0.5		6.29±0.20 a	4.18±0.20 b	45.6±3.7 a	29.9±2.8 b	0.97
TC	16	0.5	15.18±1.22					
VLDL	8	3.0		7.10±1.37 a	5.51±1.18 a	35.8±3.0 a	27.7±3.6 a	0.70
LDL	8	3.0		5.18±0.88 a	7.94±1.33 a	26.6±1.7 a	42.0±3.7 a	0.22
HDL	8	3.0		6.35±0.16 a	5.18±0.86 a	37.6±4.4 a	30.3±5.9 a	0.60
TC	8	3.0	18.63±2.18					

[a] Values (means±S.E.M.) within a row comparing as mmol/l and percent values with different letters are significantly different ($P \leqslant 0.05$) SDGU vs. FPLC. Each sample represents pooled plasma from two male hamsters.
[b] r, correlation coefficient between SDGU and FPLC methods.

respectively. The coefficient of determination (r^2), which could be considered the accuracy of predictability of LDL, was 5% with the FPLC method. The FPLC method predicted LDL values at a level 53% higher and HDL levels 18% lower than those obtained by SDGU.

In 45 total pooled (two animals/pool) plasma samples from hamsters fed either no added dietary cholesterol, low (0.05%), moderate (0.5%) or very high dietary cholesterol (3%), LDL-C determined by SDGU remained in a relatively narrow range (17.11–26.6% of total plasma cholesterol). Similar values (15–25% of TC) for hamster LDL-C have been reported using agarose electrophoresis (Sima, Bulla, & Simionescu, 1990) and SDGU (Goulinet & Chapman, 1993; Kahlon, Chow, Knuckles, & Chiu, 1993). Pooling all the data over the range of 0–3% added dietary cholesterol ($n = 45$), VLDL, LDL and HDL determined by SDGU vs. FPLC was 21, 22 and 57 vs. 22, 32 and 47% of TC, respectively, with correlation coefficients of 0.84, 0.52 and 0.91, respectively. Over this broad range of dietary cholesterol with $n = 45$, the coefficient of determination (r^2) of the most atherogenic fraction LDL was 27%; the FPLC method overestimated (+45%) LDL and the non-atherogenic cholesterol HDL were underestimated (−18%), compared with the SDGU method which separates the various lipoprotein fractions by their densities. The accuracy of predictability (r^2) of LDL in hamster plasma by particle size, i.e. the FPLC method, ranged from 4 to 27% compared with SDGU. The data suggest that LDL values determined by FPLC were inflated by 13–67%, possibly due to the inclusion of VLDL with the density range of 1.006–1.019. It may also be contaminated up to 9–34% by those HDL molecules which are of a similar size to LDL molecules (Rudel et al., 1986).

Bland and Altman (1986) suggested that it may be more appropriate to assess the agreement between two methods of clinical measurements by plotting the differences in values obtained by two methods against the mean values by the two methods rather than relying on correlation coefficients only. Data for VLDL, LDL and HDL cholesterol values ($n = 45$) obtained by SDGU and FPLC methods were also analyzed as suggested by Bland and Altman (1986). Evaluating the data by comparing the mean differences resulted in clumping of the data on one side, log transformation was used to obtain more uniform distribution of the values for VLDL and LDL. Fig. 2, shows mean log VLDL vs. difference (FPLC−SDGU) in log VLDL. For VLDL the mean difference on the log scale was 0.098 with a 95% confidence interval of −0.010–0.206. The antilogs of the limits were 0.99–1.23. Thus there was 95% probability that FPLC method would result in values between 0.99 and 1.23 times the results obtained by SDGU method. The VLDL values obtained by FPLC would be from 1% below to 23% above those obtained by SDGU.

Fig. 3, the mean difference on log scale for LDL obtained by FPLC−SDGU on log scale was 0.301 with a 95% confidence interval of 0.202–0.400. The antilogs of the limits were 1.22–1.49. Thus LDL values obtained by FPLC were 22–49% higher than those obtained by SDGU.

Fig. 4, the mean difference between FPLC minus SDGU was −1.19 with a 95% confidence interval of

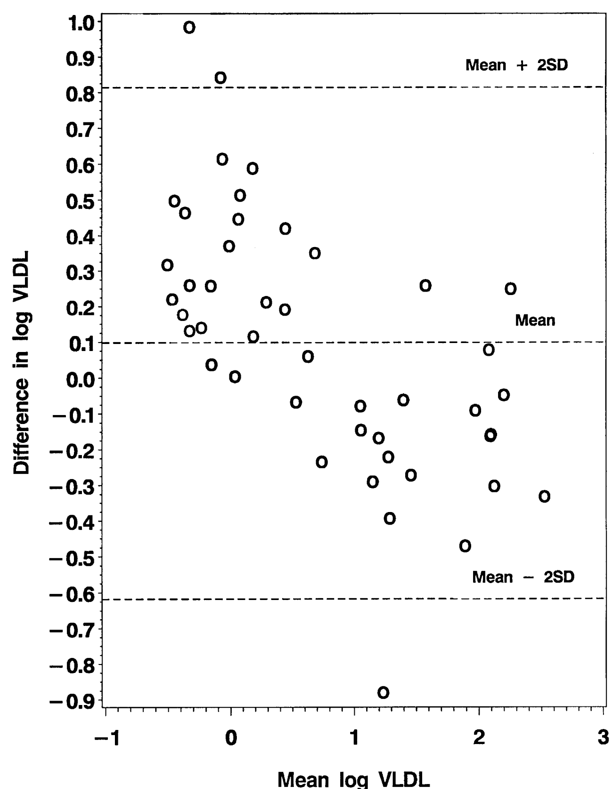

Fig. 2. Mean log very low density lipoprotein (VLDL) cholesterol vs. differences in log VLDL between means of fast phase liquid chromatography (FPLC) minus sequential density gradient ultracentrifugation (SDGU) methods. For VLDL the difference on the log scale was 0.098 with a 95% confidence interval of −0.010–0.206. The anti logs of the limits were 0.99–1.23. Thus FPLC values were from 1% below to 23% above SDGU values.

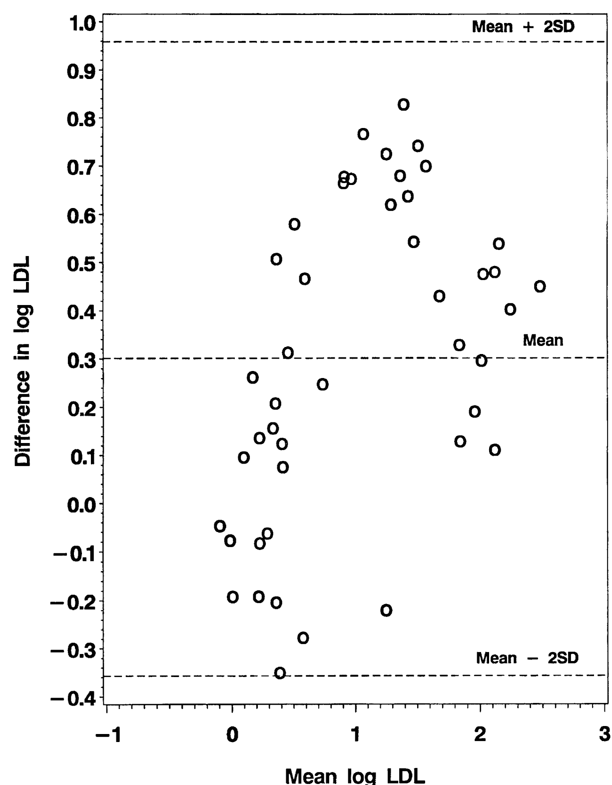

Fig. 3. Mean log low density lipoprotein (LDL) cholesterol vs. differences in log LDL between means of fast phase liquid chromatography (FPLC) minus sequential density gradient ultracentrifugation (SDGU) methods. For LDL the mean difference on log scale was −0.301 with 95% confidence interval of 0.202–0.400. The anti logs of the limits were 1.22–1.49. Thus there was 95% probability that FPLC method would result in values 22–49% higher than those obtained by SDGU.

−1.55––0.83. Thus there was 95% probability that FPLC results for HDL were from 14–27% below those obtained by SDGU method.

There was significant systematic bias with the FPLC method in VLDL, LDL and HDL with the low, moderate and high cholesterol diets. The intercept and slope of the regression lines were for VLDL, 0.27 and −0.24; LDL, 0.08 and 0.21; and HDL, −4.91 and 0.70. As observed by the slopes of the regression line and Fig. 3, for VLDL the values by FPLC method were higher at the low end and lower at the high end compared with those obtained by SDGU method. By FPLC method (slopes and Figs. 3 and 4) LDL and HDL values were lower at the low end and higher at the high end compared with those determined by the SDGU method.

FPLC is a fast chromatographic procedure that has been suggested to evaluate lipoprotein fractions by size (Kieft et al., 1991; Marzet et al., 1993; Rudel et al., 1986), however size–density relationship of VLDL, LDL and HDL are not clearly understood. This method is useful in obtaining quick answers for the intervention studies and drug therapy to make changes in the treatment in progress. The agreement between FPLC and SDGU methods was determined by correlation coefficients, regression analysis and comparing mean values by the two methods against the differences in means FPLC minus SDGU. Analyzing the data by each method resulted in the same conclusion that FPLC method should be validated for VLDL, LDL and HDL determinations with the SDGU method for critical research samples. Bland and Altman (1986) suggested that correlation coefficients between the any two methods compared showed only similarity in the methods and high correlation coefficient may lead to the wrong conclusion that the new method can replace the old method. Analyzing the data to determine agreement between two clinical measurements by plotting mean values by the two methods against the differences in values by the two methods, may show that the new method cannot be used to replace the old method even when there was high correlation between the methods compared. Using a log transformation has been advised where appropriate. In this report correlation coefficients observed for LDL were rather low and suggest that FPLC cannot replace the SDGU method under the conditions described herein. Plotting the data as suggested by Bland and Altman (1986) the same conclusion was reached that FPLC method was not in agreement

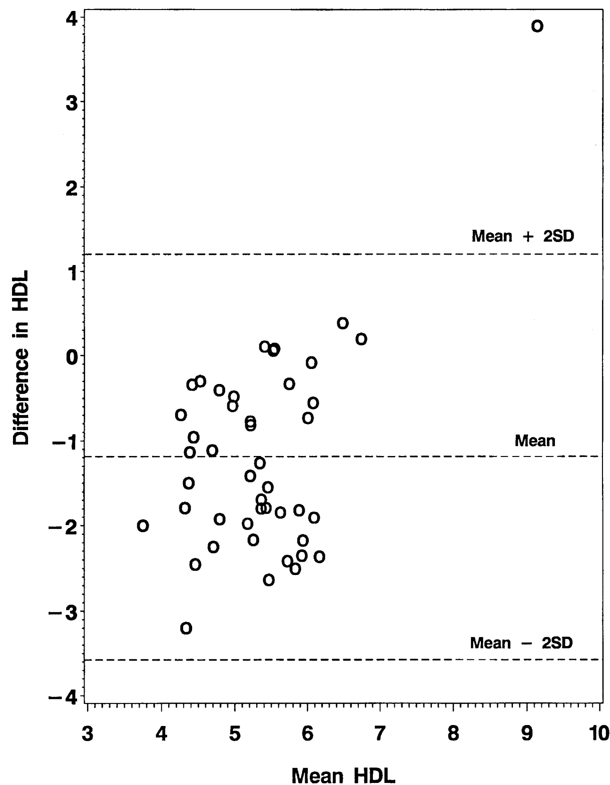

Fig. 4. Mean high density lipoprotein (HDL) vs. differences between the means of fast phase liquid chromatography (FPLC) minus sequential density gradient ultracentrifugation (SDGU) methods. For HDL cholesterol the mean difference between the two methods was −1.19 with a 95% confidence interval of −1.55–−0.83. Thus there was 95% probability that HDL value by FPLC method would be from 0.83 to 1.55 mmol/l (14–27%) below those obtained by the SDGU method.

with the SDGU method. The authors feel that it is appropriate to test the agreement between any two methods with more than one statistical procedure. When more than one procedure of statistical comparison leads to the same inference, then it would be advisable to accept or reject the new method as the case may be.

In hamsters fed 0–3% cholesterol diets lipoproteins fractions (VLDL, LDL and HDL) obtained by FPLC were compared against values by SDGU method, using correlation coefficients, FPLC methods overestimated LDL +45% and HDL was underestimated −18%. When the agreement between clinical methods was evaluated by plotting mean values against the differences between the values obtained by the two methods (Bland & Altman, 1986), FPLC method overestimated LDL 22–49% (mean 36%) and HDL was underestimated 14–27% (mean 20%). There was significant systematic bias with the level of cholesterol in the diet. As FPLC is a fast method it could be used in intervention type experiments to monitor the progress during the drug therapy, however final results for research studies may be concluded only after validation of critical values with the SDGU method.

In conclusion, in hamsters fed diets with or without added cholesterol, lipoprotein cholesterol data obtained with precipitation methods or by FPLC should be interpreted with the understanding that the values may not accurately reflect the distribution of the lipoproteins based on their density. LDL is the most atherogenic lipoprotein and its accurate measurement is critical in all evaluations and recommendations for the amelioration and prevention of atherosclerosis. When lipoprotein cholesterol fractions are determined by FPLC or by precipitation methods in critical samples in hamster studies, their validity may need to be confirmed by density gradient ultracentrifugation.

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