

# A simple and precise method for measuring HDL-cholesterol subfractions by a single precipitation followed by homogenous HDL-cholesterol assay

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**Abstract** HDL consists of two major subfractions, HDL<sub>2</sub> and HDL<sub>3</sub>. This paper describes a simple method for assaying HDL subspecies by combining a single precipitation with a direct high density lipoprotein-cholesterol (HDL-C) assay. A precipitation reagent (0.06 ml) containing 1,071 U/ml heparin, 500 mmol/l MnCl<sub>2</sub>, and 12 mg/ml dextran sulfate was added to a serum (0.3 ml). The sample was incubated and centrifuged at 10,000 rpm for 10 min. HDL<sub>3</sub>-C was measured by a homogenous HDL-C assay in the supernatant, and HDL<sub>2</sub>-C was estimated by subtracting the HDL<sub>3</sub>-C from the direct HDL-C. The HDL<sub>3</sub>-C and HDL<sub>2</sub>-C values determined by the precipitation method were identical to those determined by ultracentrifugation, and there were excellent correlations between the methods in the measurements of HDL<sub>3</sub>-C and HDL<sub>2</sub>-C ( $r = 0.933$  and  $0.978$ , respectively;  $n = 102$ ). The two methods also proved to be highly correlated in the measurement of apolipoprotein A-I and A-II in HDL subfractions. The HDL-C subfractions determined by ultracentrifugation were more closely associated with the homogenous HDL-C assay than with the total cholesterol assay, especially in the hypertriglyceridemic samples. Our method is far simpler and more precise than the classical dual precipitation method for HDL-C subfractions, and it can be easily performed in a routine chemical laboratory—Hirano, T., K. Nohtomi, S. Koba, A. Muroi, and Y. Ito. A simple and precise method for measuring HDL-cholesterol subfractions by a single precipitation followed by homogenous HDL-cholesterol assay. *J. Lipid Res.* 2008. 49: 1130–1136.

**Supplementary key words** high density lipoprotein-cholesterol • direct homogenous assay • triglyceride

High density lipoprotein-cholesterol (HDL-C) is a negative risk factor for coronary heart disease (CHD), and low HDL-C increases the risk of CHD just as powerfully as high LDL-C. HDL consists of two major

subfractions, large buoyant HDL<sub>2</sub> ( $d = 1.063$ – $1.125$  g/ml) and small dense HDL<sub>3</sub> ( $d = 1.125$ – $1.210$  g/ml). Several lines of evidence suggest that the protective effect of HDL may be better reflected in the concentrations of HDL<sub>2</sub>-C than in those of total-HDL-C or HDL<sub>3</sub>-C (1–3). Other studies, meanwhile, provide no support for this (4–6). It remains to be determined whether a shift in the distribution of HDL particles confers a greater benefit than an increase in total HDL alone. Which of the two is the more powerful negative risk factor for CHD events remains open to debate. Although studies have yet to determine which of the two is the more powerful negative risk factor for CHD events, a simple and reliable assay system for measuring the HDL subfractions will have to be developed to determine the clinical significance of the HDL species.

Research laboratories use various ultracentrifugation techniques to determine HDL<sub>2</sub> and HDL<sub>3</sub> in serum and plasma (7–9). Yet the specialized skills, time, and expensive equipment required for these techniques are disadvantageous for the processing of the large numbers of samples used in epidemiological studies. Several groups have already developed methods for measuring HDL subfractions by precipitation instead of ultracentrifugation (10–14). All of these methods, however, require dual-step precipitation: VLDL and LDL fractions are precipitated with heparin-manganese (Mn), whereupon HDL<sub>2</sub> is precipitated with dextran sulfate (DS) in the supernatant from the heparin-Mn precipitation. Dual precipitation requires two steps with the centrifuge, which prolongs the total assay time and introduces complicated steps that may compromise the accuracy of the values. Dual precipitation is also very unsuitable for the accurate measurement of HDL subfractions in severely hypertriglyceridemic samples containing chylomicrons (15). By modifying the dual precipitation method originally described by Gidez et al. (10), our group devel-

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oped a single precipitation procedure for selectively separating HDL<sub>3</sub> from other lipoproteins with a heparin/Mn/DS mixture.

In the present study, we used this single precipitation procedure and then measured cholesterol in a heparin/Mn/DS supernatant by direct HDL-C homogenous assay. Previously, we reported a successful attempt to measure small dense LDL-C by a combined method of heparin/magnesium precipitation followed by direct LDL-C assay (16). Gleaning hints from our previous study, we used direct HDL-C instead of total cholesterol (TC) assay for the measurement of cholesterol in the heparin/Mn/DS supernatant. The direct HDL-C assay is expected to eliminate the contamination of cholesterol in apolipoprotein B (apoB)-containing lipoproteins and to yield more accurate values, especially in the measurement of severely hypertriglyceridemic samples. The brief total assay time of <1 h ensures that the method can be applied easily in routine clinical laboratories.

## MATERIALS AND METHODS

Blood was sampled in a fasting state from healthy subjects and patients, some of them dyslipidemic, during visits to Showa University Hospital and affiliated hospitals. Written informed consent was obtained from all subjects, and the study was approved by the local ethics committee. **Table 1** lists the serum lipid, apoA-I, and apoA-II levels in the enrolled subjects (n = 102). Data on the TG, apoA-I, and apoA-II levels were available for 90 of the samples. The HDL-C levels ranged widely from 9 to 118 mg/dl, because a patient with Tangier disease was included in the subjects. There were also two chylomicronemic samples from subjects with severe hypertriglyceridemia (1,214 and 1,259 mg/dl). Serum was isolated within 2 h after blood sampling and stored at -80°C until analysis. The precipitation and ultracentrifugation were performed on the same day, the latter in accordance with the method described earlier (17). HDL<sub>2</sub> (d = 1.063–1.125 g/ml) and HDL<sub>3</sub> (d = 1.125–1.210 g/ml) were separated by adding sodium bromide to sequentially adjust the sera to different densities and then performing ultracentrifugation at 453,000 g for 8 h at 16°C in a Himac centrifuge with a PR80A rotor (Hitachi, Tokyo, Japan). Cholesterol concentrations in the isolated HDL<sub>2</sub> and HDL<sub>3</sub> were measured by TC assay.

### Homogenous HDL-C assay

The HDL-C was determined directly in the serum using a commercially available test kit (HDL-EX; Denka Seiken, Co., Tokyo, Japan) with a special surfactant (the homogenous method), based on a principle fully described previously (17). The HDL-C

levels determined by this method correlated excellently with those determined by ultracentrifugation (d = 1.063–1.210 g/ml; r = 0.99, n = 200) and those determined using another commercially available kit by the direct method (Determina HDL-C; Kyowa Medics, Tokyo, Japan) (r = 0.96, n = 16) (18). The within-run imprecision (coefficient of variation) was 1.25%, and the between-assay coefficient of variation was 0.79%. No significant interference was observed when bilirubin, hemoglobin, ascorbic acid, fatty acid (up to 5% soybean oil), triglyceride emulsion (Intralipid at a final concentration of up to 2,000 mg/dl), or EDTA-Na<sub>2</sub> was incubated during the measurement of HDL-C.

### Dual-step precipitation methods

The dual-step precipitation was performed according to the actual procedure described by Bachorik and Albers (15), based on the original procedure established by Gidez et al. (10). To isolate total HDL-C by precipitation, a combined precipitant consisting of 16.5 mg/ml heparin (Wako Pure Chemical Industries, Ltd., Osaka, Japan; catalog No. 081-00136, lot No. LTF0123) and 1.0 mol/l MnCl<sub>2</sub> (Wako Pure Chemical Industries, Ltd.; catalog No. 139-00722, lot No. WKN6108) was added to 0.3 ml of serum. The final concentrations of the precipitant were 91 mmol/l MnCl<sub>2</sub> and 1.5 mg/ml heparin. After 20 min of standing at room temperature, the mixture was centrifuged at 1,500 g for 1 h at 4°C. Aliquots of the resulting supernatant were taken for the assay of the HDL-C and precipitation of the HDL<sub>2</sub>. The HDL<sub>2</sub> was precipitated by dissolving DS (Sigma-Aldrich, St. Louis, MO; molecular weight 10,000, Chemical Abstract Service 9011-18-1; catalog No. D6924, lot No. 105k1216) in isotonic saline and adding a 10-fold volume of heparin/Mn supernatant. The final concentration of DS was 1.3 mg/ml. After 20 min at room temperature, the mixture was centrifuged at 10,000 rpm for 30 min at 4°C. The measured value for total HDL-C was multiplied by 1.10 and that for HDL<sub>3</sub> was multiplied by 1.2 to correct for dilution by the reagents. HDL<sub>3</sub>-C was measured by the direct HDL-C homogenous assay instead of the original TC assay, for comparison with the single precipitation method described below.

### Single precipitation method with heparin/Mn/DS

The procedure was performed in a single step by adding heparin/Mn/DS reagent to simultaneously precipitate both the apoB-containing lipoproteins and HDL<sub>2</sub>. Testing with various concentrations of DS solution (6–30 mg/ml) in the course of the studies revealed that 12 mg/ml DS was optimal for the selective precipitation of HDL<sub>2</sub> in this single precipitation (see Results). The reagent consisted of heparin (1,071 U/ml, 8.25 mg/ml), MnCl<sub>2</sub> (98.7 mg/ml), and DS (12 mg/ml). The precipitation reagent (0.06 ml) was added to 0.3 ml of serum, mixed, left at room temperature for 30 min, and centrifuged at 10,000 rpm for 10 min at 4°C. The final concentrations of heparin, MnCl<sub>2</sub>, and DS were 1.4 mg/ml (179 U/ml), 83 mmol/l (16.4 mg/ml), and 2.0 mg/ml, respectively. An aliquot of the supernatant was taken for HDL<sub>3</sub>-C measurement. HDL-C in the supernatant was determined by homogenous HDL-C assay (HDL-EX; Denka Seiken Co.). The measured value for total HDL<sub>3</sub>-C was multiplied by 1.2 to correct for dilution by the reagents. The value for HDL<sub>2</sub>-C was calculated as the difference between the total HDL-C (directly determined in the serum by HDL-EX) and HDL<sub>3</sub>-C. Two serum-based control materials were tested in 21 replicates at each of two runs using two different reagent lots. The means, standard deviations, and percentage coefficients of variation were calculated for each sample for each run and for each reagent lot. Within-run imprecision was assessed by 21 replicates in two runs, and percentage coefficients of variation

TABLE 1. Serum lipid and apoA-I and apoA-II levels in enrolled subjects

Value	TC	HDL-C	TG	ApoA-I	ApoA-II
Mean (SD)	210 (51)	62 (23)	212 (295)	146 (37)	32 (8)
Range	99–375	8–128	26–1683	15–232	6–57

ApoA-I, apolipoprotein A-I; HDL-C, high density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride. n = 102 for TC and HDL-C; n = 90 for TG, apoA-I, and apoA-II.

were 1.3–2.9% for the low-level control fluid and 1.0–2.9% for the high-level control fluid. No significant reagent lot-to-lot variation or between-run variation was observed. No significant changes in the HDL<sub>2</sub>-C and HDL<sub>3</sub>-C levels were found in serum stored at –80°C for at least 30 days. Ultimately, we decided to use the serum for the study, although the results of preliminary experiments using EDTA plasma instead of the serum were essentially the same.

The effects of severe hypertriglyceridemia on this assay were also examined. Chylomicron (Svedberg flotation index > 400) was obtained from a patient with lipoprotein lipase deficiency (19) by ultracentrifugation. Saline or chylomicron was added to normal pooled serum (1:1, v/v), and 0.2 ml of this sample was mixed with 0.04 ml of the precipitation reagent. HDL<sub>3</sub>-C in the supernatant was measured by direct HDL-C assay or TC assay.

### Biochemical analysis

Triglyceride and TC were measured by standard laboratory procedures. Serum apoA-I and apoA-II were determined by an immunoturbidimetric assay (Daiichi Chemicals Co., Tokyo, Japan). Lipoprotein(a) (Lp[a]) was measured by a commercially available test kit (Denka Seiken Co.).

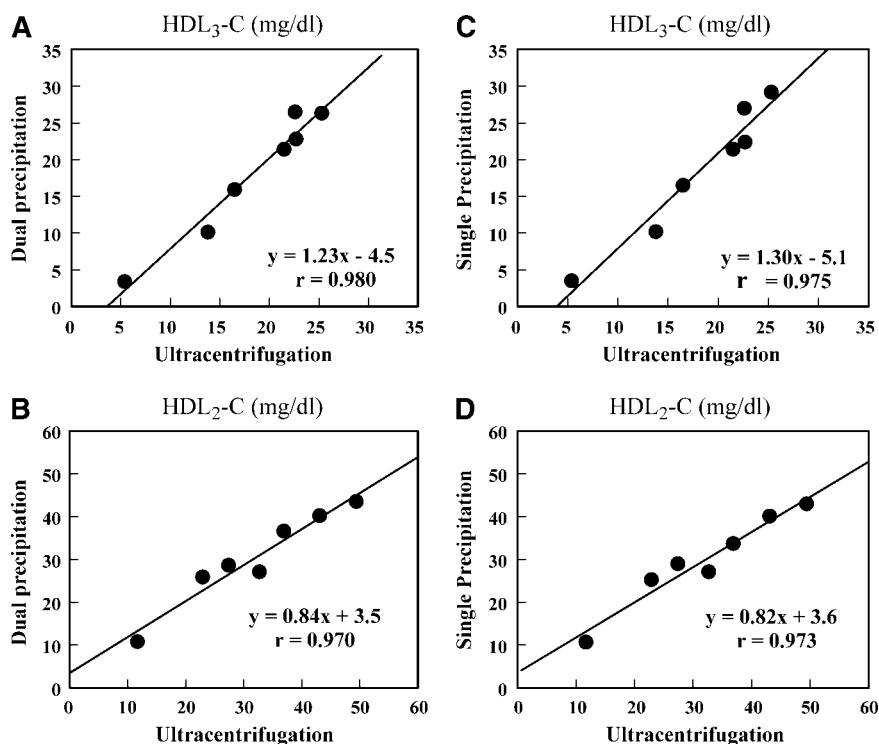
Statistical analyses were performed with Statview 5.0 software (SAS Japan, Ltd., Tokyo, Japan). Correlations between parameters were assessed by Pearson's linear regression analysis. The paired Student's *t*-test was used to assess the difference between

two methods for the measurement of the same sample. Significance was accepted at *P* < 0.05.

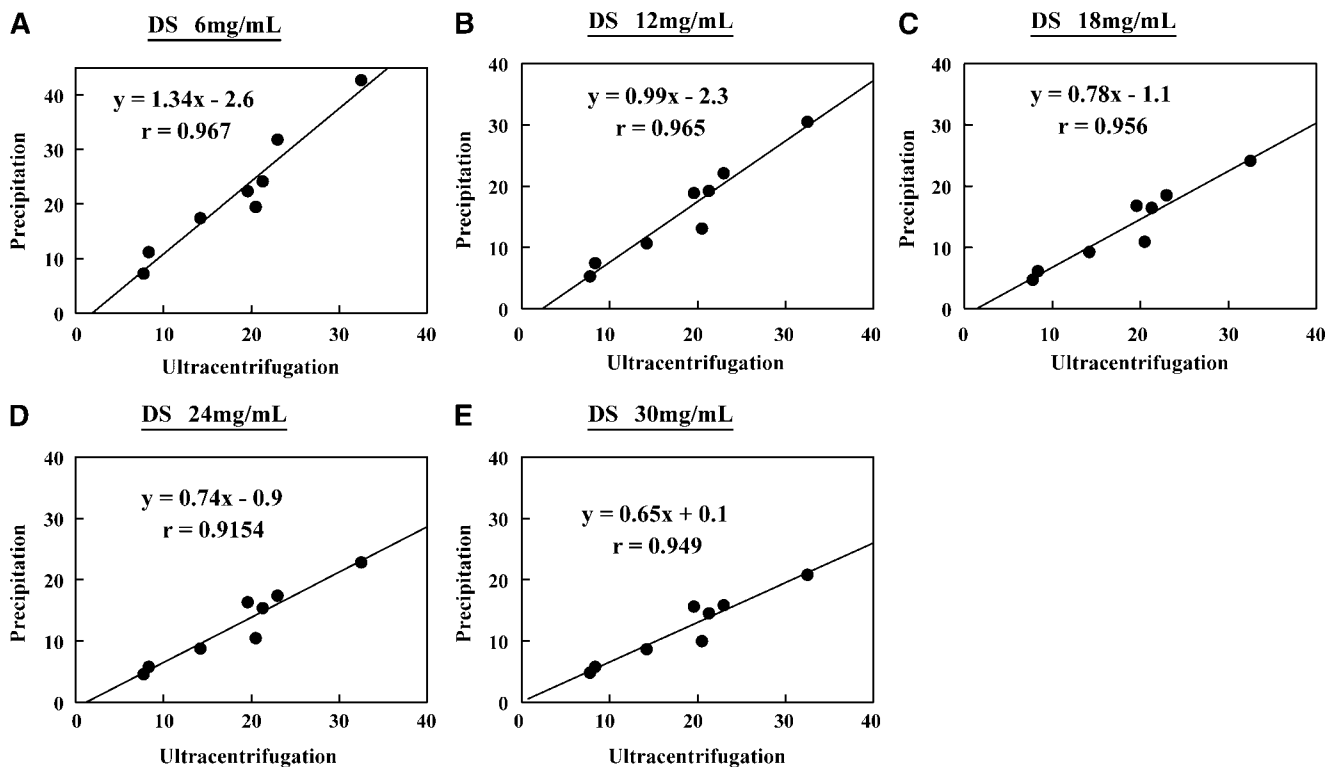
## RESULTS

### Comparison between dual-step and single-step precipitation for the measurement of HDL-C subfractions

The dual-step and single-step precipitation methods for the separation of HDL subfractions were compared. The final DS concentrations for the dual-step and single-step methods were both 1.3 mg/ml, the concentration determined to be optimal for the second precipitation for HDL<sub>2</sub> in the study by Gidez et al. (10). The HDL<sub>3</sub>-C and HDL<sub>2</sub>-C values determined by the dual and single precipitation methods correlated excellently with those determined by ultracentrifugation (Fig. 1), although the slopes for HDL<sub>3</sub>-C and HDL<sub>2</sub>-C determined by precipitation were steeper (1.23–1.29) and more sluggish (0.82–0.84), respectively. The values determined by the two precipitation methods were also well correlated with each other (*r* = 0.990 and 0.940 for HDL<sub>3</sub>-C and HDL<sub>2</sub>-C, respectively; *P* < 0.0001).



**Fig. 1.** Correlation between ultracentrifugation and the dual-step or single-step precipitation for the measurement of high density lipoprotein<sub>3</sub>-cholesterol (HDL<sub>3</sub>-C) and HDL<sub>2</sub>-C levels. The dual-step precipitation was performed according to the method of Gidez et al. (10) using heparin/manganese (Mn) as the first reagent and dextran sulfate (DS) as the second reagent (A, B). The single precipitation was performed solely with the heparin/Mn/DS mixture. An aliquot of the supernatant was taken for HDL<sub>3</sub>-C measurement determined by direct HDL-C assay (C, D). The value for HDL<sub>2</sub>-C was calculated as the difference between the total HDL-C and HDL<sub>3</sub>-C. HDL<sub>2</sub> (*d* = 1.063–1.125 g/ml) and HDL<sub>3</sub> (*d* = 1.125–1.210 g/ml) were separated by sequential ultracentrifugation, and cholesterol was determined by total cholesterol (TC) assay.



**Fig. 2.** Correlation between ultracentrifugation and the single precipitation with various concentrations of DS in the heparin/Mn/DS mixture for the measurement of HDL<sub>3</sub>-C level. The reagent consisted of heparin (1,071 U/ml, 8.25 mg/ml), Mn (500 mmol/l), and DS (6, 12, 18, 24, and 30 mg/ml) (A–E). The final concentrations of heparin, Mn, DS were 1.4 mg/ml (179 U/ml), 83 mmol/l, and 1.0–4.0 mg/ml, respectively. An aliquot of the heparin/Mn/DS supernatant was taken for HDL<sub>3</sub>-C measurement determined by homogenous HDL-C assay. HDL<sub>3</sub> ( $d = 1.125$ – $1.210$  g/ml) was separated by sequential ultracentrifugation, and cholesterol was determined by TC assay.

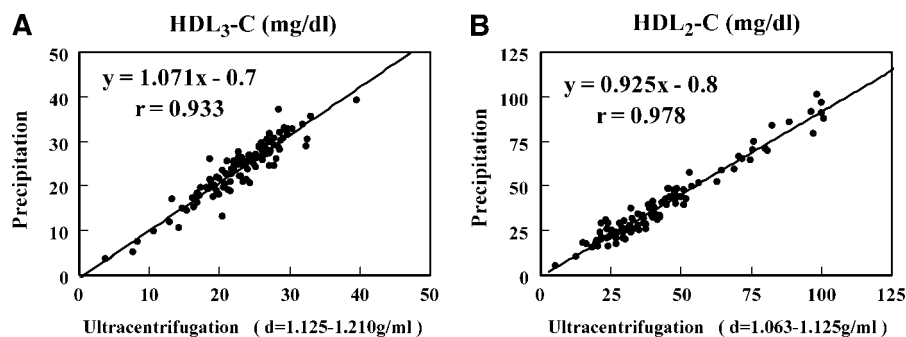
#### Determination of the optimal concentration of DS for the single-step precipitation

Having succeeded in the measurement of HDL<sub>3</sub>-C by a single-step precipitation method, we tried to determine the optimal DS concentration in the heparin/Mn/DS mixture for the separation of HDL<sub>3</sub> from HDL<sub>2</sub>. When 12 mg/ml DS (final concentration = 2.0 mg/ml) was used, the slope of the curve between the precipitation and ultracentrifugation for HDL<sub>3</sub>-C was very close to 1.0 and the y intercept was close to 0 ( $y = 0.99x - 1.54$ ) (Fig. 2). The final DS concentration was higher than that used by Gidez et al. (10) for dual precipitation (2.0 vs. 1.3 mg/ml).

The slope was steeper (1.33) at a low DS concentration (6 mg/ml) and more sluggish (0.78–0.64) at higher DS concentrations (18–30 mg/ml) (Fig. 2).

#### Correlation between the single-step precipitation and ultracentrifugation for HDL subfractions

Tests were conducted to determine the correlation between the single precipitation method and standard ultracentrifugation for HDL-C subfractions in more samples ( $n = 102$ ) (Fig. 3). The HDL<sub>3</sub>-C values determined by single precipitation with heparin/Mn/DS followed by homogenous HDL-C assay were excellently correlated with the values



**Fig. 3.** Correlation between ultracentrifugation and the single-step precipitation for the measurement of HDL<sub>3</sub>-C (A) and HDL<sub>2</sub>-C (B) in the 102 enrolled subjects. The lipid profiles of the subjects are listed in Table 1.

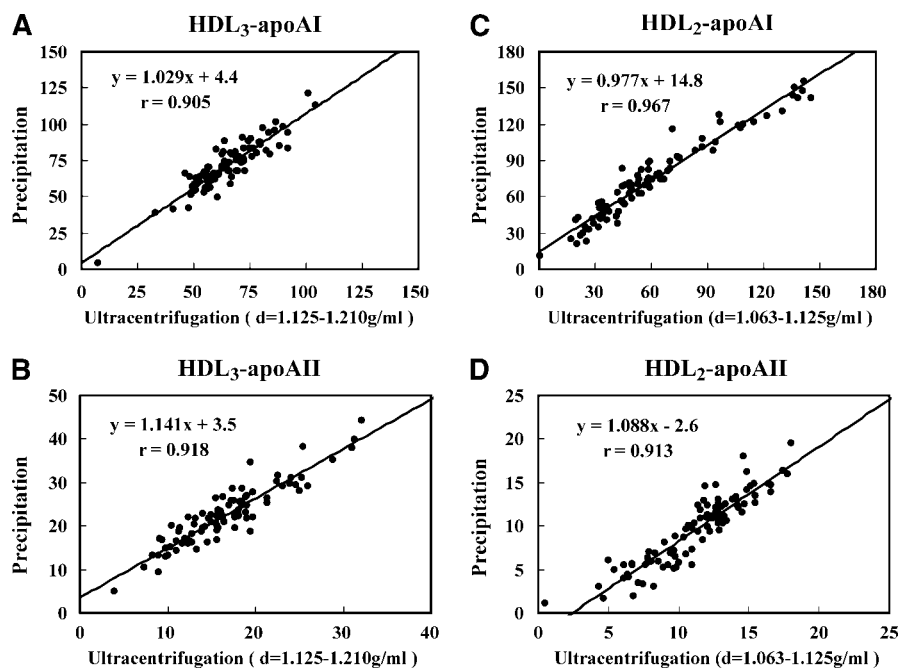


Fig. 4. Correlation between ultracentrifugation and the single precipitation for the measurement of apolipoprotein A-I (apoA-I) (A, B) and apoA-II (C, D) levels in HDL subfractions in 90 subjects.

determined by ultracentrifugation ( $r = 0.933$ ). The slope was near 1.0 (1.071) and the y intercept was almost negligible ( $-0.7$  mg/dl). A similarly strong association was observed for HDL<sub>2</sub>-C ( $y = 0.925x - 0.8$ ;  $r = 0.978$ ). As in the experiments with cholesterol, the apoA-I and apoA-II levels in HDL subfractions determined by the single precipitation method were strongly correlated with those determined by ultracentrifugation. Correlation coefficients for HDL<sub>2</sub>-apoA-I, HDL<sub>2</sub>-apoA-II, HDL<sub>3</sub>-apoA-I, and HDL<sub>3</sub>-apoA-II were 0.967, 0.931, 0.905, and 0.918, respectively ( $n = 90$ ) (Fig. 4).

#### Comparison between homogenous HDL-C and TC assay for HDL-C subfractions in the single-step precipitation method

Table 2 shows HDL<sub>3</sub>-C and HDL<sub>2</sub>-C levels determined by the ultracentrifugation technique and the single precipitation method with direct HDL-C assay or TC assay. The HDL<sub>2</sub>-C level was approximately double the HDL<sub>3</sub>-C level, as has been reported in Japanese subjects (20, 21). The HDL<sub>3</sub>-C level determined by ultracentrifugation and precipitation HDL assay was almost identical, and the difference between the two methods was  $<1$  mg/dl, whereas the difference was greater when HDL<sub>3</sub>-C was

measured by the TC assay (7 mg/dl). The HDL<sub>2</sub>-C level determined by ultracentrifugation and the precipitation HDL-C assay was also almost identical, and the difference between the two methods was only 4 mg/dl, whereas the difference became greater when HDL<sub>3</sub>-C was measured by TC assay (10 mg/dl). Figure 5 shows the correlation between the ultracentrifugation and single precipitation methods using either TC or direct HDL-C assay for the determination of HDL<sub>3</sub>-C. The values determined by the TC were not well correlated with those determined by ultracentrifugation ( $r = 0.413$ ). Four outliers along the correlation curve between the ultracentrifugation and precipitation TC assay markedly abolished a good relationship between the two variables. These outliers were from samples with severe hypertriglyceridemia (triglyceride  $> 1,000$  mg/dl).

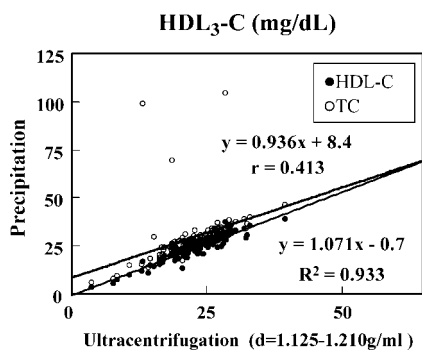
#### The effect of hypertriglyceridemia on HDL-C subfraction assay

Figure 6 shows the influence of the serum triglyceride level on the dissociation between ultracentrifugation and the precipitation method with HDL-C or TC assay. A great difference was observed in the HDL<sub>3</sub>-C values measured by

TABLE 2. Comparison between UCF and precipitation with direct HDL-C or TC assay for determination of HDL<sub>2</sub>-C and HDL<sub>3</sub>-C levels

UCF	HDL <sub>3</sub> -C		HDL <sub>2</sub> -C		
	Precipitation		UCF	Precipitation	
	HDL-C	TC		HDL-C	TC
22.8 ± 5.7	23.8 ± 6.5	29.9 ± 12.8	42.9 ± 21.8	38.9 ± 20.6	32.8 ± 24.2
Difference versus UCF	0.95	7.00	Difference versus UCF	-4.05	-10.08
Paired <i>t</i> -test value ( <i>P</i> )	4.01 ( <i>P</i> < 0.0001)	6.03 ( <i>P</i> < 0.0001)	Paired <i>t</i> -test value ( <i>P</i> )	8.82 ( <i>P</i> < 0.0001)	9.12 ( <i>P</i> < 0.0001)

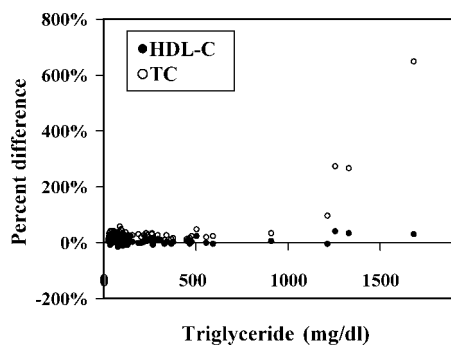
UCF, ultracentrifugation. Values shown are mg/dl ( $n = 102$ ), mean ± SD.



**Fig. 5.** Correlation between ultracentrifugation and precipitation TC or direct HDL-C assay for the measurement of HDL<sub>3</sub>-C. An aliquot of the heparin/Mn/DS supernatant was taken for HDL<sub>3</sub>-C measurement determined by homogenous HDL-C assay or conventional TC assay. There were four outliers along the correlation curve between ultracentrifugation and the precipitation TC assay. Both were from patients with severe hypertriglyceridemia.

TC assay in the samples with severe hypertriglyceridemia. No significant difference was found, however, in the measurements by the precipitation HDL-C assay. A buffy coat appeared in the supernatant after the precipitation of the chylomicronemic sample. We managed to yield stable HDL<sub>3</sub>-C values by direct HDL-C assay, however, by carefully removing the supernatant under the buffy coat through a pipette. The HDL<sub>3</sub>-C values, on the other hand, varied substantially when the TC assay was used for the chylomicronemic samples (data not shown). To explore how the direct HDL-C assay was superior to the conventional TC assay, we tested the effect of chylomicrons on HDL<sub>3</sub>-C measurement by the precipitation method. The addition of chylomicrons (19) to pooled serum increased triglycerides from 165 to 770 mg/dl. The HDL<sub>3</sub>-C value determined by the direct HDL-C assay increased slightly, from 5.8 to 6.9 mg/dl (19%), when the chylomicrons were added. In contrast, the HDL<sub>3</sub>-C value determined by the TC assay increased markedly, from 8.5 to 15.0 mg/dl (76%).

To determine the effect of Lp[a] on the precipitation method, we measured HDL-C subfractions in a sample with



**Fig. 6.** Effect of triglyceride on the percentage difference between ultracentrifugation and precipitation HDL-C or TC assay for the measurement of HDL<sub>3</sub>-C. A large difference was observed in the measurement of HDL<sub>3</sub>-C by TC assay in severely hypertriglyceridemic samples.

a high concentration of Lp(a) (90 mg/dl). The HDL<sub>3</sub>-C and HDL<sub>2</sub>-C levels determined by ultracentrifugation were identical to the corresponding levels determined by the precipitation method (20.3 vs. 19.2 and 43.0 vs. 40.7 mg/dl) in this hyper-Lp(a) case.

## DISCUSSION

We established a simple and precise assay for measuring HDL-C subfractions by a single precipitation with a heparin/Mn/DS mixture followed by a direct HDL-C assay. By measuring the total HDL-C directly in serum with a homogenous assay, we can determine the total HDL-C fraction without precipitating the apoB-containing lipoproteins. A single precipitation procedure shortens the total assay time and may minimize artificial variables. DS precipitation is widely used not only for separating HDL<sub>3</sub> from HDL<sub>2</sub> but also for the specific precipitation of apoB-containing lipoproteins (15). Noting this, we speculated that it would be theoretically possible to make a mixture of heparin/Mn and DS for the precipitation of both HDL<sub>2</sub> and apoB-containing lipoproteins simultaneously while leaving the HDL<sub>3</sub> in the supernatant. We began our experiments to test this hypothesis by preparing a heparin/Mn/DS mixture in the same final concentrations used by Gidez et al. (10). Surprisingly, we found that a single precipitation was effective for measuring HDL<sub>3</sub>-C through an approach similar to that of the classical dual-step precipitation. We also found, however, that the slopes for HDL<sub>3</sub>-C and HDL<sub>2</sub>-C were steeper and more sluggish, respectively, in the two precipitation methods. This suggested that the precipitation of HDL<sub>2</sub> was incomplete and required correction by adjustment of the DS concentration. Finally, we found that 12 mg/ml DS (2.0 mg/ml, final concentration) was the optimal concentration for the single-step precipitation. Finding a steeper slope at a low DS concentration (6 mg/ml) and a more sluggish slope at higher DS concentrations (18–30 mg/ml), we decided that DS at high concentrations precipitates some parts of the HDL<sub>3</sub> fraction. Among the various methods for precipitation, ours may be the most closely matched with the standard ultracentrifugation for the determination of HDL<sub>2</sub>-C and HDL<sub>3</sub>-C ( $r = 0.978$  and  $0.933$ , respectively). The apoA-I and apoA-II levels determined in HDL subfractions by the precipitation method were also well correlated with those determined by ultracentrifugation. This supported the accuracy of our single precipitation method for the HDL subfractionation.

When Demacker et al. (22) compared the precipitation method by the procedure of Gidez et al. (10) with density gradient ultracentrifugation, they established empirically that 0.87 mg/ml (final concentration) DS was required to obtain HDL<sub>2</sub>-C and HDL<sub>3</sub>-C values with optimal accuracy. They also observed that as the DS concentrations increased, some radiolabeled HDL<sub>3</sub> appeared to precipitate before the complete precipitation of the HDL<sub>2</sub>. Dias et al. (14) used 0.72 mg/ml DS to separate HDL<sub>3</sub> from HDL<sub>2</sub> in a total HDL fraction previously isolated by ultra-

centrifugation. Our group, however, managed to clearly separate HDL<sub>2</sub> from HDL<sub>3</sub> using higher DS concentrations than the other researchers. The mixture of heparin/Mn/DS is likely to require a higher DS concentration than the heparin/Mn supernatant for the accurate precipitation of HDL<sub>2</sub> from HDL<sub>3</sub>. Kostner, Molinari, and Pichler (11) reported a single precipitation for HDL subfractions with polyethylene glycol. Widhalm and Pakosta (23), on the other hand, did not observe a high correlation coefficient between ultracentrifugation and the polyethylene glycol method ( $r = 0.75$  for HDL<sub>2</sub>-C and  $r = 0.84$  for HDL<sub>3</sub>-C). Leino et al. (24) pointed out the inaccuracy of determinations of HDL subfractions with polyethylene glycol-based precipitation methods.

Our group applied direct HDL-C measurement instead of conventional TC assay for the determination of HDL<sub>3</sub>-C. The direct HDL-C assay by ultracentrifugation gave closer HDL-C subfraction values than the TC assay. The homogenous HDL-C assay (HDL-EX) also has the useful ability to accurately measure HDL-C without significantly affecting the serum triglyceride concentration (17). In severe hypertriglyceridemic samples, HDL<sub>3</sub>-C values determined by ultracentrifugation were substantially dissociated from those determined by TC assay but similar to those determined by homogenous HDL-C assay. The HDL-C assay also remained quite stable when we added chylomicron to the serum. Considering that the TC assay detects cholesterol in lipoproteins nonspecifically, it is possible that contamination by chylomicron in the supernatant causes the TC assay to overestimate HDL<sub>3</sub>-C.

In conclusion, the single precipitation and direct HDL-C assay is a simple and precise procedure for the measurement of HDL-C subfractions and one that performs reliably even for chylomicronemic samples. The running cost of our single precipitation method is much cheaper than that for the ultracentrifugation method. A homogenous HDL-C assay such as HDL-EX is applicable to an autoanalyzer, which cut the assay time. Our method is likely to be useful for determining the relative risk-predictive powers of different HDL subfractions in epidemiologic and clinical studies. ■

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