Clinical Efficacy of the Direct Assay Method Using Polymers for Serum High Density Lipoprotein Cholesterol

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The clinical efficacy and accuracy of the homogeneous assay method for the serum high density lipoprotein (HDL)-cholesterol determination were evaluated. The principle is as follows: low density lipoproteins (LDL) and very low density lipoproteins (VLDL) were coated by polymers and polyanion to be blocked from cholesterol esterase and cholesterol oxidase. The reaction of these enzymes for HDL cholesterol was enhanced with a detergent, and HDL cholesterol was selectively measured. Both within-run ($n = 3$, 20 times) and between-run ($n = 3$, 7 days) CVs were $< 2\%$. The repeated freezing and thawing (4 times) of three distinct sera resulted in no changes of HDL cholesterol values. Additions of lipid emulsion (Triglyceride

= 100 mg/dl) and free bilirubin (20 mg/dl) gave no effect. Linearity was found up to 300 mg/dl. Increases in HDL cholesterol values by the addition of VLDL (total cholesterol (TC) = 300 mg/dl) or LDL (TC = 300 mg/dl) to the tested sera were $< 0.5\%$. The correlation coefficient of the new method with a precipitation method was 0.995 ($n = 64$). HDL-C values for patients with hyperlipidemia (Type IIa, IIb, or III, IV, and V) by this method were comparable with those obtained by the precipitation method. From these results, we concluded that the new method meets the requirements for accuracy, precision, ease of handling massive samples, and was clinically useful. *J. Clin. Lab. Anal.* 11:82–86.

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INTRODUCTION

Since HDL cholesterol levels are considered to be an anti-atherosclerotic factor (1, 2), HDL cholesterol values have been widely measured in the clinical field. Among various methods for the HDL cholesterol assay, the precipitation methods using heparin and divalent cations or phosphotungstate are the most popular in clinical laboratories (3, 4). However, since these methods require centrifugal separation of lipoproteins other than HDL from serum specimens, routine assays of HDL cholesterol have suffered from long handling time, low throughput, and relatively poor reproducibility.

In the new method developed by Manabe et al. (Daiichi Pure Chemical, Tokyo) (5), synthetic polymers and polyanions in the first reagent adsorb to the surface of LDL, VLDL, and chylomicrons and stabilize these lipoproteins. The stabilized particles are resistant to the detergent in the second reagent, such that cholesterol esterase and cholesterol oxidase do not work on the cholesterol in these lipoproteins. In contrast, HDL particles are solubilized by a detergent, so that cholesterol in HDL is subjected to the

enzyme reactions. Consequently, HDL cholesterol is selectively determined.

Here, we evaluate the accuracy and clinical efficacy of this homogeneous assay method.

MATERIALS AND METHODS

Samples

The samples used in this study for measuring HDL cholesterol were collected from healthy control subjects ($n = 64$, males/females = 36/28, age = 51 ± 7 years old [mean $\pm$ SD]), and 55 hyperlipidemic patients (type IIa [TC > 220 mg/dl, TG < 150 mg/dl] = 15, males/females = 8/7, 49 ± 8 years old], IIb or III [TC > 220 mg/dl, TG > 150 mg/dl] = 16, males/

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females = 8/7, 51 ± 7 years old], I and V [TG = 300–2,000 mg/dl, TC < 1/5TG] = 18, males/females = 7/9, 51 ± 7 years old]). The classification of hyperlipidemia was done according to the WHO classification. The lipid levels used for classification were temporarily defined as stated above.

Homogenous Assay Method for HDL Cholesterol

The reagents of the homogeneous assay method were obtained from Daiichi Pure Chemical Co. (Tokyo, Japan). Briefly, serum samples (3 μ l) are added to the first reagent (300 μ l), containing a polyanion polymer mixture and incubated for 5 min at 37°C for measuring HDL cholesterol. During this procedure, chylomicrons, VLDL, and LDL are complexed and are stabilized. Then, the second reagent (100 μ l) containing detergents, cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine was added and incubated for 10 min at 37°C. In this step, HDL cholesterol is selectively hydrolyzed and the optical density of the developed color is measured at 660/546 nm. This study was conducted on the Hitachi 717 autoanalyzer (Tokyo, Japan).

Precipitation Method for HDL Cholesterol Measurement

The reagent of the dextran sulfate and phosphotungstate method (HDL-C, Daiichi) was obtained from Daiichi Pure Chemicals Co. (6).

Preparation of Lipoproteins by Ultracentrifugation

Very low density lipoproteins (VLDL = $d < 1.006$), low density lipoproteins (LDL = $1.019 < d < 1.063$), and high density lipoproteins (HDL = $1.12 < d < 1.21$) were separated by sequential ultracentrifugation method according to the Lipid Clinic Program (7). Obtained lipoproteins were dialyzed against the plasma density buffer containing 0.14 M NaCl, 10 mM Tris-HCl, pH 7.4, and 1 mM ethylenediaminetetraacetic acid. The purity of those lipoproteins was confirmed by the agarose electrophoresis and oil red O staining (8).

Interference Substances

Lipid emulsion as a turbid marker, ascorbic acid, hemoglobin, and free or conjugated bilirubin were checked. They were obtained from Kokusai Shiyaku (Osaka, Japan).

Sample Treatment and Handling

Freezing/thaw preparations were done in two freezers at -20°C , and -80°C .

Statistical Analysis

Comparisons were performed by analysis of variance (ANOVA) on an Apple Macintosh computer using Statview software (Abacus Concepts, Berkeley, CA).

TABLE 1. The Within-Run CVs of Low, Middle and High HDL-Cholesterol Values of Sera

	HDL-cholesterol (mg/dl)		
	low	middle	high
Number examined	n=20	n=20	n=20
mean	36.0	61.1	93.4
SD	0.65	1.29	1.45
CV (%)	1.80	1.82	1.27

RESULTS

Precision

Using three distinct sera from three individuals as specimens, within-run reproducibility was studied. As shown in Table 1, Cvs of replicate assays for low, medium, and high HDL cholesterol sera were 1.80, 1.82, and 1.27% respectively.

Assay results on the patient sera with low and high HDL cholesterol levels for 7 successive days are shown in Figure 1. The observed values for both sera were almost constant and Cvs were calculated to be 1.3% and 1.5%.

Effects of Freezing and Thawing

HDL cholesterol values of the three samples of sera were determined before and after freezing and thawing. The freezing was done at -20°C and -80°C . Thawing was done at room temperature. Repeated freezing-thawing up to four times gave no effects on the HDL cholesterol values (Fig. 2).

Interference of the Various Substances

Effects of the addition of major interfering substances to serum samples were examined. Additions of lipid emulsion

Fig. 1. HDL cholesterol values during 7 subsequent days.

Fig. 2. Effects of freezing and thawing on HDL cholesterol values by homogeneous assay. Left: serum was frozen at -20°C , right: serum was frozen at -80°C .

(equivalent to 100 mg/dl triglyceride), ascorbic acid (50 mg/dl), hemoglobin (300 mg/dl), or free bilirubin (20 mg/dl) gave essentially no effect, as shown in Figure 3, A–D. An addition of conjugated bilirubin (20 mg/dl) decreased by 13% as shown in Figure 3E.

Linearity for HDL Cholesterol

Linearity for HDL cholesterol by the homogeneous assay method was studied using purified HDL by ultracentrifugation. The assays of a dilution series of purified HDL gave a fine linearity up to 300 mg/dl, as shown in Figure 4.

Specificity for HDL Cholesterol

The specificity for HDL cholesterol by the homogenous assay method was evaluated by the addition of purified VLDL or LDL to the serum. The purity of the added lipoproteins

Fig. 3. Effects of various substances on the HDL cholesterol values by homogeneous assay method: **A**, lipid emulsion, **B**, ascorbic acid, **C**, hemoglobin, **D**, free bilirubin, **E**, conjugated bilirubin.

Fig. 4. Linearity of the HDL cholesterol value by homogeneous assay.

was confirmed by the agarose electrophoresis (data not shown). The addition of purified VLDL (total cholesterol (TC) = 300 mg/dl) resulted in an increase of 0.5% as HDL cholesterol, as shown in Figure 5A. An increase of HDL cholesterol levels by the addition of the LDL (TC = 300 mg/dl) to serum was < 0.5%, as shown in Figure 5B.

Correlation Between Homogenous Assay Method and Precipitation Method

The HDL cholesterol values measured by a new method were compared with those determined by the precipitation method on 64 samples obtained from healthy subjects. As shown in Figure 6, an excellent correlation was observed between two methods with a correlation coefficient of 0.982, a slope of 0.88 and a Y-intercept of 3.9, when a homogenous assay method is taken as Y-axis. Mean values obtained from

Fig. 5. Effect of the addition of VLDL or LDL to serum on HDL cholesterol value. **A**, Addition of VLDL. **B**, Addition of LDL.

Fig. 6. Correlation of HDL cholesterol values between homogeneous assay method and precipitation method. The sera obtained from healthy control subjects were subjected to the two methods.

the precipitation method and from this method were 57.8 and 54.8 mg/dl, respectively.

HDL Cholesterol Values in Various Hyperlipidemias

In type IIa hyperlipidemia, type IIb hyperlipidemia, or type III, type IV, and V, HDL cholesterol values measured by the homogenous assay method were not significantly different from the values obtained by precipitation method, as shown in Figure 7.

Fig. 7. HDL cholesterol values obtained by homogenous method and precipitation method in three different types of hyperlipoproteinemic patients.

DISCUSSION

The accuracy and clinical utility of the homogenous assay method for HDL cholesterol were evaluated. Fundamentally, Cvs within-run and between-run were < 2.0%. These values were accurate enough in the routine assay. Freezing and thawing had essentially no effect, indicating that this method was efficient even after the sample was once frozen.

The interfering substances observed routinely such as turbidity of the chylomicron, ascorbic acid, hemolysis, and free or conjugated bilirubin were checked. None of them interfered with the HDL value, except conjugated bilirubin. The conjugated bilirubin (20 mg/dl) was rare in routine laboratory study. In any case, HDL cholesterol evaluation in such cases should be interpreted with caution.

The linearity up to 300 mg/dl of HDL cholesterol was enough covering ranges for clinical usage, because high HDL cholesterol level observed in cholesterol ester deficient patients was usually < 300 mg/dl (8).

The specificity was studied with the addition of the purified lipoproteins. As for VLDL, the addition of VLDL (TG = 2,100 mg/dl and total cholesterol = 300 mg/dl) made 2 mg/dl increase as HDL cholesterol, which was 4%, if the basal HDL cholesterol was 50 mg/dl. This value was clinically negligible. Hypertriglyceridemia was occasionally associated with low HDL cholesterol. In such cases, the new assay method would not make trouble.

The addition of the LDL (TC = 300 mg/dl) also increased < 0.5% of the HDL cholesterol. In the familial heterozygous hypercholesterolemic patient, LDL cholesterol level was usually from 300 to 400 mg/dl (9). The difference was supposed to be from 1.5 to 2 mg/dl. This value was ~ 3% or 4% of HDL cholesterol, if the HDL value was 50 mg/dl, and was also clinically negligible.

The correlation rate between the homogenous assay method and precipitation method was 0.983. This value was good enough for clinical usage in place of the precipitation method.

Furthermore, we studied the samples of hyperlipoproteinemic patients. Three groups were studied. In type II a, II b, or III, and IV or V, HDL cholesterol values obtained by homogenous method were not significantly different from those by the precipitation method.

From these results, it is concluded that new method for measuring HDL cholesterol was accurate enough for specificity, and stability, was easy enough to be handled for massive samples, and was useful for routine measurement of serum HDL cholesterol even in various type of severe hyperlipoproteinemia.

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