New Homogeneous HDL-Cholesterol Assay Without the Influence of High TG Sample Using the Selective Detergent to Lipoproteins

Yasuki Ueda,* Masahiko Matsui, Sadao Hayashi, Yoshihisa Yamaguchi, and Yuzuru Kanakura

Laboratory for Clinical Investigation, Osaka University Hospital, Osaka, Japan.

Homogeneous HDL-cholesterol assays have been developed and used widely in routine analysis, but they have been reported to give inaccurate results in patients with hypertriglyceridemia. Recently, a new assay based on a new principle without the influence of triglycerides has also been developed and commercialized. We evaluated the basic performance of this new homogeneous HDL-cholesterol assay and compared it with the conventional polyethylene glycol/cyclodextrin-modified enzyme (PEGME) method using high-triglyceride (TG) samples (TG > 8,000 mg/L). For samples showing a discrepancy with the conventional method, other precipitation and ultracentrifugation (UC) methods were also used to confirm the values. This new homogeneous assay is based on the selective solubilizing effect of detergent on the different lipoproteins. First, non-HDL free cholesterol is consumed by enzyme and is cleared as a colorless reactant. Then, HDL-cholesterol is selectively solubilized by lipoprotein-specific detergent and reacted with the enzyme. As a result, the

precision of this new homogeneous assay was good (CV < 2%) over the wide range, and the measurement range was 0 to 2,000 mg/L. This method correlated well with the PEGME method, which is a conventional method for normolipidemic samples ($y = 0.97x - 3.1$, $r = 0.994$, $n = 424$). It also correlated well with the UC method ($y = 0.99x + 0.3$, $r = 0.989$, $n = 53$). Fourteen high-TG samples showed different results from those obtained by the PEGME method. Among these samples, one contained abnormal lipoproteins (probably due to the influence of drug therapy) and gave a significantly different result from that obtained by the PEGME method. However, the values obtained by other methods (precipitation and ultracentrifugation) agreed well with those obtained by this new method. In conclusion, this method shows a good basic performance and is useful for high-TG samples without any interference. Therefore, it is considered to be very practical for a routine test. *J. Clin. Lab. Anal.* 17:201–208, 2003. © 2003 Wiley-Liss, Inc.

Key words: HDL cholesterol; selective inhibition; homogeneous assay; hypertriglyceridemia

INTRODUCTION

HDL-cholesterol concentration is widely measured in routine tests as a risk factor for ischemic heart disease (1–3). Conventionally, it had been measured by the UC method or by the precipitation method. However, homogeneous HDL-cholesterol assay without pretreatment has recently been developed and has been rapidly popularized (4–9). Several homogeneous HDL-cholesterol assays, based on different principles, are available commercially, but they all give inaccurate results when high-TG samples are tested (10,11). Here we report a new homogeneous HDL-cholesterol assay based on a new principle—detergent selective inhibition (DSI)—that avoids the influence of TG. In the present study, the

basic performance of this new method was evaluated and was compared with that of a conventional direct method. In addition, high-TG samples that showed discrepant results when compared with this new DSI method and the conventional PEGME method were analyzed. Among these samples, one contained abnormal lipoproteins (probably due to the influence of

*Correspondence to: Yasuki Ueda, Laboratory for Clinical Investigation, Osaka University Hospital, 2-15, Yamadaoka, Suita, Osaka, 565-0871, Japan. E-mail: ueda@hp-lab.med.osaka-u.ac.jp

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miconazole nitrate) and gave a significantly different result from that obtained by the conventional PEGME method. Results analyzed by electrophoresis of this sample are also reported.

MATERIALS AND METHODS

Equipment

A Hitachi 747 automatic analyzer (Hitachi Co., Ltd., Tokyo, Japan) was used, which could be measured using one or two reagents (37°C, reaction time: 10 min). The instruments were operated using the standard protocols recommended by the manufacturer.

Serum Samples

Examination was performed using serum samples collected from 424 patients who underwent lipid profile measurement in our laboratory. Moreover, pooled serum for the basic study was prepared from samples with normal lipid profile.

Homogeneous HDL-C Assays

Two commercially available HDL-C assay kits (conventional and new methods) were used based on different principles. One was a conventional reagent kit (Lot No. 304AAK: Kyowa Medex Co., Ltd., Osaka, Japan) that selectively measured only HDL-cholesterol using polyethylene glycol and cyclodextrin sulfate together with chemically modified cholesterol oxidase and esterase, as described previously (5). The other was the new reagent kit (Lot No. 103RKX: Daiichi Pure Chemicals Co., Ltd., Osaka, Japan) that employed two different surfactants. Its measurement principle follows. In the first reaction, a lipoprotein-specific detergent allows only free-cholesterol, which is present on the surface of LDL and VLDL, to react with cholesterol oxidase and cholesterol esterase. The resultant H_2O_2 is cleared as a colorless reactant by peroxidase. In the second reaction, another detergent selectively solubilizes HDL-cholesterol and allows it to react with the enzymes. Peroxidase, 4-aminoantipyrine, and N,N-bis(4-sulfobutyl)-m-toluidine salt are used so that the

resultant H_2O_2 produces a color proportionally. Then the absorbance is determined.

HDL-C Measurement by UC Method

The β -quantification modified method was used for the UC method. The β -quantification method is proceeded by three steps involving UC, precipitation, and cholesterol assay (12). We used the enzymatic method for the cholesterol assay instead of the Abell-Kendall method.

Other Measurements

Total cholesterol (TC) was determined by the enzymatic method using cholesterol esterase and cholesterol oxidase. TG was determined by the glycerol pre-elimination enzymatic method. The phosphotungstic acid $MgCl_2$ (PTM) method (Daiichi Pure Chemicals Co., Ltd., Osaka, Japan) and the 13% polyethylene glycol (PEG) method (Daiichi Pure Chemicals Co., Ltd., Osaka, Japan) were used to determine HDL-cholesterol by the precipitation method. The PTM and PEG methods were performed according to the manufacturer's recommendation.

RESULTS

Analytical Performance

Precision was evaluated using pooled human serum of three different concentrations (low, medium, and high level). The results are shown in Table 1. Within-run reproducibility was evaluated with 20 samples, and the CV was found to be 0.73–1.06%. Between-run reproducibility was evaluated for 10 days by calculating the mean of duplicate values and the samples were stored at $-56^\circ C$. The CV was found to be 1.03–1.34%.

Linearity

High level of HDL-cholesterol control (Daiichi Pure Chemicals Co., Ltd., Osaka, Japan) was diluted with saline at 10 different concentrations, and the resulting dilutions were used as assay samples. As a result, we confirmed a linearity of at least 2,000 mg/L.

Table 1. Analytical precision of homogeneous HDL-C by DSI method

Sample, human pool serum	Within-run (n = 20)			Between-run (n = 10)		
	Mean HDL-C, mg/L	SD, mg/L	CV, %	Mean HDL-C, mg/L	SD, mg/L	CV, %
Low level	320.0	3.0	1.01	329.0	3.0	1.03
Medium level	560.0	6.0	1.06	576.0	8.0	1.34
High level	936.0	7.0	0.73	935.0	11.0	1.13

Interference

The effects of hemoglobin, conjugated bilirubin, free bilirubin, and ascorbic acid on measurement were examined. Examination was performed by preparing five serial dilutions of specimens with the interfering substances from pooled human serum of two different concentrations. As a result, no influence was observed for hemoglobin up to 5,000 mg/L, for conjugated or free bilirubin up to 500 mg/L, and for ascorbic acid up to 500 mg/L.

Correlation

Correlations with the conventional homogeneous method using the PEGME and UC methods were assessed by linear regression analysis. The samples used were taken from patients with TG levels of $\leq 8,000$ mg/L and were tested in our laboratory. The samples were measured within 1 day from blood collection for the homogeneous method and within 3 days for the UC method. As shown in Fig. 1, the linear regression curve and correlation coefficient obtained with the PEGME method were $y = 0.97x - 3.05$ mg/L ($n = 424$; 95% confidence intervals, 0.96–0.98 for the slope and -9.34 to 3.23 mg/L for the y-intercept) with $r = 0.994$. With the UC method, they were $y = 0.99x + 0.30$ ($n = 53$; 95% confidence intervals, 0.95–1.03 for the slope and -25.04 to 25.63 mg/L for the y-intercept) with $r = 0.989$.

Stability

Stability was assessed using three different levels of frozen pooled human serum for between-run reproducibility.

Measurement was performed for 2 months after a reagent bottle was opened using the analyzer with a built-in refrigerator. Calibration was only performed at the first measurement. As a result, it was confirmed that the reagent was stable at least for 2 months.

Correlation Between PEGME and DSI Methods for High-TG Samples

The correlation between the PEGME method and the DSI method was assessed using 14 samples with a TG level of 8,000 mg/L or more (Fig. 2). With these samples, the HDL-C level obtained by the PEGME method was higher than that of the DSI method (TG level, 8,220–36,660 mg/L). The bias (PEGME-DSI) was seen in one sample that showed a significant difference of the HDL-C levels between the PEGME method (1,890 mg/L) and the DSI method (150 mg/L).

Measurement of Discrepant Samples by Other Methods

In the 14 high-TG samples, eight with a difference of 30% and more between the PEGME method and the DSI method were measured to confirm the HDL-C level using the UC method, the PEG method, and the PTM method. The results are shown in Table 2, together with TC and TG levels. HDL levels determined by other methods were similar to those obtained by the DSI method. Sample 8 had a high level of chylomicron and lipoprotein fraction could not be separated. As a result, the HDL-C level for this sample could not be obtained by the UC method.

Fig. 1. Comparison of HDL-C concentration values. Correlation between the DSI and PEGME methods (A), and the UC method (B).

Fig. 2. Comparison of HDL-C concentration for high TG samples between the DSI and PEGME methods and bias plot. Correlation between the DSI and PEGME methods for 14 samples with TG levels of over 8,000 mg/L (A), and the relationship between bias plot (PEGME-DSI method) and TG levels (B).

Dilution Recovery Test

The eight discrepant samples with high-TG were diluted into 2-, 4-, and 8-fold with saline, and then were

measured by two homogeneous methods. The values obtained by the DSI method did not change after dilution as shown in Fig. 3. In contrast, the values obtained by the PEGME method for Samples 1–7 tended to agree with those obtained by the DSI method as the dilution ratio increased. On the contrary, the PEGME concentrations increased as the dilution ratio increased for Sample 8. The values obtained by the DSI method did not change as well as in other samples. The reaction curve for Sample 8 by the PEGME method was different from the curve of calibrator as shown in Fig. 4.

Confirmation of Sample 8 With Sequential Patterns by Agarose Electrophoresis

The patterns of lipoprotein fractions were confirmed for Sample 8 by electrophoresis. This sample was collected from an acute myeloblastic leukemia patient who had received an antifungal agent (miconazole nitrate) for infection treatment and had discontinued the drug 10 days later due to abnormally high lipid levels. After administration of the antifungal agent, cholesterol and TG levels began to increase and then decreased following its discontinuation. In parallel with these changes, the HDL-C level determined by the PEGME method converged with that determined by the DSI method. Figure 5 shows the agarose electrophoresis patterns of seven samples taken from this patient 2 days before and 5 days after discontinuing this drug. The TC, TG, HDL-C, and apoprotein levels in each sample are also shown in Table 3. In the electrophoresis patterns of Samples no. 1 and no. 2 (= the Sample 8), no band was noted at position α , corresponding to HDL, and a wide band was present in the range from the origin to pre- β . In the patterns of Samples no. 3 to 7, the bands were shifted in the positive direction and got closer to normal. The levels of apolipoprotein A-I and A-II, corresponding

Table 2. Results obtained by each method for samples with high TG

Sample no.	HDL-C (mg/L)					TC (mg/L)	TG (mg/L)
	DSI	PEGME	UC	PTM	PEG		
1	240	440	260	200	220	3140	24000
2	160	570	200	140	220	4530	36660
3	380	450	330	360	300	3170	9700
4	320	510	310	240	240	3310	26380
5	210	600	200	180	200	3920	33470
6	330	540	300	320	390	3540	20490
7	240	380	270	300	^a	2360	12060
8	150	1890	^a	^a	^a	4480	18530

^aAssay values were not given.

DSI, detergent selective inhibition method; PEGME, polyethylene glycol cyclodextrin modified enzyme method; UC, ultracentrifugation method; PTM, phosphotungstic acid magnesium method; PEG, 13% polyethylene glycol method.

Fig. 3. Dilution recovery test for high TG samples. Seven samples with TG levels of over 8,000 mg/L were diluted—2-, 4-, 8-fold—and then measured by the PEGME method (■) and the DSI method (●).

to HDL, were low, whereas the level of other apolipoproteins was high.

DISCUSSION

From the basic study of the DSI method, we confirmed that within-run and between-run reproducibility were good, and precise measurement was possible over the wide range.

Dilution linearity was obtained using high-concentration HDL-C of serum control, and a linearity was demonstrated up to 2,000 mg/L. Accordingly, almost all of the samples could be measured without dilution in routine testing. Linearity was also good in the low concentration of 500 mg/L and below.

The correlation between methods was assessed with samples of the TG level of 8,000 mg/L or less. As a result, a good correlation was confirmed with the result of the PEGME and UC methods. Since the PEGME method has the problem that positive errors increase with the TG level, it is recommended by the manufac-

turer that samples showing TG levels over 8,000 mg/L should be diluted and reexamined. The correlation between the PEGME and DSI methods was assessed for 14 samples showing the TG level of 8,000 mg/L and more. As shown in Fig. 2, HDL-C levels by the PEGME method were higher than that of the DSI method, while one sample showed significantly different HDL-C levels by the PEGME method (1,890 mg/L) and the DSI method (150 mg/L). Among 14 high-TG samples, eight samples with significant bias were diluted and measured in a dilution recovery test (Fig. 3). In Samples 1–7, the values determined by the PEGME method agreed with those determined by the DSI method as the dilution ratio increased. It was considered that the value by the PEGME method approached actual value because of the lowering of the TG level by diluting and, the influence of TG was stopped in the PEGME method. Furthermore, the seven samples were measured by the precipitation and UC methods, and the results were similar to those obtained by the DSI method (Table 2). Based on these facts, it was confirmed that the DSI

Fig. 4. Reaction curve for Sample 8 by the PEGME and DSI methods. Undiluted (◆), 2-fold dilution (■), 4-fold dilution (▲), 8-fold dilution (×), and calibrator (*).

method was not influenced by TG. On the other hand, Sample 8 showed results that differed from the other seven samples. As shown in Fig. 5, abnormal patterns were observed in sequential agarose electrophoresis patterns of Sample 8. The patterns of Samples no. 1 and no. 2 were different from the normal pattern in the construction and charge of lipoprotein. Because the HDL value by the PEGME method contained this abnormal lipoprotein, the HDL value showed a significantly high level. The reaction curve (Fig. 4) of this sample, prepared by the PEGME method, was also different from the curve of calibrator, and it was evident that the reaction was incomplete. As the HDL value exceeded the measurable upper limit value, the reaction of this sample did not finish within reaction time. Since the reaction was not enough, the HDL value by the

PEGME method was lower in appearance than actual value. Because the HDL levels were lowered by diluting and went into the measurable range, it was considered that the values by the PEGME method were higher as the dilution ratio increased. These results are shown in the dilution recovery test (Fig. 3). The HDL-C level for Sample 8 could not be obtained by the UC method as lipoprotein fraction could not be separated. The electrophoresis pattern of Samples no. 1 and no. 2 initially showed few bands at position α and the level of the apolipoprotein A-I, corresponding to HDL, was low. Therefore it was considered that the HDL was actually low and the value obtained by the DSI method was correct. Administration of the antifungal agent might induce hepatopathy in this patient, resulting in abnormal lipid metabolism. Based on these results there

Fig. 5. Sequential agarose-gel electrophoresis pattern for Sample 8: during administration of miconazole nitrate (No. 1); the day not administered (No. 2); 5 days after stopping the miconazole nitrate (No. 3–7).

Table 3. Sequential lipid data in sample number eight

Pattern no.	TC (mg/L)	TG (mg/L)	DSI method HDL-C (mg/L)	PRGME method HDL-C (mg/L)	apo A I (mg/L)	apo A II (mg/L)	apo B (mg/L)	apo CII (mg/L)	apo CIII (mg/L)	apo E (mg/L)
1	4300	14240	90	1280	890	182	2170	270	332	644
2	4480	18530	150	790	1890	178	2670	276	358	648
3	2730	8640	220	910	230	205	1630	220	251	178
4	2680	7790	260	1050	290	236	1560	215	263	156
5	2470	6120	310	1080	330	263	1450	183	238	128
6	2480	8700	280	1100	340	309	1390	200	259	146
7	2190	4530	440	1310	430	336	1240	146	224	95

is the possibility that the PEGME method may measure lipoproteins other than HDL in samples containing abnormal lipoproteins. Thus, administration of miconazole nitrate may induce the increase of cholesterol and TG levels caused by an adverse reaction (13,14) and produce abnormal lipoproteins in some cases. Therefore, we must be cautious with those cases when the PEGME method is used.

The precision of the DSI method was good, and a problem was not noted as in the PEGME method. In conclusion, the DSI method is considered to be a very useful routine test because it avoids the problems found in high -TG sample.

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