Evaluation of precipitation and direct methods for HDL-cholesterol assay by HPLC

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HDL-cholesterol (HDL-C) values measured by precipitation (sodium phosphotungstate–MgCl₂) and direct methods were compared with those obtained by HPLC with a new column (TSKgel Lipopropak) and an eluent (TSKeluent LP-1). The HDL-C values determined by the precipitation method were significantly \( P < 0.001 \) lower than those by the HPLC method, whereas the HDL-C values by the direct method were slightly but significantly higher \( P < 0.02 \) than those by the HPLC method. A quantitative HPLC analysis of the cholesterol concentration in HDL and non-HDL fractions in the supernatant of serum separated by precipitation reagents with different MgCl₂ concentrations ranging from 7.3 to 44 mmol/L revealed that reagents with \( > 22 \) mmol/L MgCl₂ precipitated part of HDL as well as non-HDL lipoproteins. The HPLC method providing quantitative and qualitative information with high precision was regarded as being a reliable approach for HDL-C assay. The HPLC can be also used to evaluate alternative methods for cholesterol assay.

Since 1980, when we reported the HPLC method for lipoprotein cholesterol analysis with gel permeation column [1–3], many investigators have reported the usefulness of this technique [4–9]. The older HPLC method, however, has remained an alternative approach to lipoprotein cholesterol assay because of the difficulties in eliminating the nonspecific adsorption of lipoproteins to the gel materials (TSKgel SW and PW types, Tosoh).

Recently, we reported a simple, rapid, and accurate analysis of cholesterol in lipoproteins particularly suitable for HDL-cholesterol (HDL-C) assay by using an HPLC with an improved gel permeation column and eluent for lipoprotein separation (TSKgel Lipopropak and TSKeluent LP-1, Tosoh) [10–14]. With this new HPLC method, we have now evaluated the values of HDL-C measured by precipitation and direct methods, which are popular in Japanese clinical laboratories. Further, the effect of MgCl₂ concentration in the precipitation reagent on fractionation of HDL was examined by analysis of the supernatants by using the HPLC method.

Materials and Methods

Specimens

One hundred twenty-nine sera from healthy subjects together with sera from patients with various diseases were analyzed, and informed consent from all the subjects participating in this study was obtained. HDL-C in 74 sera were assayed for comparison of three methods: HPLC, precipitation (sodium phosphotungstate–MgCl₂), and direct methods. The supernatants of 55 sera treated with the reagents for the precipitation method were examined by HPLC.

Precipitation Method for HDL-C

A commercial precipitation reagent (Determiner HDL, Kyowa Medex) composed of 7.2 g/L sodium phosphotungstate, 0.3 g/L dextran sulfate, and 44 mmol/L MgCl₂ was used as a standard fractionation reagent for HDL-C assay. We also prepared a series of reagents with the same compositions as commercial reagents except MgCl₂ at the following concentrations: 7.3, 12.2, 14.7, 17.1, 19.6, 22, 24.4, 29.3, and 44 mmol/L. Equal volumes of serum and precipitation reagent were mixed, and centrifuged at 1500g for 10 min. The cholesterol values in supernatants were measured by an automated chemical analyzer (Hitachi 7150) with a certified reference serum [509AIJ, total cholesterol (TC) = 5.741 mmol/L, Kyowa Medex] as a calibrator.

Direct Method for HDL-C

A commercial reagent kit (Determiner HDL-C, Kyowa Medex) for direct HDL-C assay [15] that contained cho-
lesterol esterase and cholesterol oxidase modified by polyethylene glycol, sulfated α-cyclodextrin, dextran sulfate, and MgCl₂ was used in this experiment. The concentration of HDL-C was measured by the automated chemical analyzer with a certified reference serum (115AEK, HDL-C = 1.361 mmol/L, Kyowa Medex) as a calibrator.

**HPLC METHOD FOR HDL-C**

A routine HPLC apparatus (CCP&8010, Tosoh) with two connected columns (TSKgel Lipopropak, 7.5 mm in diameter, 300 mm in length) made specially for lipoprotein analysis, together with the TSKeluent LP-1 (Tosoh), also made for lipoprotein analysis, were used at a flow rate of 0.6 mL/min. Each 20 μL of serum diluted with an equal volume of buffer (20 mmol/L HEPES, 0.15 mol/L NaCl, pH 7.5) or the supernatant of the mixture of an equal volume of serum and precipitation reagent was applied to the HPLC. The effluent from the columns was mixed with reagent 1 [30 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 6.75), containing 3000 U/L ascorbic acid oxidase, 1000 U/L cholesterol oxidase, 2500 U/L peroxidase, 30 mmol/L N-ethyl-N-(3-methyl phenyl)-N'-succinyl ethylenediamine] at a flow rate of 0.225 mL/min and reagent 2 [30 mmol/L MOPS buffer (pH 6.75) containing 5000 U/L cholesterol oxidase, 25 000 U/L peroxidase, 2.2 mmol/L 4-aminoantipyrine] at a flow rate of 0.075 mL/min in the reaction tube (Teflon, 0.4 mm in diameter and 7.5 m in length) at a temperature of 45 °C and the amount of a colored substance produced was measured at 550 nm. TC and HDL-C were calculated automatically from the total area and HDL area, respectively, on the HPLC pattern monitored by cholesterol with a certified reference serum (WCHL 952 M, TC = 4.896 mmol/L, Health Care Technology Foundation, Standard Reference Center, Kawasaki, Japan) as a calibrator. The linearity and detection limit for the HDL-C assay with the HPLC method were examined by applying 20 μL of diluted (20 mmol/L HEPES, 0.15 mol/L NaCl, pH 7.5) serum samples. The linearity was confirmed in the HDL-C concentration range between 0.074 and 2.42 mmol/L, and the detection limit was as low as 0.5 mol/L. Identification of the HDL peak was done by comparing the elution pattern of the HDL fraction separated by ultracentrifugation.

**STATISTICS**

Quantitative variables are reported as mean ± SD. Paired Student’s t-test was used to determine the significance levels of the differences. Relations between variables were evaluated by Spearman’s correlation. \( P < 0.05 \) was considered significant.

**Results**

**ELUTION PATTERNS OF SERUM MONITORED BY CHOLESTEROL IN HPLC METHOD**

Representative elution patterns of serum monitored by cholesterol for four subjects with different triglyceride (TG) values are shown in Fig. 1. All the sera used in this study, including those with TG values 11.29 mmol/L, showed clearly separated peaks of HDL (peak 4) from apolipoprotein (apo) B-containing lipoproteins (peaks 1, 2, and 3).

![Fig. 1. HPLC profiles for serum and their supernatants treated with precipitation reagents of various concentrations of MgCl₂.](image)
COMPARISON OF HDL-C VALUES ASSAYED BY THE THREE METHODS

Within-run precision (CV) of the HPLC, precipitation, and direct methods with a sample with a normal TG value (TC = 5.33 mmol/L, TG = 0.97 mmol/L) were 0.55%, 1.95%, and 0.56%, respectively (Table 1).

Serum HDL-C of 74 subjects (TC = 5.36 ± 1.43 mmol/L, TG = 1.64 ± 2.35 mmol/L) was measured by the three methods. The average values of HDL-C by the three methods are summarized separately for the three groups classified by the TG values (Table 2).

The values of HDL-C both by precipitation and direct methods showed good correlations with those by the HPLC method, as shown in Fig. 2A. However, the paired t-test (n = 74) revealed that the values by the precipitation method were significantly (P < 0.001) lower than those by HPLC. Those by the direct method, on the contrary, were slightly but significantly higher (P < 0.02) than those by the HPLC method. Moreover, the differences of HDL-C between the precipitation method and the HPLC method (HDL-C) showed a significant negative correlation (r = -0.755, P < 0.001) with HDL-C values by the HPLC method, but in the case of the direct method, no significant correlation (r = 0.218) was observed, as shown in Fig. 2B.

EFFECT OF MgCl₂ CONCENTRATIONS ON SEPARATION OF HDL

The effect of MgCl₂ in the precipitation reagent on the separation of HDL was examined by using a series of reagents with different MgCl₂ concentrations. As shown in Fig. 1, peak height for the lipoproteins larger than HDL (peaks 1, 2, and 3) in the elution patterns for each serum sample (pattern a) gradually decreased in supernatants proportional to the increase in MgCl₂ concentration, from pattern b, 7.3 mmol/L MgCl₂ reagent to pattern g, 44 mmol/L (commercial) MgCl₂ reagent. Moreover, MgCl₂ concentrations enough to precipitate non-HDL fractions depended on TG values of each subject. As for all subjects, including those with high TG values, peaks of non-HDL lipoproteins were not observed in the supernatants at 22

| Table 1. Within-run precision of HDL-C quantification by three methods (n = 5). |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | HPLC            | Precipitation   | Direct          |
| Mean, mmol/L                   | 1.461           | 1.309           | 1.459           |
| SD, mmol/L                     | 0.008           | 0.026           | 0.008           |
| CV, %                          | 0.55            | 1.95            | 0.56            |

| Table 2. Comparison of HDL-C values (mmol/L) obtained by the three methods. |
|---------------------------------|-----------------|-----------------|-----------------|
| Group                           | TG conc., mmol/L | n   | HPLC          | Precipitation   | Direct          |
| A                               | <1.694          | 55  | 1.99 ± 0.89   | 1.81 ± 0.74**   | 2.02 ± 0.89**   |
|                                 | (0.61–4.80)      |     | (0.61–3.69)   | (0.61–4.81)     | (0.66–4.81)     |
| B                               | 1.694–4.517     | 14  | 1.11 ± 0.35   | 1.02 ± 0.34*    | 1.11 ± 0.37     |
|                                 | (0.45–2.01)      |     | (0.43–1.99)   | (0.37–2.02)     | (0.37–2.02)     |
| C                               | ≥4.517          | 5   | 1.04 ± 0.29   | 0.90 ± 0.27*    | 0.94 ± 0.30*    |
|                                 | (0.65–1.33)      |     | (0.55–1.11)   | (0.54–1.22)     | (0.54–1.22)     |
| All                             | 74              |     | 1.76 ± 0.88   | 1.60 ± 0.75**   | 1.78 ± 0.89*    |
|                                 | (0.45–4.80)      |     | (0.43–3.69)   | (0.37–4.81)     | (0.37–4.81)     |

Values are mean ± SD (range).

* P < 0.05; ** P < 0.01.
Percentages of cholesterol values in the HDL and non-HDL fractions in supernatants relative to the respective values in the original serum determined by the HPLC method are plotted against concentration of MgCl₂ (mmol/L) in precipitation reagents for two groups of subjects with normal and high TG values. As for the normal TG group (TG = 0.84 ± 0.14 mmol/L, n = 5), non-HDL lipoproteins disappeared at a concentration of 7.3 mmol/L (Fig. 3A). However, in the high TG group (9.18 ± 0.37 mmol/L, n = 5), >70% of non-HDL cholesterol in the supernatant at the same concentration of MgCl₂ and non-HDL fractions decreased rapidly to zero as the concentration of MgCl₂ was increased to 22 mmol/L, corresponding to 50% of that contained in commercial reagent (Fig. 3B).

The cholesterol values in HDL fractions in the supernatants of sera with normal and high TG values were almost the same, between 7.3 and 24.4 mmol/L MgCl₂ concentrations. The values in both groups, however, decreased gradually as the concentration of MgCl₂ exceeded 24.4 mmol/L (Fig. 3A, B).

### Recovery of HDL Fraction in Supernatants

The concentration of cholesterol in the HDL and non-HDL fractions in supernatants by 44 mmol/L (commercial) and 22 mmol/L MgCl₂ reagents were measured by HPLC method for serum samples from 55 subjects (TC = 5.83 ± 1.18 mmol/L, TG = 3.12 ± 3.63 mmol/L); those in non-HDL fractions (n = 55) were negligible (0.0024 ± 0.0068 and 0.0099 ± 0.0165 mmol/L, respectively). On the contrary, the cholesterol value in the HDL fraction was higher in the supernatants by 22 mmol/L MgCl₂ reagent (1.45 ± 0.86 mmol/L) than those by 44 mmol/L (commercial) reagent (1.58 ± 1.00 mmol/L). The recovery of the HDL fraction in the supernatant was calculated as a percentage of cholesterol values in the HDL fraction of the supernatant to those in the HDL fraction of original serum, both obtained by the HPLC method. The results obtained by both reagents are plotted against HDL-C values of original serum measured by the HPLC method (Fig. 4). The average recovery of HDL fraction in the supernatant by 44 mmol/L (commercial) reagent was 91.5% ± 4.9%, and that by 22 mmol/L MgCl₂ reagent 97.9% ± 3.6%.

A significant negative correlation (r = −0.45, P < 0.001)
was found between the percentage recovery of the HDL fraction in the supernatants by commercial reagent and HDL-C values of original serum (Fig. 4). On the contrary, no such correlation was found in the supernatants treated with 22 mmol/L MgCl₂ reagent.

Discussion

HDL-C has been routinely measured in Japan since its role as a risk factor for coronary atherosclerosis was reported [16]. The precipitation method [17] has been most popular in Japan, but recently it has largely been replaced by the direct method [15].

We reported [10–12] that HPLC with an improved gel permeation column and new eluent has become a reliable determination method for HDL-C assay, with high precision (CV <0.5%) due to complete separation of the HDL peak from non-HDL lipoproteins, as well as elimination of nonspecific adsorption of lipoproteins to column materials. The elimination of nonspecific adsorption was confirmed by >99.8% recovery of lipoproteins from the column [10]. The ultracentrifugation method is time consuming and also requires correction of the data on the basis of percentage recovery and contamination by apo B lipoproteins [17] to obtain accurate values of HDL-C, whereas the HPLC method is free from such factors.

Previously, we reported [13, 14] that the HDL-C values assayed by the precipitation method with different commercial reagent kits available in Japan were always lower than those by the HPLC method. In line with this assertion, Chiba et al. [18] reported that the precipitation reagent containing dextran sulfate, sodium phosphotungstate, and MgCl₂ readily precipitates apo E-rich HDL as compared with 130 g/L polyethylene 6000. We realized that one of the reasons for lower values by commercial reagent as opposed to the HPLC method was attributable to apo E-rich HDL, often found in large-size HDL, which is readily precipitated. We could not, however, explain all the reasons for the lower values obtained with the precipitation method. From the present examinations of the supernatants obtained by the precipitation method, we speculate that the commercial reagents precipitate part of HDL with normal size, even in routine samples, because of the high concentration of MgCl₂, which was necessary to precipitate non-HDL lipoproteins completely in samples with high TG values.

The effects of concentration of metal ions in the reagents for precipitation methods on the HDL-C values were already pointed out by Warnick et al. [17] by comparison with the ultracentrifugation method, and the present results confirm their results.

Unlike the precipitation method, the values of HDL-C measured by the direct method were very close to those by the HPLC method, despite a significant difference by statistics. Okamoto et al. [19] reported, using agarose gel chromatography, that the apo A-I peak was completely measured by the direct method, whereas partly by the precipitation method. This finding was also confirmed by our present work. The difference between HPLC and the direct method is suspected to be caused by the difference in calibrator, and for this the direct method has much advantage with respect to accuracy compared with the precipitation method.

We believe that the present HPLC method is most reliable for HDL-C assay among the methods available at present. The HPLC method is especially useful as a comparative method to evaluate other methods, because it gives not only quantitative information with excellent precision but also qualitative information through the elution patterns.

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References


