Surfactant-Based Homogeneous Assay for the Measurement of Triglyceride Concentrations in VLDL and Intermediate-Density Lipoprotein

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**Background:** Existing studies have demonstrated the clinical significance of triglyceride content in VLDL (VLDL-TG) and intermediate-density lipoprotein (IDL-TG). We developed a homogeneous assay protocol to directly measure VLDL-TG.

**Methods:** Possible reagents and conditions for measuring VLDL-TG were comprehensively tested, and the “best” combination was determined. Healthy persons were instructed to consume a fatty meal after 15-h overnight fasting. Serum VLDL-TG + IDL-TG concentrations were measured using the proposed method. Patients with serum LDL-cholesterol concentrations ≥3.62 mmol/L (140 mg/dL) were administered simvastatin at a daily dose of 5 mg, and serum VLDL-TG concentrations were then measured.

**Results:** The combination of 2 nonionic surfactants played an important role in differentiating VLDL and IDL from other lipoproteins, probably via specific interactions with phospholipids and apolipoproteins. The regression line of the proposed method (y) and the ultracentrifugal assay (x) was: y = 0.98x + 0.31 mmol/L ($r = 0.98$; n = 73; $P < 0.05$). The difference between postprandial total TG and VLDL-TG concentrations was statistically significant ($P < 0.05$). After 8 weeks of therapy with simvastatin, total TG and LDL-cholesterol concentrations were 13.6% and 26.3% lower, respectively ($P < 0.05$), whereas VLDL-TG did not show any significant decrease.

**Conclusion:** Our homogeneous method can measure TG content in VLDL and IDL.

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The National Cholesterol Education Program’s Adult Treatment Panel III report (ATP III) identified the metabolic syndrome as a multiplex risk factor for cardiovascular disease that is deserving of more clinical attention (1). The emerging risk factors include increased triglyceride (TG)$^5$ concentrations (2–9). TGs originate from dietary and nondietary sources, and the vast majority of circulating TGs are carried in lipoproteins with a density <1.019 kg/L. These lipoproteins include chylomicrons, VLDL, and intermediate-density lipoproteins (IDL). Schonfeld (10) reported that patients with hyperchylomicronemia do not appear to have any increased risk for coronary heart disease (CHD). In contrast, the clinical significance of the TG content of VLDL and IDL has been increasingly shown (11–14). TG concentrations, therefore, may be measured for VLDL + IDL independently from other TG-rich lipoproteins, such as chylomicrons, not only for epidemiologic studies but also for routine lipid analysis.

In this report, we propose a homogeneous assay protocol to directly measure the TG content in VLDL and IDL. In this method, a specific combination of surfactants plays a key role in stabilizing the polar lipids of VLDL and IDL via exclusive interaction with the apolipoproteins. This method is suitable for clinical laboratories and can be used on automated analyzers (we refer to these

$^5$ Nonstandard abbreviations: TG, triglyceride; IDL, intermediate-density lipoprotein; CHD, coronary heart disease; LPDS, lipoprotein-deficient serum; FPLC, fast protein liquid chromatography; LPL, lipoprotein lipase; and apo, apolipoprotein.
measurements as VLDL-TG and conventional TG measurement as total TG in the text). We examined the performance of the proposed method, VLDL-TG concentrations in the postprandial state, and VLDL-TG concentrations after a lipid-lowering regimen.

**Materials and Methods**

**STUDY POPULATIONS**

EDTA-plasma samples for evaluating the proposed method were taken from an antecubital vein of 73 volunteers in nonfasting state (group A). Fasting sera for determining reference intervals were taken from 150 healthy volunteers (90 men and 60 women; 21–64 years of age) who were recruited from Shin-Test Corporation, Tokyo, Japan (group B). Six healthy individuals participated in the test for examining postprandial response to a fatty meal (group C). To examine the response to a lipid-lowering drug [simvastatin (15)], 45 patients were recruited (group D). The patients in group D had type IIa hyperlipidemia; their sera were taken in the fasting state. The exclusion criteria for groups A, B, and C were current or previous treatment with lipid-lowering drugs and age <20 or >60 years. Patients currently or previously taking lipid-lowering drugs; currently pregnant; having a history of hypertension, diabetes mellitus, or cardiovascular diseases; and <40 or >69 years of age were excluded from group D.

Samples were stored at 4°C and were analyzed within 2 days except for analysis of interassay variations. Samples for lipoprotein fractionation were prepared from dipotassium EDTA (4.82 mol/L)-anticoagulated venous blood. The study design was approved by the Ethics Committee on Human Research of Niigata University (Niigata, Japan), and written informed consent was obtained from all participants.

**LIPOPROTEIN PREPARATIONS AND ANALYSES**

Samples from group A were processed as follows: Chylomicrons were isolated in the upper layer of samples after centrifugation for 1 h at 10 000g in a Kubota 1710 centrifuge. Samples (2 mL) were mixed with 0.3 mmol/L disodium EDTA and 1 mmol/L benzamidine hydrochloride. VLDL (d < 1.006 kg/L), IDL (1.006 < d < 1.019 kg/L), LDL (1.019 < d < 1.063 kg/L), and HDL (1.063 < d < 1.210 kg/L) were separated by sequential adjustment of the plasma density with NaBr followed by sequential ultracentrifugation at 400 000g and 16°C for 16 h in a Himac centrifuge with an RP80AT rotor (Hitachi Koki). We isolated each fraction by aspirating the supernatant (interassay CV of our procedure is 5.8%) after each centrifugation. The bottom fractions after the final ultracentrifugation were collected, desalted, and used as lipoprotein-deficient serum (LPDS) for the recovery test. Gel-permeation chromatography and electrophoresis on agarose gels showed no cross-contamination among these fractions.

Gel-permeation chromatography was performed on a fast protein liquid chromatography (FPLC) system equipped with a Superose-6 column (Pharmacia). Samples (500 μL) were loaded and eluted at room temperature in phosphate-buffered saline supplemented with disodium EDTA. The eluate was monitored by ultraviolet absorbance at 280 nm, and 25 fractions (0.5 mL each) were collected. Each FPLC peak was identified by comparison with samples subjected to ultracentrifugation and by their apolipoprotein content.

Electrophoretic patterns of samples were determined on 10% agarose gel films (Titan Gel Lipo; Helena) in a 0.06 mol/L barbital buffer (pH 8.6) for 30 min at 90 V. TGs in each band were visualized by nitrotetrazolium blue.

Total TG concentrations were determined enzymatically by use of Quick Auto Neo TGII assay reagents (Shino-Test) on an H-7170S automatic analyzer (Hitachi) in 2 steps: Briefly, free glyceride, which exists natively in serum or in plasma, was first converted to glycerol 3-phosphate by glycerol kinase (EC 2.7.1.30) and degraded into dihydroxyacetone 3-phosphate plus hydrogen peroxide by glycerol-3-phosphate oxidase (EC 1.1.3.21). The hydrogen peroxide was further degraded into H2O and O2 by peroxidase (EC 1.11.1.6). In the second step, TG was degraded into glycerol by lipoprotein lipase (LPL) in surfactant, followed by the same reactions as in the first step. The generated hydrogen peroxide was measured quantitatively by colorimetric assay. The reagents used for the first- and second-step reactions are subsequently designated as reagents 1 and 2, respectively.

**PROTOCOL FOR VLDL-TG MEASUREMENTS**

We tested possible reagents and conditions for measuring VLDL-TG extensively and determined the “best” combination as follows: We investigated the interactions of the 5 subspecies of lipoproteins with 159 surfactants, such as ether or ester compounds of polyoxylalkylenes. Each surfactant was added to either reagent 1 or reagent 2 (from which the original surfactant had been excluded) at various concentrations (usually 1 g/L and 5 g/L), and total TGs were measured.

We also investigated various LPL preparations, buffers, and other supplements. Candidates for LPL were LP-BP and LPL from Asahi Kasei and LPL-311 and LPL-314 (EC 3.1.1.34) from Toyobo. In addition, we evaluated 13 “good” buffer formulations, including MES and Bis-Tris, at different concentrations for controlling pH in each appropriate range. Because it is well known that albumin, metal ions, and sugars can affect the specificity of surfactants against lipoprotein subspecies (16, 17), we evaluated bovine serum albumin, 2 metal ions, and 8 cyclodextrin derivatives, such as hydroxypropyl-cyclodextrin. These evaluations were performed independently for reagents 1 and 2.

The automatic analyzer was calibrated with a VLDL solution obtained from Chemicon International. The ma-
Material was prepared in bulk and then stored as aliquots at 4 °C. The material was also used in the recovery test. We confirmed that this material had the expected electrophoretic mobility even after storage.

**RECOVERY AND INTERFERENCE TESTS**

VLDL solution [TG content, 12.74 mmol/L (1128 mg/dL)] was mixed with LPDS in different volumes; the percentage of the added VLDL was then measured and expressed as recovery. TG content in the LPDS was almost 0. Interference was determined according to the protocol recommended by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS). Briefly, we mixed different amounts of each suspected interfering material (bilirubin and hemoglobin) with a plasma pool prepared from patient samples. We then analyzed the plasma samples by the proposed method and checked for differences in values attributable to the addition of the suspected interferent.

**POSTPRANDIAL ANALYSIS**

Persons in group B were instructed to consume a fatty meal (Chinese cuisine; 1200 calories, 35% fat) after a 15-h overnight fast. Blood samples were taken immediately before and at 2 and 4 h after the meal.

**EFFECT OF MEDICATION**

For the study of the effect of lipid-lowering medications, patients were eligible if their serum LDL-cholesterol concentrations were ≥3.62 mmol/L (140 mg/dL) and if they exhibited at least one of the following risk factors: age ≥45 years for males or ≥55 years for females; history of hypertension, diabetes mellitus, or cigarette smoking; serum HDL-cholesterol concentration <1.03 mmol/L (40 mg/dL); or familial history of CHD. Eligible patients were enrolled in a 12-week study during which they were given lipid-lowering dietary advice (group D). Lipoprotein profiles were obtained immediately before and after this period. Patients whose serum LDL-cholesterol concentrations remained ≥3.62 mmol/L (140 mg/dL) after this period were given simvastatin (5 mg/day). Patients were seen, on average, at 4-week intervals. During each visit, dietary advice was reinforced and a fasting lipoprotein profile was obtained. On the second visit, patients whose serum LDL-cholesterol concentrations remained ≥3.62 mmol/L (140 mg/dL) were randomly assigned to either 5 or 10 mg/day simvastatin.

**STATISTICAL ANALYSIS**

The Student *t*-test and correlation coefficient statistics were applied to the experimental results, and unless otherwise specified, the significance of the values was evaluated at the 0.05 confidence level (*P*). Paired Student *t*-tests were used to compare lipid profiles in samples collected from patients in the fasting and nonfasting state as well as patient samples obtained before and after administration of medication. Correlation coefficients (*r*) were used for evaluation of the proposed method. Data are reported as the mean (SD).

**Results**

**ASSAY REAGENTS AND PROTOCOL**

The relative reactivity of each surfactant against the 5 subtypes of lipoprotein in relation to total TG measurements are shown in Table 1. The nonionic surfactant nonylphenol ethoxylate (Emulgen 911; Kao) reacted specifically against LDL and HDL. This surfactant depleted the TG content of LDL and HDL and was suitable for the first-step reaction. In contrast, another nonionic surfactant, alkylpolyoxyethylene (Tergitol NP-10; Nikko Chemicals), reacted highly specifically against the 4 subtypes other than chylomicrons. This surfactant was therefore ideal for the second-step reaction, in which LPL reacted exclusively against VLDL and IDL. Use of various concentrations of the surfactants altered the reactivity.

Although the specificity did not depend on buffer components, the pH was crucial. At higher pH values, co-measurement of TGs originating from certain subtypes, such as LDL, increased.

LPL was another determinant that could be used to

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differentiate among VLDL, IDL, and other lipoprotein subspecies. We evaluated LP-BP and LPL at concentrations of 10, 50, 100, 250, and 500 kU/L. We also evaluated LPL-311 and LPL-314 at concentrations of 10, 50, 100, 250, and 500 U/L. Because LP-BP is a lipophilic enzyme, it requires a surfactant to react with the target molecules. We found that LP-BP lowered the specificity of Emulgen 911, whereas LPL-314 did not.

We also evaluated 13 “good” buffer formulations, including MES and Bis-Tris, at various concentrations (10, 25, 50, 100, 150, and 200 mmol/L) for controlling pH in the appropriate ranges: e.g., 6.0, 6.5, and 7.0; 6.5, 7.0, and 7.5; and 7.0, 7.5, and 8.0.

In the final assay protocol, reagent 1 was composed of 100 U/L LPL-314, 150 U/L glycerol kinase, 3 kU/L glycerol-3-phosphate oxidase, 600 U/L peroxidase, 1 g/L Emulgen 911, 1 mmol/L MgCl2, 1 g/L sodium azide, and 1.5 mmol/L N-[(2-carboxyethyl)-(N-ethyl-3-methylaniline) hydrochloride (as chromogen) in 50 mmol/L MES buffer (pH 6.5). We added 2.5 μL of each sample to 200 μL of reagent 1 and incubated the mixture at 37 °C for 4 min. To this mixture, we added 100 μL of reagent 2, which contained 500 kU/L LPL, 5 g/L NP-10, 1 g/L sodium azide, 600 U/L peroxidase, and 0.75 mmol/L 4-aminoantipyrine (as chromogen) in 50 mmol/L MES (pH 6.0). The resulting color was measured immediately before and at 5 min after the addition of reagent 2 at 600 nm (main) and 700 nm (subsidiary).

**Validations**

To assess the linearity of the proposed method, we measured a series of aqueous solutions containing VLDL [TG content, 17.5 mmol/L (1551 mg/dL)] diluted 5:5, 4:5, 3:5, 2:5, and 1:5 with phosphate-buffered saline. The results showed that the method was linear up to 17.5 mmol/L (1551 mg/dL; y = 1.0x + 0.034 mmol/L, r = 1.00, P <0.001). To assess the imprecision of the method, we assayed serum samples containing low [0.71 mmol/L (63 mg/dL)], medium [1.49 mmol/L (132 mg/dL)], and high VLDL-TG concentrations [2.98 mmol/L (264 mg/dL)]. Intrasay imprecision (CV) was 1.1%, 0.61%, and 0.83% at the low, medium, and high VLDL-TG concentrations, respectively (n = 20). The interassay CV was 1.8%, 3.1%, and 4.1%, respectively, for aliquots with low, medium, and high VLDL-TG concentrations evaluated on 5 consecutive days. These results fulfill the precision criteria for acceptable performance of total TG measurement, as specified by the National Cholesterol Education Program recommendations (18). We also assessed analytical recovery by adding VLDL to an aliquot of LPDS and then assaying the sample. The recovery, calculated as measured VLDL/added VLDL × 100%, was 100 (0.7%). Bilirubin up to 356 mmol/L (20.8 mg/dL) and hemoglobin up to 4.82 mmol/L (0.482 g/dL) did not interfere with VLDL-TG measurements.

We investigated the effects of freezing by storing aliquots of 2 samples [VLDL-TG content, 0.93 mmol/L (82 mg/dL) and 5.05 mmol/L (447 mg/dL)] at −40 °C and 5 °C for 14 days, after which we measured VLDL-TG. The measured values decreased by 72.5 (0.28)% after freezing and 92.1 (0.02)% after storage at 5 °C (P <0.05).

The automatic analyzer was calibrated with a commercially available VLDL solution (Chemicon International). The interassay CV of this material stored at 4 °C for 19 days was 1.0%.

**Analytical Specificity**

To examine specificity, we prepared 25 lipoprotein fractions by FPLC of plasma of 2 patients with type IIA hyperlipidemia [total TG, 1.75 mmol/L (136 mg/dL)] and type IV hyperlipidemia [total TG, 3.50 mmol/L (310 mg/dL)], respectively. VLDL-TG was measured in each fraction by the proposed method. Total TG and total cholesterol concentrations were also measured for comparison. The FPLC profiles are shown in Fig. 1.

Because chylomicrons and IDL cannot be separated by FPLC, we again obtained all fractions by sequential ultracentrifugation of plasma samples from 4 patients. Each fraction was analyzed by the proposed method (a) and total TG assay (b). The cross-reactivity, as defined by

\[
\frac{a}{b} \times 100\% ,
\]

is shown in Table 2.

**Method Comparison with Ultracentrifugation Procedure**

We examined the comparability of the proposed method with other analytical methods by preparing VLDL fractions from plasmas of group A [VLDL-TG, 0.12–12.7 mmol/L (11–1125 mg/dL)] by ultracentrifugation. Comparison between the proposed method (y) and ultracentrifugal assay (x) revealed a regression line of y = 0.98x + 0.31 mmol/L (r = 0.98; n = 73; P <0.05). The regression line for samples with VLDL-TG <2.82 mmol/L (250 mg/dL) was y = 1.07x + 0.11 mmol/L (r = 0.93; n = 48; P <0.05).

**Results for Normotriglyceridemic Individuals**

Serum VLDL-TG concentrations in the healthy volunteers (group B) ranged from 0.12 mmol/L (11 mg/dL) to 4.93 mmol/L (437 mg/dL). The distribution of serum VLDL-TG concentrations in these normotriglyceridemic individuals [<1.69 mmol/L (150 mg/dL); n = 125] is shown in Fig. 2. Also indicated in Fig. 2 is the distribution of total serum TG concentrations.

**Differences Between VLDL-TG and Total TG**

We investigated the differences in VLDL-TG concentrations between the fasting and nonfasting states in group B. As expected, total TG concentrations had increased by 247 (65.1)% at 4 h after volunteers had consumed the fatty meal (Fig. 3A), whereas VLDL-TG concentrations in-
creased by 176 (19.0)% (Fig. 3B). The difference between postprandial concentrations of total TG and VLDL-TG was statistically significant \( (P < 0.05) \). The electrophoretic patterns of 2 typical cases are shown in Fig. 4; case 1 shows a clear increase in VLDL-TG with less chylomicron TG at 4 h after the meal, whereas case 2 shows a clear increase in chylomicron TG with less VLDL-TG. Differences between the types could not be distinguished based on total TG concentrations.

Of the 45 patients recruited for the diet period (group D), 18 fulfilled the entry criteria and were given simvastatin. Their initial total TG concentrations ranged from 0.75 to 3.56 mmol/L (66–315 mg/dL). Median follow-up time was 8 weeks. After 8 weeks of therapy with simvastatin, total TG and LDL-cholesterol were 13.6% and 26.3% lower, respectively \( (P < 0.05) \), than the baseline values, whereas VLDL-TG was not significantly lower. There were no significant differences in either total TG or VLDL-TG concentrations between patients receiving simvastatin doses of 5 and 10 mg/day.

**Discussion**

We found that the combination of 2 nonionic surfactants improved differentiation of VLDL and IDL from other lipoproteins, probably via specific interactions with phospholipids and apolipoproteins.

Compared with LDL and HDL, biological and technologic difficulties hamper the measurement of VLDL and IDL. Circulating VLDL rapidly loses TG and is converted to IDL by LPL, which is activated by apolipoprotein (apo)C-II present on the surface of VLDL, and IDL is then converted to LDL \( (19) \). The in vivo half-life of each VLDL particle in healthy persons is \( \approx 2.5 \) h \( (20) \). Because the metabolism of VLDL and IDL are regulated not only by LPL but also by apolipoproteins, insulin, and other factors, the half-life varies widely among individuals and different disease states. Therefore, samples should be taken after overnight fasting, not frozen, and measured within a few days.

VLDL particles also continuously decrease in size,
hence reducing the difference in physicochemical characteristics between VLDL and IDL or between IDL and LDL (19). The crucial factor is the composition of apolipoproteins. Chylomicrons contain apoA, apoC, and apoB-48 and lack apoB-100. The compositions of VLDL, IDL, and LDL, which all contain apoB-100 as the structural protein, are similar to each other but differ from those of chylomicrons and HDL, which contain apoB-48 and apoA, respectively. Thus, in vitro differentiation of chylomicrons and HDL from the other TG-rich lipoproteins is relatively easy. In contrast, most methods for detecting VLDL and IDL may also detect some LDL particles. If all LDL particles are excluded, then some IDL will be missed. A trade-off therefore exists in the differentiation of lipoprotein subspecies. This problem has also been recognized to a lesser extent in methods for measuring LDL- and HDL-cholesterol (16, 17). In this study, we found that ~30% of TG in LDL was erroneously present in the results obtained by the proposed method (Table 2). However, because the amount of TG in LDL is not significant compared with that presents in VLDL, this limitation does not significantly affect the applicability of this method.

Although factors determining interindividual variation are not well defined, many researchers have reported that higher postprandial TG concentrations can be considered an important factor in the pathogenesis of metabolic syndrome or CHD (21, 22). Schneeman et al. (21) reported that accumulation of predominantly VLDL rather than chylomicron particles after feeding is relevant to the potential atherogenicity of postprandial hypertriglyceridemia. Our method is the first to enable the possibility of easily measuring this process.

In the Scandinavian Simvastatin Survival Study, after 6 weeks of therapy with 20 mg/day simvastatin, total TG concentrations were decreased on average by 15%, whereas little change was seen in the placebo group (15). In our study, the patients were taking simvastatin at doses of 5 or 10 mg/day and obtained equally significant reductions (14%; \( P \leq 0.05 \)) in total TG concentrations. With simvastatin treatment, VLDL-TG concentrations showed a slight decrease (11.4%), which was not statistically significant. These results show that VLDL-TG concentrations may provide different information from those of total TG and that the 2 species respond differently to medication. The proposed method could provide an alternative means of evaluating TG concentrations during lipid-lowering therapy.

In conclusion, the data presented here offer evidence that our homogeneous method can measure TG content exclusively in VLDL and IDL. This method does not require use of previous sample preparation techniques such as immunoprecipitation or centrifugation and enables clinical laboratories to measure serum samples cost-effectively on an automatic analyzer. This is the first approach to directly measure VLDL- and IDL-TG.
This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (No. 14370792, 2002, 2003, and 2004).

References