methods

# A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC

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**Abstract** **We describe an on-line dual detection method using HPLC for lipoprotein analysis that allows simultaneous determination of cholesterol and triglyceride profiles from a single injection of sample. Two different gel permeation columns, TSKgel LipopropakXL and Superose 6HR, were applied to the dual detection system, evaluating analytical performance of the proposed method and the columns by analyzing serum samples from human and nonhuman subjects. Both TSK and Superose columns produced good within-day imprecision values less than 4.7% for cholesterol and 4.2% for triglyceride determination. Linear regression analysis showed the results from the Superose column (y) correlated well with those from the TSK column (x): $y = 0.969x + 5.44$ ($r = 0.990$) for total cholesterol (mg/dl), $y = 1.08x - 11.14$ ($r = 0.985$) for total triglycerides (mg/dl), and $y = 1.093x - 0.06$ ($r = 0.978$) for the ratios of triglycerides to cholesterol (mg/mg). Furthermore, the cholesterol and triglyceride profiles elucidated the differences in the resolution ability of the columns, which have not been apparent from a single lipid profile. We conclude that the dual detection concept with proper choice of column and enzymic reagents specific to the objectives of the particular study can facilitate studies of lipoprotein metabolism.—** Usui, S., Y. Hara, S. Hosaki, and M. Okazaki. **A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J. Lipid Res.* 2002.** 43: **805–814.**

**Supplementary key words** gel permeation column • lipoprotein profile • hyperlipidemia • chylomicron

Plasma lipoproteins have been classified on the basis of their hydrated density, particle size, and electrophoretic mobility. Ultracentrifugation is considered to be the traditional method used to define major lipoprotein classes according to their density (1) and is widely used for separation and fractionation of lipoproteins from plasma or serum samples. Ultracentrifugation, however, is time-consuming and requires large sample volumes and great care to precisely recover lipoproteins from a centrifugal tube.

HPLC with gel permeation columns is an alternative method for classifying and quantifying lipoproteins on the basis of differences in particle size (2, 3). We previously reported HPLC methods with an on-line single detection technique combined with a selective enzymatic reaction by which lipid constituents of lipoproteins in the column effluent were detected and monitored without subsequent analyses of the column fractions collected (4–13). The on-line detection technique eliminated laborious and time-consuming procedures accompanied with fraction collection produced a high throughput of samples, and improved analytical precision and detection sensitivity. Other researchers also reported on-line cholesterol determination methods by high-performance gel chromatography and fast lipoprotein chromatography (14–17), and Garber et al. recently observed that the on-line detection was much preferable to fraction collection for determination of plasma cholesterol profiles from individual plasma samples with very low sample volumes (16).

On the other hand, all of the previous systems suffered from the disadvantage that only one kind of lipid could be measured in a single analytical run. We and other investigators have used common enzymatic reagent systems for cholesterol (4–6, 14–16), triglycerides (7, 8), phospholipids (9), or unesterified cholesterol (17) in several studies to examine lipid profiles of lipoproteins from human and non-human subjects, but were required to complete a separate injection with each enzymic reagent, a practice both

Abbreviations: 4-AA, 4-aminoantipyrine; apo, apolipoprotein; CHO, cholesterol oxidase; CM, chylomicron; CV, coefficient of variation; EMSE, *N*-ethyl-*N*-(3-methylphenyl)-*N*′-succinylethylendiamine; FG, free glycerol; GPO, glycerol-3-phosphate oxidase; POD, peroxidase; R1, reagent 1; R2, reagent 2; TBA, Tris-buffered acetate.

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inefficient and wasteful of samples. Multiple injections may be impossible for samples with small volumes, e.g., those from individual mouse models, supernatants of cell culture media in lipoprotein metabolism studies, and large series of samples in epidemiological studies. Although Kieft et al. proposed a simultaneous determination of not only lipoprotein cholesterol but also other constituents by splitting the postcolumn line (14), there has been no precedent for simultaneous dual analysis with HPLC in lipid analysis of serum lipoproteins.

In this study, we describe a new dual detection HPLC system for lipoprotein analysis that made it possible to monitor and obtain simultaneously cholesterol and triglyceride profiles in a single injection of samples, reducing the number of analytical runs and tests needed. Two different kinds of gel permeation column, TSKgel LipopropakXL (Tosoh Co., Tokyo, Japan) and Superose 6HR (Pharmacia, Uppsula, Sweden), were studied for analytical performance and separation characteristics using samples from humans and animals.

## MATERIALS AND METHODS

### Reagents

For the detection of cholesterol and triglycerides with the HPLC method, we used enzymatic reagents that were previously described but subsequently modified by the manufacturer (Kyowa Medex Co., Tokyo, Japan) (6, 8). The reagent system for cholesterol detection consists of reagent 1 (R1-C) and reagent 2 (R2-C). R1-C contains peroxidase (POD, 10,000 U/l), *N*-ethyl-*N*-(3-methylphenyl)-*N*′-succinylethylendiamine (EMSE, 1.1 mmol/l), MOPS buffer (20 mmol/l, pH 7.0), detergents, and stabilizer. R2-C contains cholesterol esterase (300 U/l), cholesterol oxidase (CHO, 2,000 U/l), POD (10,000 U/l), 4-aminoantipyrine (4-AA, 1.5 mmol/l), $CaCl_2 \bullet 2H_2O$ (0.68 mmol/l), MOPS buffer (20 mmol/l, pH 7.0), detergents, and stabilizer. Similar to the reagent system for cholesterol, the triglyceride reagent system includes reagent 1 (R1-TG) and reagent 2 (R2-TG). R1-TG contains glycerol kinase (3,000 U/l), glycerol-3-phosphate oxidase (GPO, 15,000 U/l), ATP (4.9 mmol/l), POD (5,000 U/l), EMSE (1.1 mmol/l), $MgSO_4 \bullet 7H_2O$ (2 mmol/l), PIPES buffer (50 mmol/l, pH 6.2), detergents and stabilizer. R2-TG contains LPL (3,000 U/l), POD (5,000 U/l), 4-AA (1.5 mmol/l), $MgSO_4 \bullet 7H_2O$ (2 mmol/l), PIPES buffer (50 mmol/l, pH 6.2), detergents, and stabilizer.

Equal amounts of R1 and R2 were mixed before use. After mixing, the cholesterol reagent was used within 4 weeks and the triglyceride reagent within 2 weeks.

### Lipoprotein analysis by a dual detection HPLC system

The HPLC system consisted of an AS-8020 auto-injector, CCPS and CCPM-II pumps, and two UV-8020 detectors (Tosoh) (6). A SC-8020 system controller (Tosoh) was used for instrument regulation and data collection. Lipoproteins were separated on two tandem connected TSKgel LipopropakXL columns (300 × 7.8 mm, Tosoh) with 0.05 mol/l Tris-buffered acetate (TBA, pH 8.0) containing 0.3 mol/l sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 ml/min or a single Superose 6HR column (300 × 10 mm, Pharmacia) with 0.05 mol/l PBS (pH 7.4) containing 0.15 mol/l NaCl at a flow rate of 0.5 ml/min. The TSK column medium is composed of porous polymer matrices with a nominal bead size of 10 μm and a pore size of 100 nm, which is expected to exclude most of chylomicron (CM) to the void volume. On the other hand, the Superose medium is formed from cross-linked agarose matrices with a nominal bead size of 13 μm, giving the exclusion limits of ~40,000,000 Da and the optimal separation ranges of 5,000 to 5,000,000 Da (globular proteins) according to the manufacturer's instructions (cat. no. 52-1768-00, Pharmacia). The TSK column has a smaller column volume than the Superose column, but tolerates higher back-pressure (2.5 MPa vs. 1.5 MPa). For that reason, two TSK columns were connected in tandem and used to obtain higher resolution within a relatively short analytical time. Both running buffers were filtered through a 0.22 μm filter (Millipore Co., Bedford, MA) before use and continuously degassed with a SD-8022 on-line degasser (Tosoh) during analysis. The column effluent was split equally into two lines by a Micro-Splitter P-460 (Upchurch Scientific Inc., Oak Harbor, WA), one mixing with cholesterol reagent and the other with triglyceride reagent, thus achieving simultaneous profiles from a single injection (**Fig.1**). The two enzymatic reagents were each pumped at a flow rate of 0.35 ml/min for the TSK column and 0.25 ml/min for the Superose column. Both enzymatic reactions proceeded at 37°C in a reactor coil (Teflon, 15 m × 0.4 mm id). Ten microliter samples, unless stated otherwise, were injected by an AS-8020 auto-injector with a pre-suction volume of 25 μl at intervals of 24 min for the TSK column and 35 min for the Superose column. When an increased back-pressure or loss of resolution was observed, the Superose column was washed according to the manufacturer's instructions.

The final step of the enzymatic determination of cholesterol and triglycerides involved the detection of hydrogen peroxide produced by CHO and GPO enzymes, respectively. One molecule of hydrogen peroxide is theoretically produced from one molecule of cholesterol or triglycerides, and the measurement of hydrogen oxide is carried out by the use of the same chromogenic substrates (EMSE and 4-AA) in both the cholesterol and triglyceride reagent systems. Therefore, the ratio (mol/mol) of triglycerides to cholesterol in the lipoproteins is directly determined based on the relative area under the chromatographic curves. The molar ratios are converted to weight ratios by multiplying the triglyceride molar values times 2.3, consistent with the molecular weight ratio of triglycerides (as triolein) to cholesterol of approximately 2.3. Total cholesterol and triglyceride concentrations (in mg/dl) were calculated by comparison with total area under the chromatographic curves of a calibration material of known concentration.

### Effects of running buffers on enzymatic reactions

Enzymatic reagents were diluted to 2-fold with an equal amount of TBA or PBS. A 10 μl serum sample was added to 1.1 ml of the diluted enzyme solution in a glass curvet at 37°C, and the developed color was measured at 550 nm for 300 s on a UV1650-PC spectrophotometer (Simadzu, Kyoto, Japan).

### Separation of standard lipoproteins by ultracentrifugation

Standard lipoprotein fractions (CM plus VLDL with a density <1.006 kg/l, IDL with a density from 1.006 to 1.019 kg/l, LDL with a density from 1.019 to 1.063 kg/l, $HDL_2$ with a density from 1.063 to 1.125 kg/l, and $HDL_3$ with a density >1.125 kg/l) were prepared by sequential ultracentrifugation (1, 18). A 1 ml serum sample was placed in a polycarbonate centrifuge tube (cat. no. 343778, Beckman Instruments, Inc., Palo Alto, CA) and centrifuged on a Beckman Optima-TLX preparative ultracentrifuge with a fixed-angle TLA 120.2 rotor at 110,000 rpm for 3 h at 16°C, to obtain a 1.006 kg/l top fraction containing VLDL and

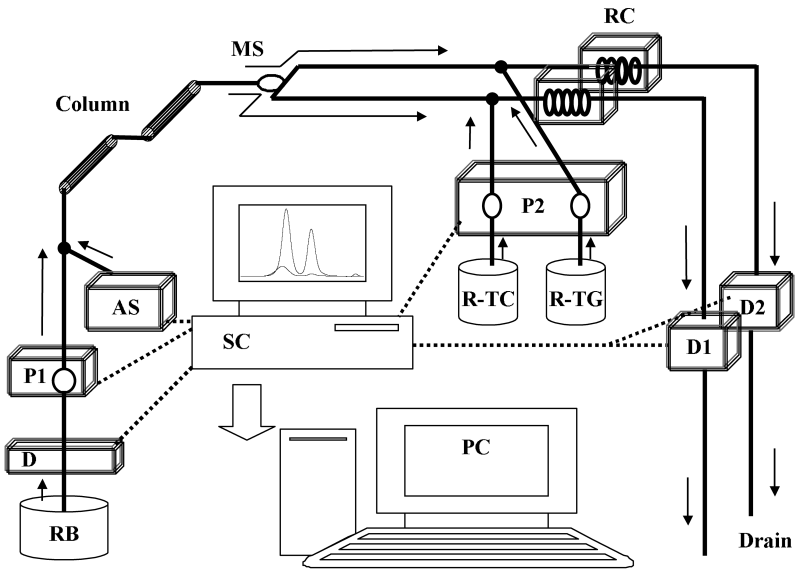


**Fig. 1.** HPLC system with on-line enzymatic dual detection of cholesterol and triglycerides of serum lipoproteins. Separations were obtained with two tandem connected TSK columns. AS, auto-sampler; D, degasser; D1, detector 1; D2, detector 2; MS, micro splitter; P1, pump 1; P2, pump 2; PC, personal computer; RB, running buffer; RC, reaction coil; R-TC, reagent for cholesterol; R-TG, reagent for triglycerides; SC, system controller. Arrows indicate flow directions.

CM using a tube slicing technique (Beckman CentriTube Slicer). Density of the ultracentrifugal 1.006 kg/l bottom fraction was adjusted to 1.019 kg/l with addition of a concentrated NaBr solution and then centrifuged at 110,000 rpm for 3 h at 16°C to obtain a 1.019 kg/l top fraction containing IDL. LDL was separated by centrifugation at 110,000 rpm for 3 h at 16°C from the 1.019 kg/l bottom fraction after adjustment to density 1.063 kg/l. $HDL_2$ was separated by centrifugation at 110,000 rpm for 6 h at 16°C from the 1.063 kg/l bottom fraction after adjustment to density 1.125 kg/l.

### Study subjects and blood sampling

For comparison of serum total cholesterol and triglyceride values obtained by both TSK and Superose columns, 60 apparently healthy firemen, aged 25–57 years (mean age, 42 years), at Kashiwa firehouse participating in a study of health care and physical fitness (19) were included in this comparison. Serum obtained from a healthy male volunteer was used to prepare standard lipoprotein fractions by ultracentrifugation in order to identify the peaks on the chromatographic patterns. To further investigate characteristics and resolution of the columns, two patients with either type I hyperlipidemia or apolipoprotein E-2/2 (apoE-2/2), and wild-type CETP-expressed as well as apoE-deficient mice were studied. Blood samples from the human subjects were kindly provided by Dr. Hideki Asakawa at Itami Municipal Hospital, Dr. Minoru Ohkubo at Toranomon Hospital, and Dr. Nobuo Yamami at Tokyo Medical and Dental University, and mice specimens were obtained from Dr. Shinji Yokoyama at Nagoya City University and Dr. Tokuo Yamamoto at Tohoku University. All human subjects gave informed consent to participate in this study under the permission of the ethics committees of the individual institutions. Animal experiments were performed according to the protocols that are designed under the guidelines by the individual institutions and approved by their animal welfare committees.

The blood samples were allowed to clot at room temperature, and then centrifuged at 3,000 rpm for 15 min to obtain serum samples. All serum samples were stored at 4°C and analyzed within 10 days after blood collection.

## RESULTS

### Effects of running buffers on enzymatic reactions

The TSK and Superose columns each required different buffers to obtain good resolution and recovery of lipoproteins. We examined the effects of running buffers TBA and PBS on enzymatic reactions and determined an optimum reaction time for the detection of cholesterol and triglycerides. Although no significant difference depending on the buffers was found in the cholesterol detection as shown in **Fig. 2**, the reaction rate for triglyceride detection was slower in PBS than TBA. Maximum absorbance, however, was constant from 220 to 300 s in the detection of cholesterol and triglycerides. Therefore, the tube length of 15 m (0.4 mm id), which corresponded to a reaction time of 162 s for the TSK column and 228 s for the Superose column, was sufficient and suitable to carry out the on-line enzymatic reactions.

### Precision

It was important to split the column effluent equally into two lines in order to achieve simultaneous dual detection. We controlled flow volumes of each branch line by adjusting a Micro-Splitter P-460 (Upchurch Scientific Inc.) and confirmed that each volume of the split lines was almost identical when the same triglyceride reagent system was pumped into each line as shown in **Fig. 3**.

Precision studies are summarized in **Table 1**. One frozen serum-based standard material (Kyowa Medex, Japan) with a total cholesterol concentration of 152 mg/dl and a total triglyceride concentration of 70 mg/dl was analyzed in four replicates per day for 5 days to assess the precision of the dual detection system equipped with two kinds of columns. The TSK column produced within-day imprecision values (coefficients of variation, CVs) of 1.1% for cholesterol and 1.7% for triglyceride measurement. Within-day imprecision CVs for elution time of chromatographic peaks were less than 0.27% for cholesterol and 0.21% for triglyceride detection. With the Superose column, CVs for within-day imprecision were 4.7% for cholesterol, 4.2% for triglyceride, and less than 0.29% for elution time of chromatographic peaks. CVs for between-day imprecision were 2.3% and 3.3% for cholesterol, 2.2% and 2.5% for triglyceride, and less than 0.32% and 1% for elution times

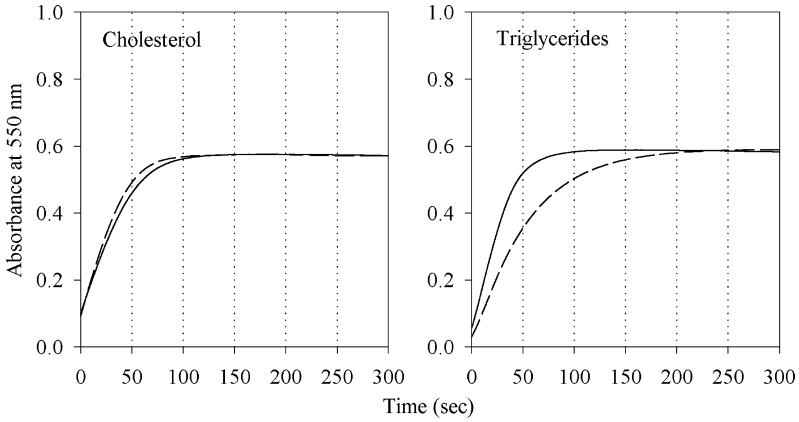


**Fig. 2.** Effects of running buffers on enzymatic reactions for cholesterol (left panel) and triglycerides (right panel). Enzymatic reagents were diluted to 2-fold with an equal amount of tris-buffered acetate (TBA) or PBS, and reacted with serum samples. Developed color was monitored at 550 nm for 5 min at 37°C. Solid line, TBA; Dashed line, PBS.

of chromatographic peaks with the TSK and Superose columns, respectively.

### Analytical run time

Approximately 37 min per sample was required for total elution from the TSK column and 50 min from the Superose column. However, because void volumes were not immediately eluted after sample injection, it was possible to load the next sample on columns before the previous sample was completely eluted. Within-day imprecision CVs described, obtained with injection intervals of 24 min for the TSK column and 35 min for the Superose column, were comparable to those obtained with longer times for intervals of 37 min for the TSK column (0.3% for total cholesterol and 1.5% for total triglycerides) and 50 min for the Superose column (2% for total cholesterol and 1.9% for total triglycerides). This overlapping sample injection technique dramatically decreased analytical time and enzyme reagent volumes without compromising analytical precision.

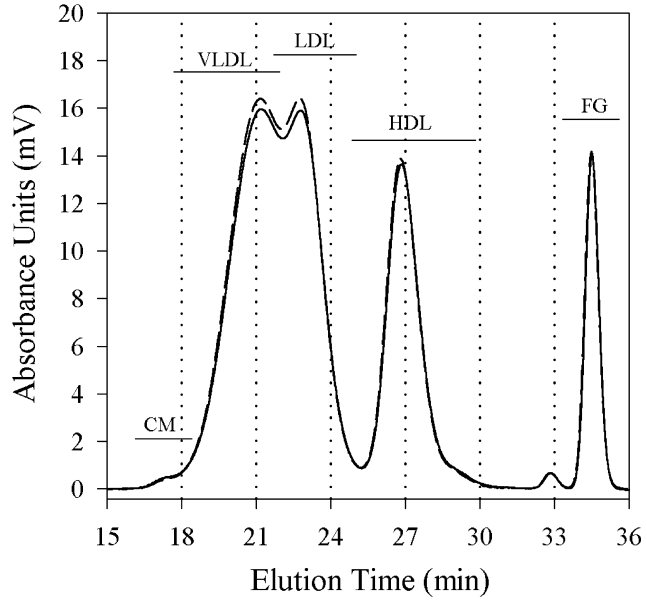


**Fig. 3.** Dual detection profiling by TSK columns with the same triglyceride reagent pumped into each branch line split by a Micro-Splitter. Solid and dashed lines are simultaneous signals produced by detectors 1 and 2 (as illustrated in Fig. 1), respectively.

### Comparison between total cholesterol and triglyceride values obtained from TSK and Superose columns

**Table 2** shows the results of total cholesterol and triglyceride concentrations and the ratios of triglycerides to cholesterol obtained by the dual detection system with TSK or Superose columns on serum samples from 60 healthy firemen. Total cholesterol values obtained by the TSK column were slightly but significantly higher than those obtained by the Superose column when judged by paired Student's *t*-test (213.8 ± 38.8 mg/dl vs. 212.6 ± 37.8 mg/dl,

TABLE 1. Summary of analytical imprecision of a dual detection HPLC system*

| | TSLgel LipopropakXL | | Superose 6HR | |
|---|---|---|---|---|
| | Within-day, CV% | Between-day, CV% | Within-day, CV% | Between-day, CV% |
| Total concentration | | | | |
| Cholesterol | 1.1 | 1.2 | 2.0 | 2.8 |
| Triglycerides | 1.7 | 2.0 | 1.6 | 0.9 |
| VLDL concentration | | | | |
| Cholesterol | ND | ND | 4.7 | 2.3 |
| Triglycerides | ND | ND | 4.2 | 2.5 |
| LDL concentration | | | | |
| Cholesterol | 0.6** | 2.3** | 4.1 | 3.3 |
| Triglycerides | 0.7** | 2.1** | 1.8 | 1.3 |
| HDL concentration | | | | |
| Cholesterol | 0.6 | 1.3 | 1.1 | 1.4 |
| Triglycerides | 1.3 | 2.2 | 2.2 | 1.9 |
| VLDL elution time | | | | |
| Cholesterol | ND | ND | 0.29 | 1.00 |
| Triglycerides | ND | ND | 0.28 | 0.91 |
| LDL elution time | | | | |
| Cholesterol | 0.27 | 0.32 | 0.22 | 0.75 |
| Triglycerides | 0.21 | 0.31 | 0.24 | 0.74 |
| HDL elution time | | | | |
| Cholesterol | 0.16 | 0.10 | 0.18 | 0.62 |
| Triglycerides | 0.16 | 0.13 | 0.19 | 0.62 |

* The results were obtained from a dual HPLC system with TSKgel LipopropakXL or Superose 6HR columns, analyzing a serum-based frozen standard material (Kyowa Medex) in four replicates for 5 days. The standard material had a total cholesterol concentration of 152 mg/dl and a triglyceride concentration of 70 mg/dl. ND means no peak was detected.

** LDL peaks included VLDL because of no detection of VLDL peaks.

TABLE 2. Comparison between total cholesterol and triglyceride values obtained from TSKgel LipopropakXL or Superose 6HR columns, analyzing serum samples from 60 healthy firemen (mean ± SD)

| | TSLgel LipopropakXL | Superose 6HR | *P** |
|---|---|---|---|
| Total Cholesterol (mg/dl) | 213.8 ± 38.8 | 212.6 ± 37.8 | 0.018 |
| Total Triglycerides (mg/dl) | 136.7 ± 67.3 | 136.3 ± 73.1 | 0.761 |
| Triglyceride/Cholesterol Ratio (mg/mg) | 0.633 ± 0.274 | 0.632 ± 0.303 | 0.899 |

* Judged by paired Student's *t*-test.

n = 60, $P = 0.018$). On the other hand, the TSK and Superose columns produced similar values for total triglycerides and the ratios of triglycerides to cholesterol. To further examine the agreement among the values obtained by different columns with the same dual detection system, we calculated linear regression equations by the least-squares method as shown in **Fig. 4**. The results from the Superose column (y) correlated well with those from TSK column (x): y = 0.969x + 5.44 ($r = 0.990$, n = 60) for total cholesterol (mg/dl), y = 1.08x − 11.14 ($r = 0.985$, n = 60) for total triglycerides (mg/dl), and y = 1.093x − 0.06 ($r = 0.978$, n = 60) for the ratios of triglycerides to cholesterol (mg/mg).

### Peak identification

A whole serum sample from a healthy subject analyzed on the dual detection HPLC system with two connected TSK columns gave two separated peaks in the cholesterol profile and three peaks in the triglyceride profile (**Fig. 5**, left panel). Comparison with ultracentrifugal lipoprotein fractions revealed that the first peak eluted at about 20–25 min contained VLDL, IDL, and LDL and the second peak at 25–30 min contained $HDL_2$ and $HDL_3$. The third peak eluted at 34–35 min on the triglyceride profile was identified as endogenous free glycerol (FG), because pure glycerol solution was detected only on the triglyceride profile at the same position (data not shown). Cholesterol profiles of the ultracentrifugal fractions had one additional peak at 33–34 min (indicated as arrows in Fig. 5), which was not present on the whole serum profile. This peak was found at the same position even with a concentrated salt solution without lipoproteins, indicating that the peak eluting after $HDL_3$ was an artifact from the salt used for density adjustment. The peak eluting at 20–23 min in the $HDL_2$ fraction was consistent with lipoprotein[a] (Lp[a]), because most of the Lp[a] has a density between 1.050 and 1.100 kg/l (20), and is enriched in cholesterol similar to LDL but is larger than LDL. The subject used here had an Lp[a] value of 16 mg/dl, determined by an immunoturbidimetric method.

On the other hand, the Superose column gave three and four distinct peaks for the cholesterol and triglyceride profiles, respectively, of the whole serum sample on the same dual detection HPLC system (Fig. 5, right panel). The first peak eluting at 17–20 min contained almost all of the VLDL and the second peak at 20–28 min contained all of the IDL and LDL and the rest of the VLDL. Both $HDL_2$ and $HDL_3$ fractions were contained in the third

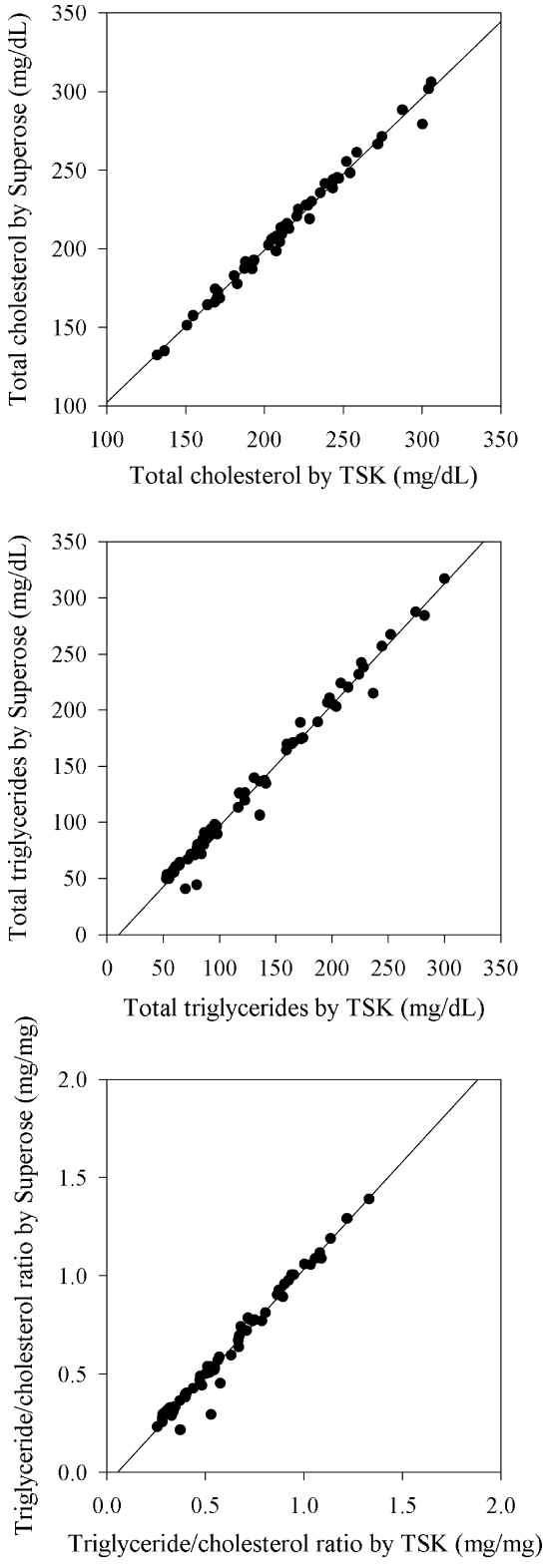


**Fig. 4.** Comparison between TSKgel LipopropakXL column and Superose 6HR column for determination of total cholesterol (top panel), triglyceride (middle panel), and the triglycerides to cholesterol ratio (bottom panel) on the dual detection system, analyzing serum samples from 60 healthy firemen. The results from the Superose column are plotted as a function of those of TSK column. Regression line is presented in each panel (n = 60): top panel, y = 0.969x + 5.44 ($r = 0.990$); middle panel, y = 1.08x − 11.14 ($r = 0.985$); bottom panel, y = 1.093x − 0.06 ($r = 0.978$).

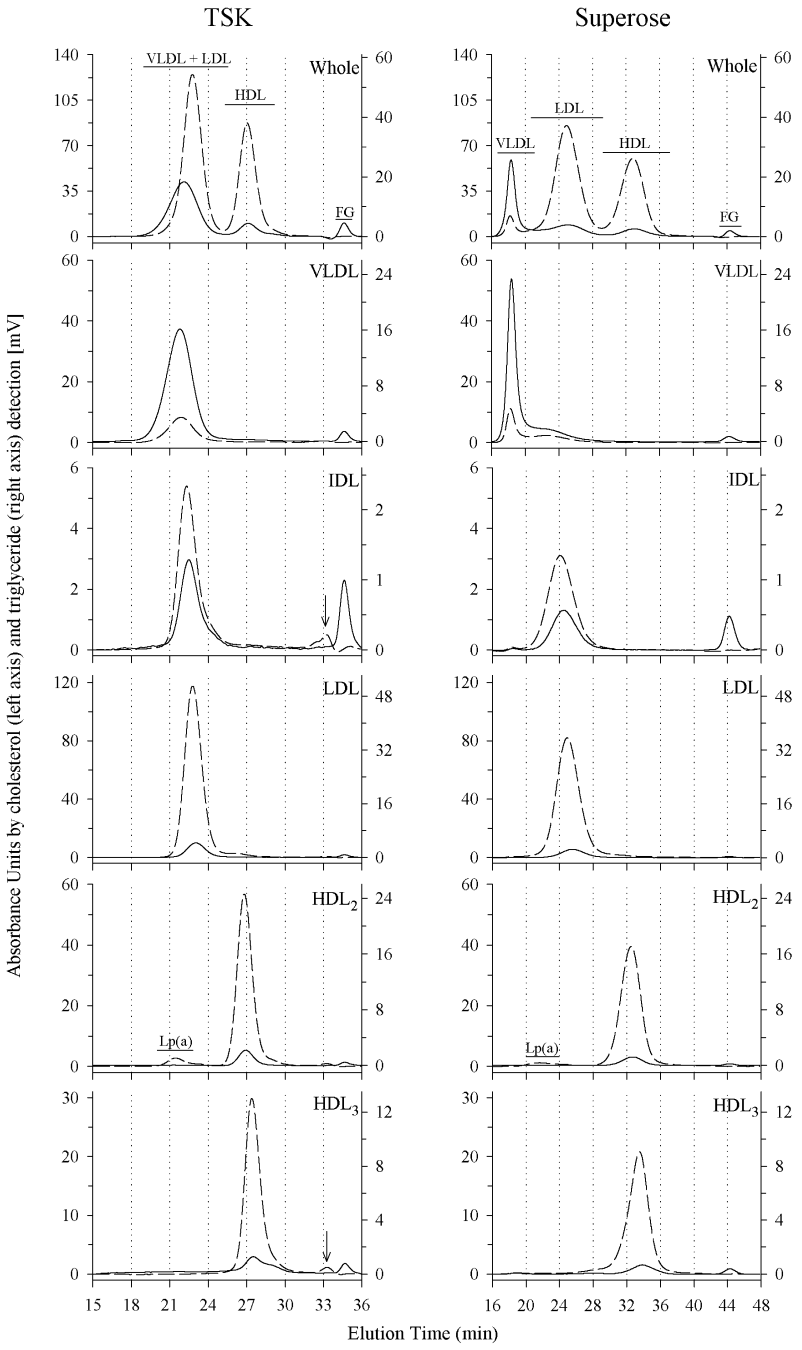


**Fig. 5.** Representative chromatographic patterns by the dual detection of cholesterol (dashed line) and triglycerides (solid line) of whole serum and its ultracentrifugal fractions from a healthy subject with a total cholesterol concentration of 132.9 mg/dl and a total triglyceride concentration of 63.9 mg/dl. Ten microliters of whole serum and ultracentrifugal fractions were injected into two tandem connected TSK columns or a single Superose column. The results from TSK and Superose columns are presented in left and right panels, respectively. Axis scales are constant (cholesterol axis-triglyceride axis = 2.3:1) to visualize the cholestrol-triglyceride ratio (mg/mg). Arrows indicate a background of salt for density adjustment. VLDL, density <1.006 kg/l; IDL, density from 1.006 to 1.019 kg/l; LDL, density from 1.019 to 1.063 kg/l; $HDL_2$, density from 1.063 to 1.125 kg/l; $HDL_3$, density >1.125 kg/l.

peak eluting at 28–37 min. The fourth peak eluting at around 44 min, observed only on the triglyceride profile, was identified as FG for the same reasons described above. The $HDL_2$ fraction had an Lp[a] peak eluting at 20–24 min in the cholesterol profile.

Except for the 1.006 kg/l top fraction containing VLDL, other ultracentrifugal fractions were detected as a single peak in both cholesterol and triglyceride profiles by TSK and Superose columns. In the VLDL fraction, the Superose column produced two separated peaks at 17–20 min and 20–27 min, while the TSK column gave one asymmetric peak at 20–24 min. This finding suggests that the Superose column could separate a 1.006 kg/l top fraction into triglyceride-rich large VLDL and cholesterol-rich small VLDL subfractions, although the small VLDL peak could not be detected in the whole serum.

### Application in human subjects with lipid abnormalities and animal models

The proposed dual detection HPLC system was used to determine cholesterol and triglyceride profiles of a type I hyperlipidemic patient without LPL activity, who generally has a severely elevated CM concentration. Profiles are shown in **Fig. 6** (top panel). As expected, another distinct peak, not present in a healthy subject, was detected at 17–18 min in the whole serum by the TSK column but not by the Superose column. This peak eluted faster and with proportionately more triglycerides than the VLDL peak,

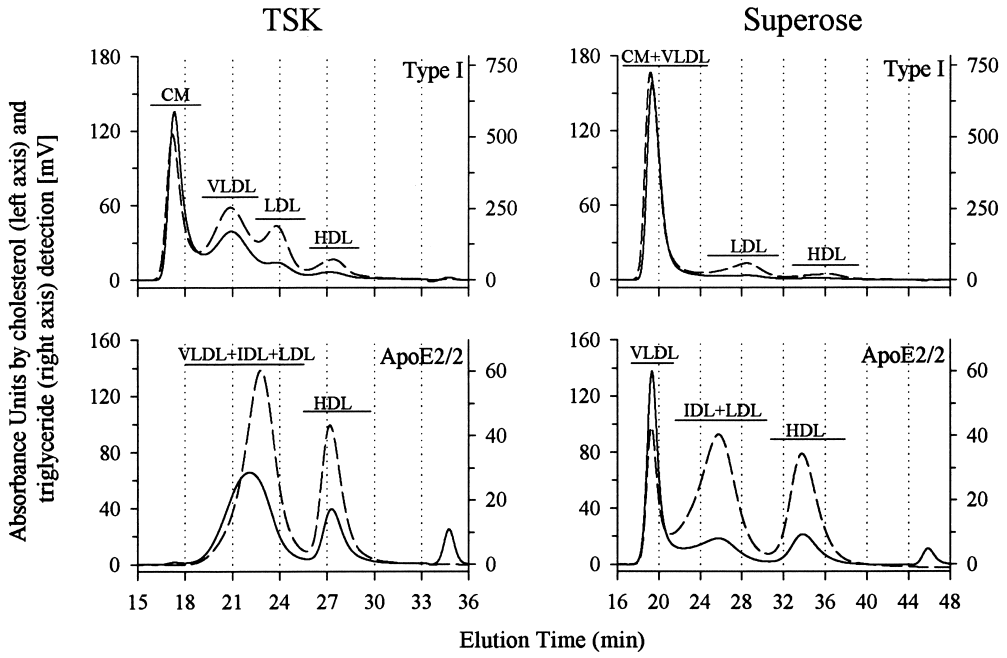


**Fig. 6.** Representative chromatographic patterns by the dual detection of cholesterol (dashed line) and triglycerides (solid line) of whole serum samples from type I hyperlipidemic (top panel) and apoE-2/2 (bottom panel) patients. The results from TSK and Superose columns are presented in left and right panels, respectively. Axis scales are constant (cholesterol axis-triglyceride axis = 2.3:1) to visualize the cholestrol-triglyceride ratio (mg/mg), except for the top panels. The type I hyperlipidemic patient had a total cholesterol concentration of 132.7 mg/dl and a total triglyceride concentration of 1227.9 mg/dl. The apoE-2/2 patient had a total cholesterol concentration of 193.5 mg/dl and a total triglyceride concentration of 147.3 mg/dl.

consistent with the presence of CM. The TSK column, therefore, was able to detect the CM peak in whole serum without prior ultracentrifugation. Other important observations were made, specifically that both chromatographic patterns produced by TSK and Superose columns showed all lipoprotein particles increased in the triglycerides to cholesterol ratio and the LDL peak eluted much later than a healthy subject, indicating the presence of small dense LDL (Fig. 6, top panel). Small dense LDL is often associated with hypertriglyceridemia and enriched in triglycerides relative to normal LDL (21). In addition, the VLDL peak of the type I hyperlipidemic patient was eluted faster by the TSK column than that of a healthy subject, indicating the presence of large VLDL particles. When large VLDL and small LDL coexisted in a sample, their peaks appeared clearly in the TSK profile. By contrast, VLDL particle sizes were impossible to determine using the Superose column, because VLDL eluted at the void volume.

Figure 6 (bottom panel) shows cholesterol and triglyceride profiles by the dual detection HPLC analysis of a whole serum sample from a patient with apoE-2/2. Most apoE-2/2 patients are either normolipidemic or hypocholesterolemic, but have characteristic elevated levels of β-migrating VLDL and/or IDL (22). The apoE-2/2 subject described here had a total cholesterol concentration of 193.5 mg/dl and a total triglyceride concentration of 147.3 mg/dl. Compared with a healthy subject as shown in Fig. 5, both TSK and Superose columns produced wider LDL peaks, indicating an elevation of IDL. Furthermore, the VLDL peak produced by the Superose column showed an increase in the absolute cholesterol level and an elevation in the ratio of cholesterol to triglycerides relative to a healthy subject. This result indicates the potential of the dual detection system to characterize abnormal lipoproteins qualitatively as well as quantitatively.

**Figure 7** shows cholesterol and triglyceride profiles of wild-type, CETP-expressed, and apoE-deficient mice. In the CETP-expressed mouse (Fig. 7, middle panel), a reduction of HDL-cholesterol (HDL-C) level and an elevation of HDL-triglyceride level were clearly observed on both chromatographic patterns obtained by the TSK and Superose columns. The reduction in the HDL-C level of the CETP-expressed mouse seemed to be associated with that of $HDL_2$-C level, because the HDL-peak was eluted slightly later than that of the wild-type mouse. As for the apoE-deficient mouse (Fig. 7, bottom panel), an elevation of cholesterol-rich VLDL was clearly found in both TSK and Superose profiles. The apoE-deficient mouse was hypercholesterolemic (824.6 mg/dl) but not accompanied by a significant increase of triglycerides (110.7 mg/dl), consistent with results previously reported (22).

## DISCUSSION

We report the development of an on-line dual detection HPLC system for simultaneously obtaining both cholesterol and triglyceride profiles of serum lipoproteins and describe

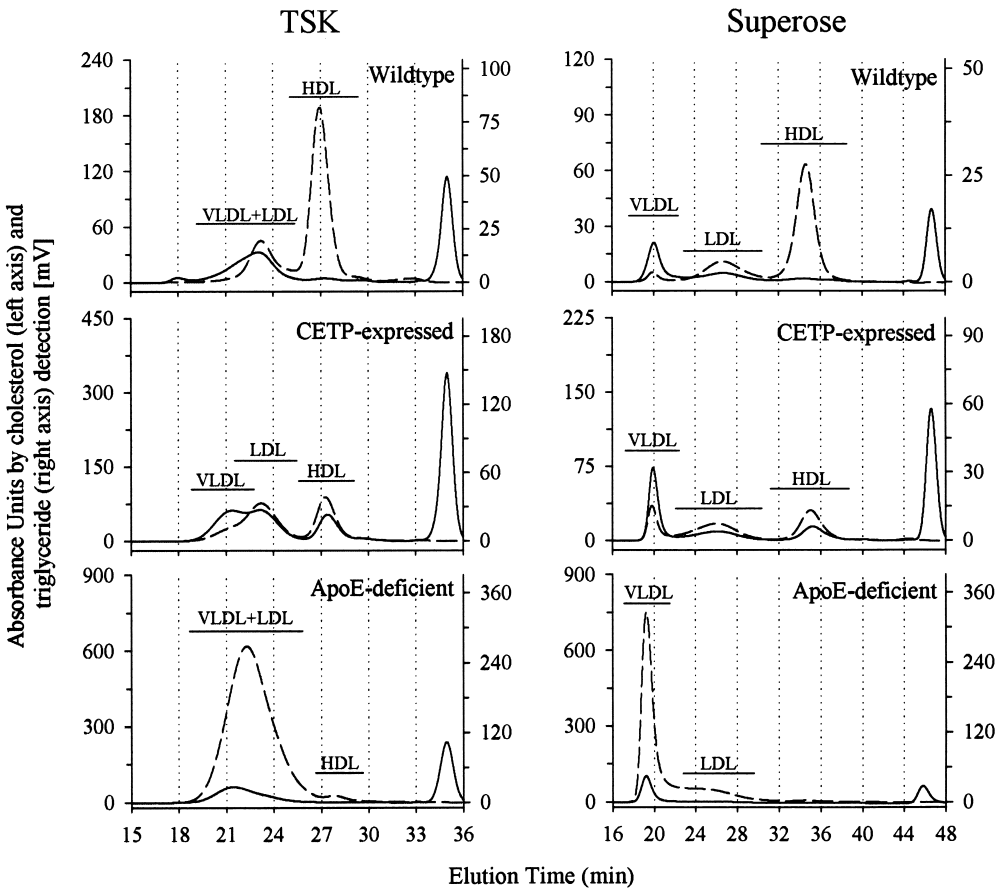


**Fig. 7.** Representative chromatographic patterns by the dual detection of cholesterol (dashed line) and triglycerides (solid line) of whole serum samples from wild-type (top panel), CETP-expressed (middle panel), and apoE-deficient (bottom panel) mice. The results from TSK and Superose columns are present in left and right panels, respectively. Axis scales are constant (cholesterol axis-triglyceride axis = 2.3:1) to visualize the cholestrol-triglyceride ratio (mg/mg). Serum samples from wild-type (total cholesterol, 116.3 mg/dl; total triglycerides, 46.4 mg/dl) and CETP-expressed (total cholesterol, 120.8 mg/dl; total triglycerides, 135.3 mg/dl) mice were diluted to 20-fold with saline, and 200 μl and 100 μl of the diluted sera were applied to TSK and Superose columns, respectively. A serum sample from an apoE-deficient mouse (total cholesterol, 824.6 mg/dl; total triglycerides, 110.7 mg/dl) was diluted to 10-fold with saline, and 100 μl and 50 μl of the diluted serum were applied to TSK and Superose columns, respectively.

analytical performance using two kinds of gel permeation columns, TSKgel LipopropakXL and Superose 6HR.

One of the advantages of the dual detection system is the reproducibility. In our precision study, both TSK and Superose columns produced good within-day imprecision with CVs less than 4.7% and 4.2% in the simultaneous detection of cholesterol and triglycerides, respectively. For between-day imprecision, CVs were less than 3.3% for cholesterol and 2.5% for triglycerides, indicating that the dual HPLC system provides reliable data with two types of columns. The imprecision is comparable to that reported previously for single detection systems (6, 8). In addition, elution time of each lipoprotein peak, which is an important indicator of the lipoprotein particle size, was highly reproducible in spite of the absence of an internal standard material. The endogenous FG peak, however, may be used as an internal standard for elution time, because it has a constant molecular weight and is present in most serum samples.

Total cholesterol and triglyceride values obtained with the Superose column were highly correlated and in good agreement with those of the TSK column. These results suggest that recovery of lipoproteins from the columns might be similar with further optimization of running buffers. The dual detection system could be adapted as well for analysis of other lipid constituents of lipoprotein, e.g., free cholesterol and phospholipids, using appropriate enzymatic reagents.

Another advantage is that the dual profiling system requires only small sample volumes. This method is suitable for obtaining lipid profiles of infants, children, the elderly, and experimental small animals from which large volume of serum are simply not practical. Injection of only a 10 μl sample will avoid overloading, prevent peaks from broadening, and extend column life. The Superose column needed washing after injection of about 200 samples to reduce back-pressure, reobtain resolution, and correct peak tailing, but recovered almost com-

pletely to the initial condition after washing. The loss of resolution was signaled, not always but usually, by the peak tailing of FG, which was an important indicator of column deterioration that was not always apparent from the lipoprotein profiles. On the other hand, the TSK column maintained good performance up to about 5,000 samples (6).

We made it clear by the dual lipid profiles that there were several significant differences in the resolution and separation ability of serum lipoproteins based on column types. The main differences were found in separation of CM, VLDL, and LDL (plus IDL), as shown in Figs. 5–7. The Superose column excluded most VLDL (plus CM) with elution in the void volume and separated LDL (plus IDL and a part of VLDL) and HDL from whole serum for both cholesterol and triglyceride profiles. When the Superose column was used to examine ultracentrifugal fractions, only a 1.006 kg/l top fraction was further resolved into two subfractions (large VLDL and small VLDL), and each of the other lipoprotein fractions was eluted as a single peak on both cholesterol and triglyceride profiles. The two subfractions in a 1.006 kg/l top fraction were apparently different in the ratio of triglycerides to cholesterol, demonstrating that the dual detection was more useful and advantageous to qualitative analysis than a single detection system. In addition, cholesterol-rich VLDL, which cannot be demonstrated by a single lipid profile, was also found in an apoE-2/2 subject by the dual lipid profiling. By contrast, the TSK column excluded CM to the void volume using a whole serum sample from a patient with type I hyperlipidemia. However, VLDL was apparently overlapping and eluted together with IDL and LDL on both cholesterol and triglyceride profiles even in a healthy subject as shown in Fig. 5. Because increased CM concentrations occur generally with non-fasting status and type I and type V hyperlipidimia, the TSK column will be more useful for evaluation of lipid profiles in lipoprotein metabolism studies than in a clinical setting.

In conclusion, the on-line dual enzymatic detection technique was confirmed to be highly reproducible on the HPLC system and decreased the number of analytical runs and tests needed to fully characterize samples. We demonstrated that the dual detection of cholesterol and triglycerides provides reliable, qualitative, and quantitative information on serum lipoproteins, allowing the determination of abnormal lipoprotein profiles. Furthermore, the dual lipid profiles revealed several important differences in the resolution ability of the columns. Exclusion of CM from a whole serum sample by the TSK column will be especially useful because the need for additional experimental procedures is avoided. The Superose column may be effective for analysis of whole serum samples not likely to have CMs. In summary, this dual detection HPLC system will facilitate convenient characterization of lipoproteins and contribute to a better understanding of lipoprotein metabolism with appropriate selection of gel permeation columns and detection enzymes depending on the particular study objectives.

The authors thank Dr. Hideki Asakawa at Itami Municipal Hospital, Dr. Minoru Ohkubo at Toranomon Hospital, Dr. Nobuo Yamami at Tokyo Medical and Dental University, Dr. Shinji Yokoyama at Nagoya City University, and Dr. Tokuo Yamamoto at Tohoku University for their assistance with blood collections. We gratefully acknowledge Kyowa Medex, Japan, for providing enzymic cholesterol and triglyceride reagents, and Associate Professor Tetsuya Mizuno at Tokyo Medical and Dental University and Dr. G. Russel Warnick at Pacific Biometrics Research Foundation for useful discussion.

*Manuscript received 2 October 2001 and in revised form 30 January 2002.*

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