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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN SERUM LIPOPROTEINS

SELECTIVE DETECTION OF TRIGLYCERIDES BY ENZYMATIC REACTION

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SUMMARY

A simple and convenient method for detection and quantitation of triglycerides in each lipoprotein fraction (chylomicron, VLDL, LDL and HDL) has been developed by high-performance liquid chromatography followed by enzymatic reaction using a high-speed reaction type chromatograph.

Triglycerides in serum lipoproteins eluted from the gel permeation column (TSK GEL) could be sensitively and selectively detected by the absorbance at 550 nm using a commercial enzyme reagent kit.

The distribution of triglycerides in each lipoprotein fraction could be examined with a small amount of serum (10–50 μl) in less than 50 min by this method. Moreover, free glycerol could be detected as a sharp peak at the elution volume of total permeation of the column.

This technique was found to be suitable for the study of triglyceride-rich lipoproteins such as chylomicron and VLDL.

INTRODUCTION

Serum lipoproteins are usually defined according to their density: very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL_2 and HDL_3). However, it is well known that there is a high correlation between lipoprotein density and particle size, owing to their chemical composition and structure.

We have succeeded in applying high-performance liquid chromatography (HPLC) with gel permeation columns for serum lipoprotein analysis[1,2]. The direct quantitation method for cholesterol in each lipoprotein fraction from a small amount of serum (10–20 μl) was developed by combining two methods: separation by HPLC with gel permeation columns and selective detection of cholesterol by enzymatic

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reaction[3,4]. This technique can be applied to the selective detection of other lipid components, such as triglycerides and phospholipids, using an appropriate reagent. In fact, we have established the quantitation method of choline-containing phospholipids in serum lipoproteins using a commercial enzyme reagent kit[5].

In this paper, we describe a procedure of selective detection for triglycerides by a method combining HPLC and enzymatic reaction in post-column effluent. Elution patterns of triglyceride are examined for human sera from normal and pathological subjects. A few examples of monitoring a change of lipoprotein distribution are also reported. Evaluation of the gel permeation column for lipoprotein analysis and the effect of elongation of the column on the separation are also investigated.

EXPERIMENTAL

Apparatus

HPLC was carried out using the high-speed chemical derivatization chromatograph (HLC 805, Toyo Soda, Yamaguchi, Japan), as described previously[3,4] except that the enzymatic reaction was done by using a stainless-steel tube (20 m × 0.5 mm I.D.) in a thermostatted water bath (Thermo Mini TM-100, Tokyo Rikakikai, Tokyo Japan).

Ultracentrifugation for the separation of the standard lipoprotein fractions from human serum was carried out using an RP 55 rotor in an Hitachi 55P-2 ultracentrifuge. After centrifugation, lipoproteins in the top layer were collected with an Hitachi tube slicer (Model TSU2, Hitachi, Tokyo, Japan).

Materials and methods

Samples. Human sera used in this experiment were obtained from normolipidemia, hyperlipidemia and patients with various diseases after 12–16 h of fasting. Standard lipoprotein fractions for analysis by HPLC were prepared from the serum by the sequential flotation method of Havel *et al.*[6]. Ascitic chylomicron was prepared from abdominal ascites as the $d < 1.006$ fraction after centrifugation at 10.500 *g* for 24 h.

Standard proteins (thyroglobulin, γ-globulin, β-lactoglobulin, cytochrome *c* and albumin) dissolved in 0.15 *M* NaCl at a concentration of 0.1–1.0% were used to obtain the relationship between molecular weight and elution volume for the gel permeation column.

Reagents. The concentration of triglycerides in the samples subjected to HPLC or in the fraction separated by HPLC was enzymatically determined using a commercially available reagent kit (Determiner TG, Kyowa Medex, Tokyo, Japan). This reagent was obtained in premixed lyophilized vials together with buffer solution. When reconstituted with (per vial) 85 ml of 0.1 *M* Good buffer (pH 6.75) containing detergents and aldehyde trapping reagent, the individual components were present in the following concentrations: lipoprotein lipase, 1 unit/ml; glycerol oxidase, 33.4 units/ml; peroxidase, 10 units/ml; 4-amBin 4-aminoantipyrine, 0.5 m*M*; N-ethyl-N-(3-methylphenyl)-N′-acetylethylendiamine, 0.9 m*M*. The concentration of cholesterol in the loaded samples or in the fraction separated by HPLC was determined using a commercial enzyme reagent kit (Determiner TC"555", Kyowa Medex) as described previously[3,4].

Separation of lipoproteins by HPLC. The separation of lipoproteins was performed by HPLC with gel permeation columns (TSK GEL, G5000PW and G4000PW; Toyo Soda). Experimental conditions were as follows: column, G5000PW, G4000PW, G5000PW + G5000PW (each column, 600 × 7.5 mm I.D.); eluent, 0.15 *M* NaCl; flow-rate, 0.50 ml/min.

Detection of triglycerides in flow diagram. Triglycerides were detected by measuring the A_{550} of the post-column effluent, described previously for cholesterol quantitation[3,4]. The A_{550} of the mixed eluate and enzyme solution (Determiner TG kit) was monitored after passage through a stainless steel reaction tube (20 m × 0.5 mm I.D.) at 45°C using the high-speed chemical derivatization chromatograph. The flow-rate of the enzyme solution (Determiner TG) was 0.40 ml/min.

RESULTS AND DISCUSSION

Recently, highly sensitive and simple quantitation methods for serum triglycerides have been developed using various enzymatic reaction systems[7–10], and the determination of triglycerides can be precisely and reproducibly performed with a very small amount of serum (20 μl) in an aqueous system.

For the detection of triglycerides in the eluate from the gel permeation column, we used a commercial enzyme reagent kit (Determiner TG), which had been developed using a new enzyme, glycerol oxidase[11,12]. The enzymatic reaction schemes for the selective detection of triglycerides are as follows:

$$\text{triglycerides} + H_2O \xrightarrow[\text{lipoprotein lipase}]{} \text{free glycerol} + \text{fatty acid}$$

$$\text{free glycerol} + O_2 \xrightarrow[\text{glycerol oxidase}]{} \text{glyceraldehyde} + H_2O_2$$

$$\text{4-aminoantipyrine} + \text{EMAE} + 2H_2O_2 \xrightarrow[\text{peroxidase}]{} \text{quinone diimine dye} + 4H_2O$$

where EMAE is N-ethyl-N-(3-methylphenyl)-N′-acetylethylendiamine.

Triglycerides can be measured by the A_{550} of the quinonediimine dye (λ_{max} = 555 nm) which is produced by the above reaction schemes using this reagent kit. The end-point of this reaction can be obtained within 5 min after incubation at 37°C in a test-tube. We successfully applied this reagent kit for the detection of triglycerides in the flow diagram by HPLC using the following experimental conditions: temperature of the reaction bath, 45°C; dimensions of the reactor, 20 m × 0.5 mm I.D.; flow-rate of the main path (*i.e.* the pathway of the eluate from the column), 0.50 ml/min; flow-rate of the enzyme solution (Determiner TG), 0.40 ml/min. The A_{550} of the mixed eluate and enzyme solution was monitored after passage through the reactor.

The elution patterns monitored by the A_{550} using the G5000PW column are

shown in Fig. 1 for the standard lipoprotein fractions (chylomicron, VLDL, LDL, HDL_2 and HDL_3) which were prepared by the sequential flotation methods[6]. The elution profile of free glycerol is also presented in the same figure (Fig. 1j). Free glycerol in the applied samples to HPLC was detected as a sharp peak at the elution volume of total permeation of the column.

The relationship of the molecular weights of proteins and lipoproteins to elution volumes was investigated by monitoring the A_{280} using the standard proteins and lipoproteins. The results for the G5000PW column are shown in Fig. 2. All the plots for proteins and lipoproteins in the molecular weight range from 10^4 to 10^6 are almost on the straight line.

Fig. 3 presents typical elution patterns monitored by the A_{550} for various amounts of human serum applied to the HPLC apparatus. Sample is the whole serum from the patient with primary biliary cirrhosis, which contains 173 mg/dl of triglycerides. A very small amount of serum (5 μl) gave five clearly separated peaks: chylo-

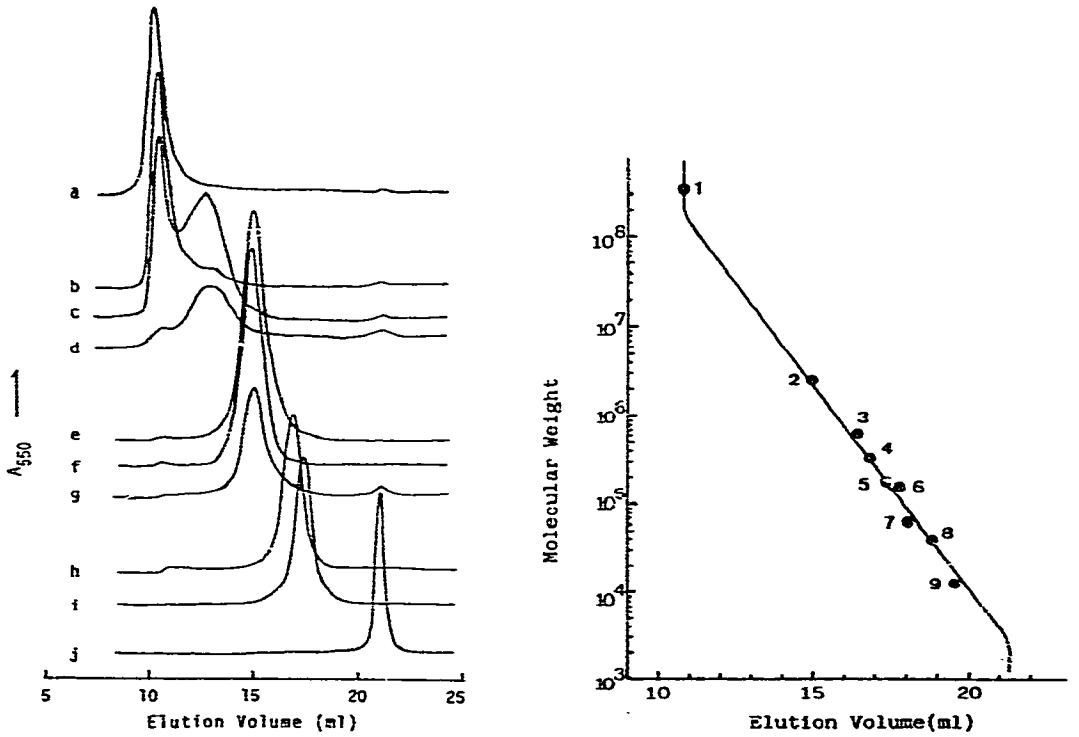

Fig. 1. Elution patterns of the A_{550} for standard samples. Column, G5000PW (600 × 7.5 mm I.D.); eluent, 0.15 *M* NaCl, flow-rate, 0.50 ml/min (main path), 0.40 ml/min (enzyme solution, Determiner TG kit); temperature of the reactor (20 m × 0.5 mm I.D., stainless steel tube), 45°C. Samples: a, ascitic chylomicron; b–d, chylomicron + VLDL fraction (d < 1.006); e–g, LDL (d 1.006–1.063); h, HDL_2 (d 1.063–1.125); i, HDL_3 (d 1.125–1.210); j, free glycerol.

Fig. 2. Relationship between molecular weight and elution volume for G5000PW column. Column, G5000PW (600 × 7.5 mm I.D.); eluent, 0.15 *M* NaCl; flow-rate, 0.50 ml/min; detector, A_{280}. Samples: 1, ascitic chylomicron; 2, LDL; 3, thyroglobulin (bovine); 4, HDL_2; 5, HDL_3; 6, γ-globulin (human); 7, albumin (human); 8, β-lactoglobulin (bovine); 9, cytochrome *c*.

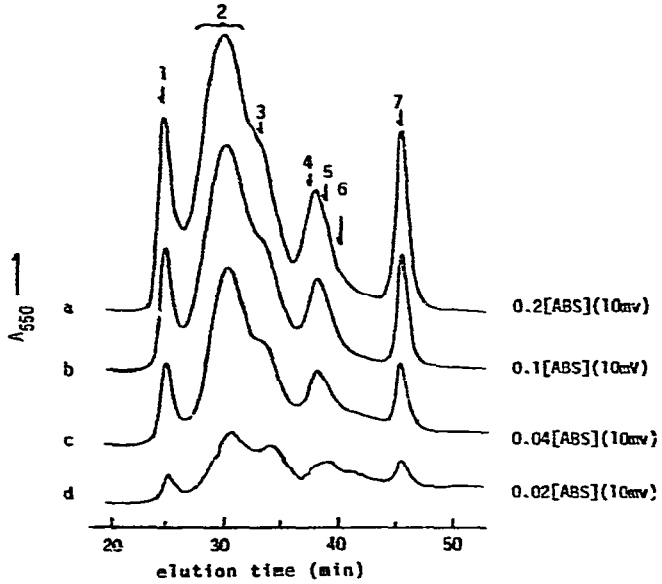

Fig. 3. Elution curves monitored by the A_{550} for various amounts of human serum applied to HPLC. Sample: serum of patient with primary biliary cirrhosis (173 mg/dl of triglycerides). Loaded volume: a, 40 μl; b, 20 μl; c, 10 μl; d, 5 μl. Elution position: 1 = chylomicron; 2 = VLDL; 3 = LDL; 4 = HDL_2; 5 = HDL_3; 6 = albumin; 7 = free glycerol. HPLC conditions as in Fig. 1.

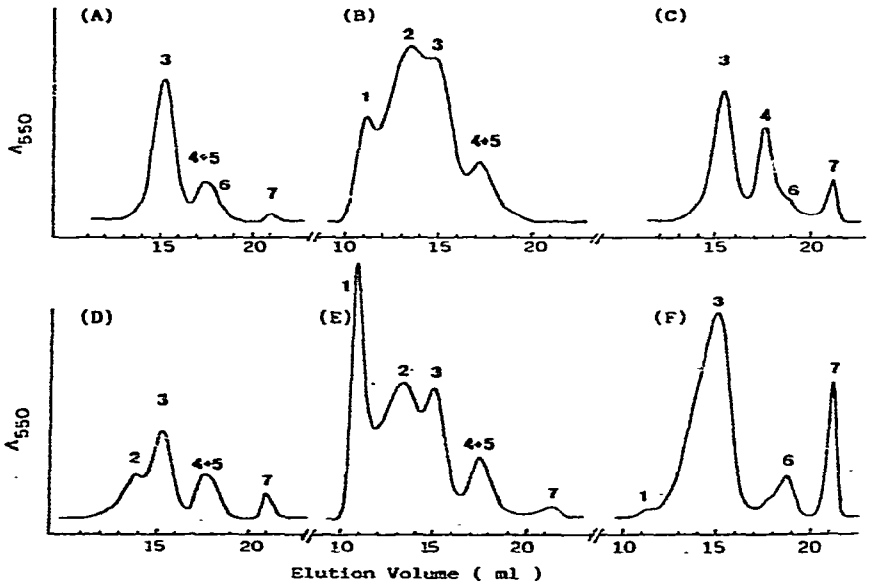

Fig. 4. Elution patterns of the A_{550} for various human sera. Sample: A, normal male; B, hyperlipidemia; C, liver cirrhosis; D, normal child; E, fatty child; F, baby one day after birth. Loaded volume: 10–50 μl. Peaks: 1 = chylomicron; 2 = VLDL; 3 = LDL; 4 = HDL_2; 5 = HDL_3; 6 = bilirubin; 7 = free glycerol. HPLC conditions as in Fig. 1.

micron, VLDL, LDL, HDL and free glycerol. The arrow in Fig. 3 indicates the elution position of each lipoprotein fraction, serum albumin and free glycerol. The elution positions were determined from the results shown in Fig. 1 and Fig. 2. These fractions are designated by the numbers: 1, chylomicron; 2, VLDL; 3, LDL; 4, HDL_2; 5, HDL_3; 6, albumin; 7, free glycerol. As shown in Fig. 3, the G5000PW column was found to be useful for the separation of the large-particle-size fractions of serum lipoproteins such as chylomicron, VLDL and LDL. On the other hand, small-particle-size fractions, such as HDL_2 and HDL_3, eluted as one peak.

The detection limit for triglycerides by this method was found to be 1 μg per one separated peak (see Fig. 3d). This indicates that *ca.* 50 μg of triglycerides per millilitre of serum can be detected when 20 μl of serum is analysed. Although the peak respence of the A_{550} increased slightly with increased serum volume, the elution patterns obtained for serum volumes from 10 to 40 μl were very similar. This shows that the quantitation of triglycerides in each lipoprotein and that of free glycerol can be performed from the peak area of the A_{550} and the concentration of triglycerides in applied samples with use of 10–40 μl of whole serum.

In Fig. 4, typical elution patterns monitored by the A_{550} are presented for six examples of human sera: A, normal male; B, hyperlipidemia; C, liver cirrhosis; D, normal child; E, fatty child; F, baby one day after birth. Six distinct peaks of the A_{550} were observed. They were identified from the results shown in Fig. 1 and Fig. 2 as follows: 1, chylomicron; 2, VLDL; 3, LDL; 4, HDL_2; 5, HDL_3; 6, pigments such as bilirubin which adsorb to serum albumin; 7, free glycerol. Among these peaks, the elution position of VLDL (peak 2) varied with the individual subjects. This result is consistent with the fact that the VLDL fraction has a wide distribution of particle size and has many subclasses[13–17]. The hyperlipidemic sera (subjects B and E) present a larger amount of large-particle-size lipoprotein fractions, such as chylomicron and VLDL, than that of normolipidemic sera (subjects A, C and D). This method was found to give much information about large-particle-size lipoproteins that contained a high amount of triglycerides.

In the case of liver cirrhosis (subject C), a sharp peak of HDL_2 and a shoulder peak of bilirubin are observed. We have reported the decrease in serum HDL_3-cholesterol level in the case of liver cirrhosis using our HPLC method[18]. In the case of the one-day-old baby (subject F), which contains a high amount of bilirubin, a large peak is detected at the elution position of serum albumin (peak 6). These results indicate that this method can give qualitative data about small-particle-size fractions.

It is well known that there is an appropriate amount of free glycerol in human serum[19,20]. By our HPLC method, free glycerol can be detected as a sharp peak at the elution volume of total permeation as shown in Fig. 1. The content of free glycerol was found to vary with the individual subjects (see peak 7 of Fig. 4).

Fig. 5 shows two examples of the application of this method for monitoring a change of lipoprotein distribution. One is a change of elution patterns due to fasting time in the case of an obese woman (Fig. 5A). The other is the comparison of lipoprotein distribution between the fresh serum and the freezing–thawing serum for a triglyceride-rich subject (Fig. 5B) and a triglyceride-poor subject (Fig. 5C). The latter example indicates that large-particle-size fractions, such as chylomicron and VLDL, increase on freezing–thawing. On the other hand, in the case of the subject containing a very low level of VLDL, no change was observed as shown in Fig. 5C.

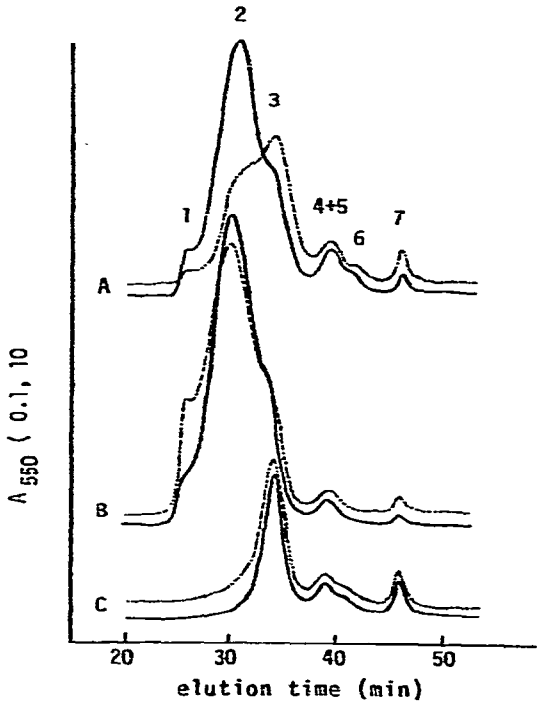

Fig. 5. Elution patterns of the A_{550} for human sera: examples of monitoring pattern change. Samples: A, serum of obese woman, ——— (6 h of fasting), ——— (16 h of fasting); B, hyperlipidemia, ——— (fresh serum), ——— (freezing–thawing serum); C, normolipidemia, ——— (fresh serum), ——— (freezing–thawing serum). Loaded volume: A, 30 μl; B, 10 μl; C, 30 μl. Peaks as in Fig. 4. HPLC conditions as in Fig. 1.

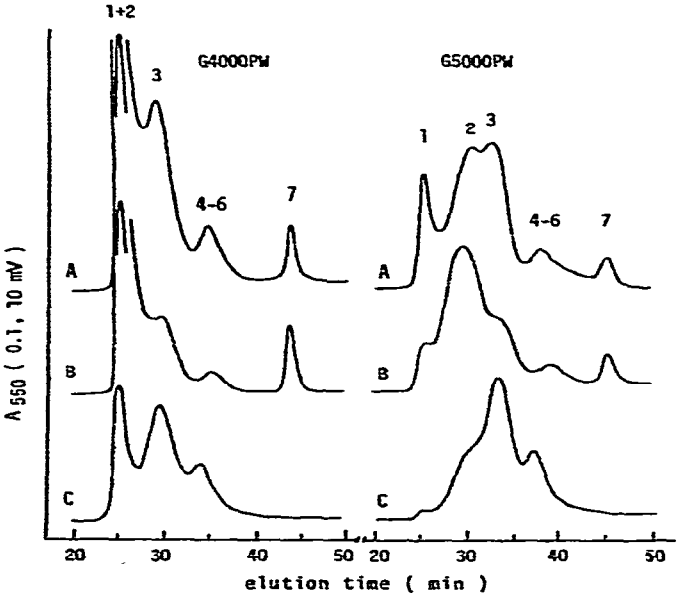

Fig. 6. Comparison of elution pattern of the A_{550} between G4000PW and G5000PW columns. Samples: human serum (A, acute liver hepatitis; B and C, hyperlipidemia). Loaded volume: A, 30 μl; B, 10 μl; C, 30 μl. Peaks as in Fig. 4. HPLC conditions as in Fig. 1.

Thus our results are consistent with the fact that evaluation of lipoproteins of frozen serum by electrophoretic procedures is possible only for the subject of a very low level of VLDL[21,22].

Elution patterns of the A_{550} for the same subject were compared on the G4000PW and G5000PW columns (see Fig. 6). In the case of G4000PW, chylomicron and VLDL elute as one peak at the void volume, and LDL is completely separated from VLDL. Therefore, the G4000PW column is preferable for evaluating the level of LDL.

Lastly, the effect of elongation of the column on the separation was examined using a single column of G5000PW and a combined column of G5000PW + G5000PW. Elongation of the column was found to increase the resolution of the separation for all lipoprotein fractions, as shown in Fig. 7. Detailed examination of subclasses of the VLDL fraction can be performed by using the G5000PW + G5000PW system (see peak 2 of Fig. 7A). The elution patterns of cholesterol monitored by the A_{550} using Determiner TC "555" are also presented in the same Figure for reference. From these experiments, it can be seen that the combination of the elution patterns of triglycerides and cholesterol give much information on the nature of serum lipoproteins in normal and pathological subjects.

The establishment of the selective detection method for triglycerides in serum lipoproteins by HPLC should help to make progress rapid in the study of lipoprotein metabolism and diagnosis of the various diseases. We are now examining the pattern analysis of the Fredrickson *et al.* typing system for familial hyperlipidemia[23] using our HPLC method.

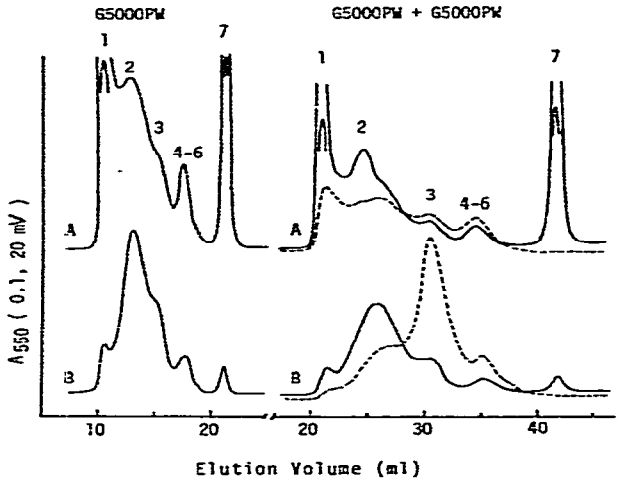

Fig. 7. Elution patterns of triglycerides (———) and cholesterol (———) for human serum. Columns, G5000PW, G5000PW + G5000PW (each column, 600 × 7.5 mm I.D.); sample, human serum of hyperlipidemia; loaded volumes, 20 μl HPLC conditions for triglyceride monitor as in Fig. 1. HPLC conditions for cholesterol monitor: flow-rate, 0.50 ml/min (main path) and 0.20 ml/min (enzyme solution, Determiner TC "555"); temperature of the reactor (20 m × 0.25 mm I.D., stainless steel tube), 40°C. Peaks as in Fig. 4.

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