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HIGH-PERFORMANCE AQUEOUS GEL PERMEATION CHROMATOGRAPHY OF SERUM LIPOPROTEINS: SELECTIVE DETECTION OF CHOLESTEROL BY ENZYMATIC REACTION

MITSUYO OKAZAKI, KEIKO SHIRAISHI, YOSHIMI OHNO* and ICHIRO HARA*

Laboratory of Chemistry, Department of General Education, Tokyo Medical and Dental University, Kohnodai, Ichikawa-shi, Chiba Prefecture 272 (Japan)

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SUMMARY

A rapid method for the quantitation of cholesterol in each lipoprotein fraction has been developed which utilizes high-performance aqueous gel permeation chromatography followed by enzymatic reaction using reaction-type high-performance chromatography.

Cholesterol in serum lipoproteins eluted from the column could be sensitively and selectively detected by the absorbance at 550 nm following the enzymatic reaction. The sensitivity of the detection for cholesterol measured by A_{550} was compared with that for protein measured by A_{280} using the standard lipoprotein fractions: low-density lipoprotein (LDL) and high-density lipoproteins (HDL_2 and HDL_3). The effects of changing the flow-rate and lengthening the column on the resolution of LDL and HDL were examined. Analyses of serum protein and cholesterol were performed with this method for human and animal subjects.

INTRODUCTION

A new application of high-performance gel permeation chromatography (GPC) for analysis of human serum lipoproteins has been developed using a combination of TSK GEL columns [1, 2]. In a previous paper [1] the best combination of the column for the separation of human serum lipoproteins and the effects of pH or salt concentration of the eluent on their separation were examined by monitoring the peaks of lipoproteins by A_{280} using the total lipoprotein mixture prepared by ultracentrifugation.

The protein levels in each lipoprotein fraction could be analyzed by monitoring A_{280} with the total lipoprotein fraction ($d < 1.210$) prepared from

*Present address: Sanyo Gakuen Junior College, Okayama, Okayama Prefecture, Japan 703.

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individual sera by ultracentrifugation [1]. On the other hand, the cholesterol in each lipoprotein fraction could be directly measured by the enzymatic reaction after separation by high-performance GPC with whole serum [3, 4]. The study of the optimum conditions for the enzymatic reaction given in the flow diagram (Fig. 1) using the reaction-type high speed liquid chromatograph (HLC 805, Toyo Soda Manufacturing Co., Tokyo, Japan) will be reported in another paper [5].

In this paper, the sensitivity of the detection for cholesterol by A_{550} was compared with that for protein by A_{280} with use of the standard lipoprotein fractions: low-density lipoprotein (LDL) and high-density lipoproteins (HDL_2 and HDL_3). The effects of changing the flow-rate and lengthening the column on the resolution of LDL and HDL subfractions were examined. The elution patterns of protein and cholesterol for individual subjects including human and animal sera were examined using this flow system.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography (HPLC) was carried out using the high-speed chemical derivatization chromatograph (HLC 805, Toyo Soda) equipped with a reactor (20 m × 0.25 mm I.D. stainless-steel tube) and detectors for A_{280} and A_{550} as shown in Fig. 1.

Ultracentrifugation for the separation of the standard lipoprotein fractions from human serum was performed using an RP55 rotor in an Hitachi 55 P-2 ultracentrifuge (Hitachi, Tokyo, Japan).

Materials and methods

Samples. Human sera used in this experiment were obtained from normal men and women or patients with various diseases after 12—16 h of fasting. Standard lipoprotein fractions for analysis by high-performance GPC were prepared from the sera by the sequential flotation method [6]. HDL_2 was isolated from serum as the d = 1.063—1.125 fraction. The d > 1.063 fraction obtained after centrifugation at 105,000 g for 24 h in the RP55 rotor was recentrifuged under the same conditions. After density adjustment to 1.125 with solid sodium bromide and 24 h of centrifugation at 105,000 g, the top fraction was recentrifuged at the same density. HDL_3 and LDL were prepared as the d = 1.125—1.210 and the d = 1.006—1.063 fractions, respectively, in the same way.

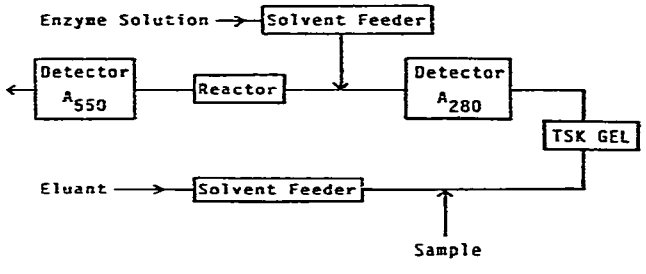

Fig. 1. Flow diagram for the enzymatic detection of cholesterol by high-performance GPC.

Reagents. The concentration of total cholesterol in the samples subjected to high-performance GPC or in the fraction separated by high-performance GPC was enzymatically determined using commercially available reagent kits (Determiner TC"555", Kyowa Hakko Co., Tokyo, Japan). The reagent was supplied in premixed lyophilized vials, together with buffer solution. When reconstituted with (per vial) 80 ml of 0.025 *M* potassium hydrogen phthalate buffer (pH 6.0) containing detergents the individual components were present in the following concentrations: cholesterol esterase, 1 unit/ml; cholesterol oxidase, 1.8 units/ml; peroxidase, 6.7 units/ml; 4-aminoantipyrine, 1 m*M*; N-ethyl-N-(3-methylphenyl)-N′-acetylethylenediamine, 0.3 m*M*. The reconstituted reagent has to be used within three days.

Separation of lipoproteins by high-performance GPC. The separation of lipoproteins was performed by HPLC with gel permeation columns (TSK GEL, G5000PW and G3000SW; Toyo Soda). Experimental conditions in this study were as follows. Columns: G5000PW + G3000SW (size of each column, 600 mm × 7.5 mm), G5000PW + G3000SW + G3000SW, G5000PW + G3000SW + G3000SW + G3000SW. Eluent, 0.15 *M* NaCl; flow-rate, 0.46—1.06 ml/min.

Detection of protein and cholesterol. The protein peaks were directly monitored by A_{280}. Cholesterol can be determined by measuring the A_{550} of the post-column effluent using the Determiner TC"555" kit after measurement of the A_{280}. The absorbance at 550 nm of the mixed eluate and enzyme solution (TC"555") was monitored after passage through the reactor (20 m × 0.25 mm I.D.) at 40°C using a high-speed chemical derivatization chromatograph (see Fig. 1).

RESULTS AND DISCUSSION

Recently, highly sensitive and selective methods have been developed for the quantification of cholesterol by the enzymatic reaction [7—10]. With these methods, the quantification of cholesterol in a very small amount of serum (10 μl) can be precisely performed in an aqueous system. In this experiment, the detection of cholesterol in the eluate from the column was achieved using the commercial kit (Determiner TC"555") with the following reaction schemes.

$$\text{cholesterol ester} + H_2O \xrightarrow[\text{cholesterol esterase}]{} \text{free cholesterol} + \text{fatty acid}$$

$$\text{free cholesterol} + O_2 \xrightarrow[\text{cholesterol oxidase}]{} \text{choleste-4-en-3-one} + H_2O_2$$

$$\text{4-aminoantipyrine} + \text{N-ethyl-N-(3-methylphenyl)-N′-acetylethylenediamine} + 2H_2O_2 \xrightarrow[\text{peroxidase}]{} \text{quinone diimine dye } (\lambda_{max.}\ 555\ \text{nm}) + 4H_2O$$

Using this reagent, cholesterol can be measured by the absorbance at 550 nm of the quinone diimine dye which is produced by the enzymatic reaction.

The absorbance at 550 nm of the mixed eluate and enzyme solution (TC"555") was determined after passage through the reactor at constant temperature. The optimum conditions for cholesterol measurement used in this experiment are as follows: temperature of the reactor, 40°C; dimensions of the reactor, 20 m × 0.25 mm I.D.; flow-rate of the main path (i.e. the pathway of the eluate from the column), 1.0 ml/min; flow-rate of the enzyme solution (TC"555"), 0.35 ml/min. Under these conditions, the concentration of the enzyme solution in the reaction solution and the reaction time were experimentally confirmed to be sufficient for completion of the reaction [5].

The elution profiles of cholesterol and protein are shown in Fig. 2—4 for the standard lipoprotein fractions (LDL, HDL_2 and HDL_3) which were prepared by the repeated ultracentrifugation described in Experimental.

The sensitivity of cholesterol detection by measuring A_{550} was compared with that for protein by A_{280} using the standard lipoprotein fractions, LDL, HDL_2 and HDL_3. The elution curves were monitored by A_{280} and A_{550} by applying various amounts of each lipoprotein fraction to the GPC apparatus. With LDL fraction, the sensitivity for cholesterol was fifteen times higher than that for protein, as shown in Fig. 2. For example, LDL fraction containing 2.6 μg of cholesterol could be detected by A_{550} but not by A_{280} (Fig. 2d). The detection limit for cholesterol was found to be 0.5 μg per one sepa-

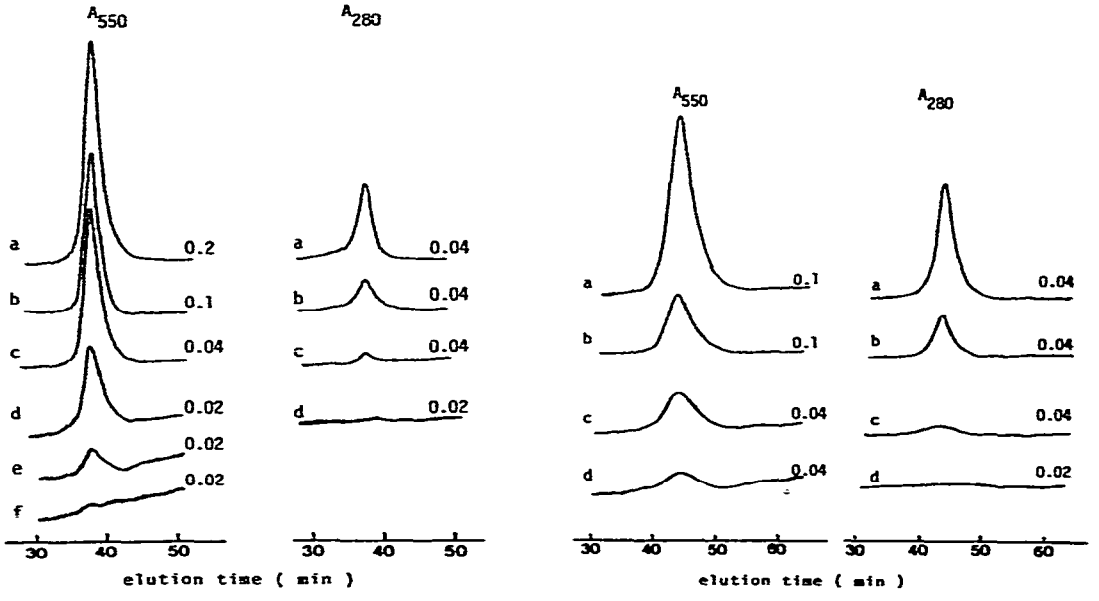

Fig. 2. Elution patterns of cholesterol (A_{550}) and protein (A_{280}) for LDL fraction. Column, G5000PW + G3000SW + G3000SW; eluent, 0.15 *M* NaCl; flow-rate, 1.0 ml/min (main path), 0.35 ml/min (enzyme solution); temperature of the reactor, 40°C; sample, LDL fraction (d = 1.006—1.063). Cholesterol contents of loaded samples: a, 27.0 μg; b, 10.8 μg; c, 4.9 μg; d, 2.6 μg; e, 1.0 μg; f, 0.5 μg. Numbers on the curves are the ranges of absorbance in the detector (10 mV).

Fig. 3. Elution patterns of cholesterol (A_{550}) and protein (A_{280}) for HDL_2 fraction. Sample, HDL_2 fraction (d = 1.063—1.125). Cholesterol contents of loaded samples: a, 23.5 μg; b, 9.0 μg; c, 2.5 μg; d, 1.5 μg. Conditions as in Fig. 2.

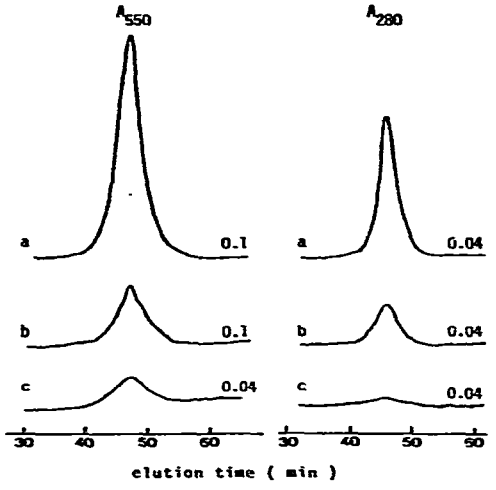

Fig. 4. Elution patterns of cholesterol (A_{550}) and protein (A_{280}) for HDL_3 fraction. Sample, HDL_3 fraction (d = 1.125—1.210). Cholesterol contents of loaded samples: a, 32.0 μg; b, 9.6 μg; c, 3.0 μg. Conditions as in Fig. 2.

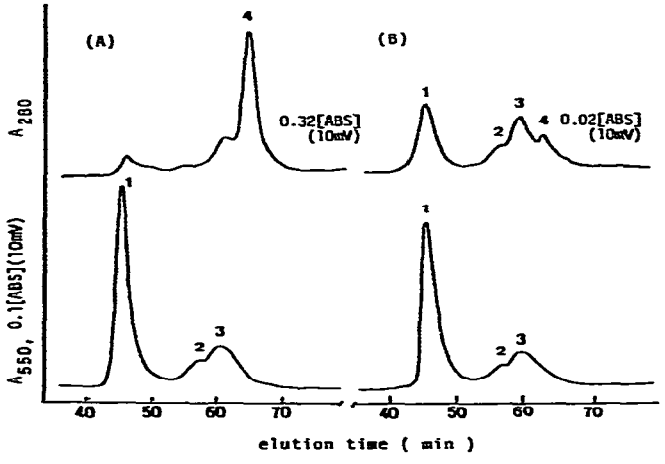

Fig. 5. Elution patterns of protein (A_{280}) and cholesterol (A_{550}) for human serum. Sample (normal male subjects): (A) 20 μl of whole serum; (B) 20 μl of total lipoprotein fraction (d < 1.210). Column: G5000PW + G3000SW + G3000SW + G3000SW. Peaks: 1, LDL; 2, HDL_2; 3, HDL_3; 4, albumin. Other conditions as in Fig. 2.

rated peak. Therefore, 25 μg of cholesterol per ml of serum can be detected by this method using 20 μl of serum for analysis. The respective sensitivities of detection for cholesterol were four times higher than that for protein in the case of HDL_2 (Fig. 3) and three times in the case of HDL_3 (Fig. 4). Therefore, even with HDL fractions, detection by A_{550} was superior to that by A_{280}.

The reproducibility of this method was ± 0.06 ml for the elution volume and ± 0.10 μg for cholesterol quantification [3].

The elution patterns of protein and cholesterol for whole serum and total lipoprotein fraction (d < 1.210) are shown in Fig. 5. In the case of the total

lipoprotein fraction (Fig. 5B), the peaks of lipoproteins can be monitored both by A_{280} and A_{550}. The elution curves of A_{280} for whole serum show that a large amount of serum proteins are eluted in the same fraction as the lipoproteins. Therefore, the protein level in each lipoprotein fraction can only be analyzed using the total lipoprotein fraction prepared from each individual serum by the ultracentrifugation as described in the previous paper [1]. On the other hand, the lipoprotein peaks can be monitored by the selective detection of cholesterol by A_{550} using whole serum. As presented in Fig. 5, the elution patterns of A_{550} with whole serum was not quite different from those with the total lipoprotein fraction. This suggests that the presence of serum proteins does not affect the separation of lipoproteins by GPC and the quantitation of cholesterol by the enzymatic reaction.

The effects of lengthening the column and reducing the flow-rate on the separation of lipoprotein by GPC were examined with use of whole serum (school children, female). Fig. 6 shows the elution patterns of protein and cholesterol by various combined column systems while keeping the flow-rate of the main path constant (1.0 ml/min). In the case of G5000PW + G3000SW, the LDL and HDL fractions could be separated but the HDL subfractions (HDL_2 and HDL_3) eluted as one peak. In the best working combined column system (G5000PW + G3000SW + G3000SW), the separation of LDL and HDL fractions was complete and the subfractions of HDL could be analyzed in less than 50 min. By adding one more G3000SW column to this system, the resolution of each lipoprotein fraction is improved, as shown in Fig. 6C.

It has been demonstrated in this study that the resolution of LDL, HDL_2 and HDL_3 can be improved by lengthening the G3000SW column. The effect of the flow-rate in the main path on the resolution of LDL and HDL_2 was examined for each column system using the same subjects as indicated in Fig. 6. The plots of the resolution values against the flow-rate are presented

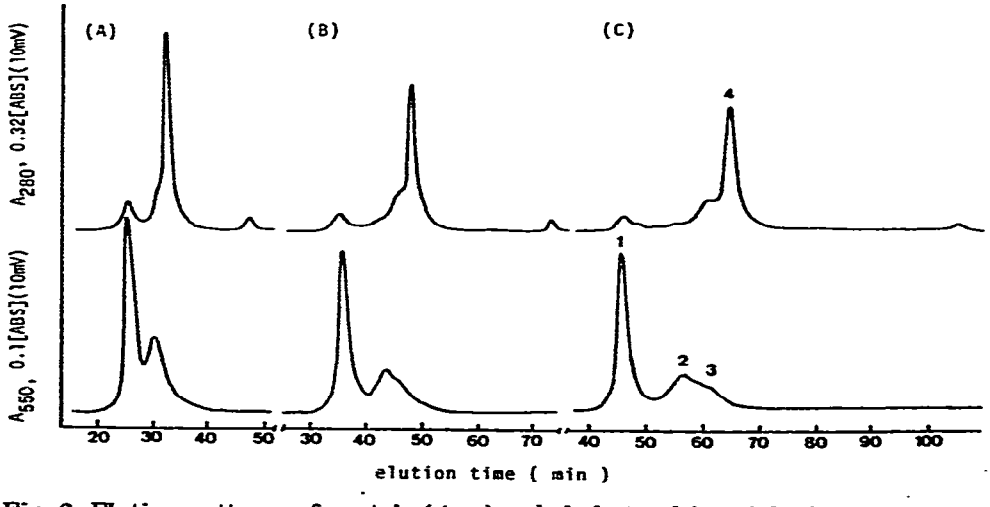

Fig. 6. Elution patterns of protein (A_{280}) and cholesterol (A_{550}) for human serum by various combined column systems. Sample: 20 μl of whole serum (school children, female). Columns: (A) G5000PW + G3000SW; (B) G5000PW + G3000SW + G3000SW; (C) G5000PW + G3000SW + G3000SW + G3000SW. Peaks as in Fig. 5. Other conditions as in Fig. 2.

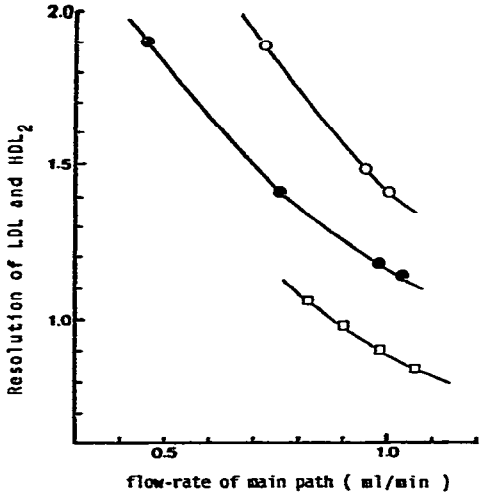

Fig. 7. Effects of flow-rate and lengthening of column on the resolution of LDL and HDL_2 fractions. Columns: (□), G5000PW + G3000SW; (●), G5000PW + G3000SW + G3000SW; (○), G5000PW + G3000SW + G3000SW + G3000SW. Sample as in Fig. 6. Other conditions as in Fig. 2.

in Fig. 7, from which it is clear that with the decrease in the flow-rate the resolution of LDL and HDL_2 increased significantly. Improvement in the separation can be achieved not only by lengthening the column but also by reducing the flow-rate of the main path. Until now the best conditions for analysis of lipoproteins, including the separation of the HDL subfractions, have been found to be: column system, G5000PW + G3000SW + G3000SW; flow-rate of main path, 1.0 ml/min.

Analyses of lipoproteins in individual human and animal sera were performed using the flow system (Fig. 1) under the optimum experimental conditions described above. In Fig. 8 typical elution patterns of protein and cholesterol are presented for three examples of human sera: a normal subject (young female, A) and two pathological cases (liver cirrhosis, B; and hyperlipidemia, C). Six distinct peaks of A_{550} are observed. Using standard lipoprotein fractions they were identified as: peak 1, VLDL; peak 3, LDL; peak 4, HDL_2; peak 5, HDL_3. Peak 2, observed between VLDL and LDL, and peak 6, seen after HDL_3, are assumed to be those of IDL (intermediate-density lipoprotein) and VHDL (very-high-density lipoprotein), respectively. Many serum protein peaks were observed. Two of them were identified as: peak 7, γ-globulin; peak 8, albumin. The levels of both serum protein and serum lipoprotein were found to vary with individual subjects. The pathological subjects in particular presented quite different patterns from those of normal subjects. In the case of liver cirrhosis, total lipoprotein levels were low, HDL fractions were mainly composed of HDL_2, the level of γ-globulin increased and that of albumin decreased compared with those of normal subjects. The hyperlipidemia serum (Fig. 8C) had a large amount of the high molecular weight lipoprotein fraction between VLDL and LDL.

Fig. 9 shows the elution patterns shown by animal sera under the same experimental conditions as in Fig. 8. Although the characteristic patterns

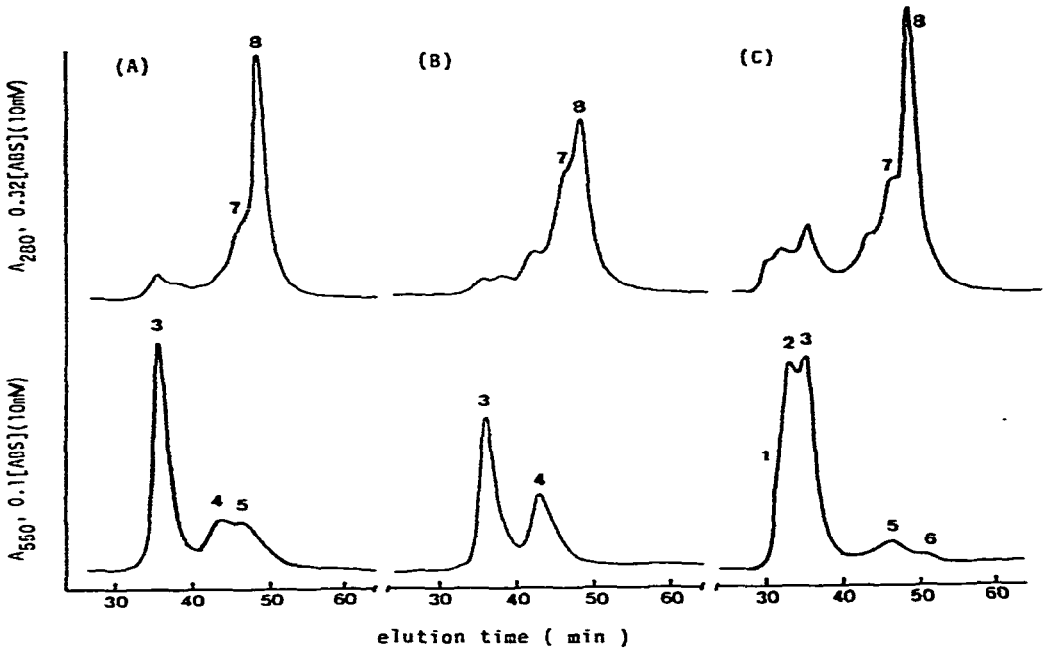

Fig. 8. Analyses of protein and cholesterol for human sera. Samples: (A) normal female subject; (B) liver cirrhosis subject; (C) hyperlipidemic (type III) subject. Loaded volume: 20 μl of whole serum. Peaks: 1, VLDL; 2, IDL; 3, LDL; 4, HDL_2; 5, HDL_3; 6, VHDL; 7, γ-globulin; 8, albumin. Conditions as in Fig. 2.

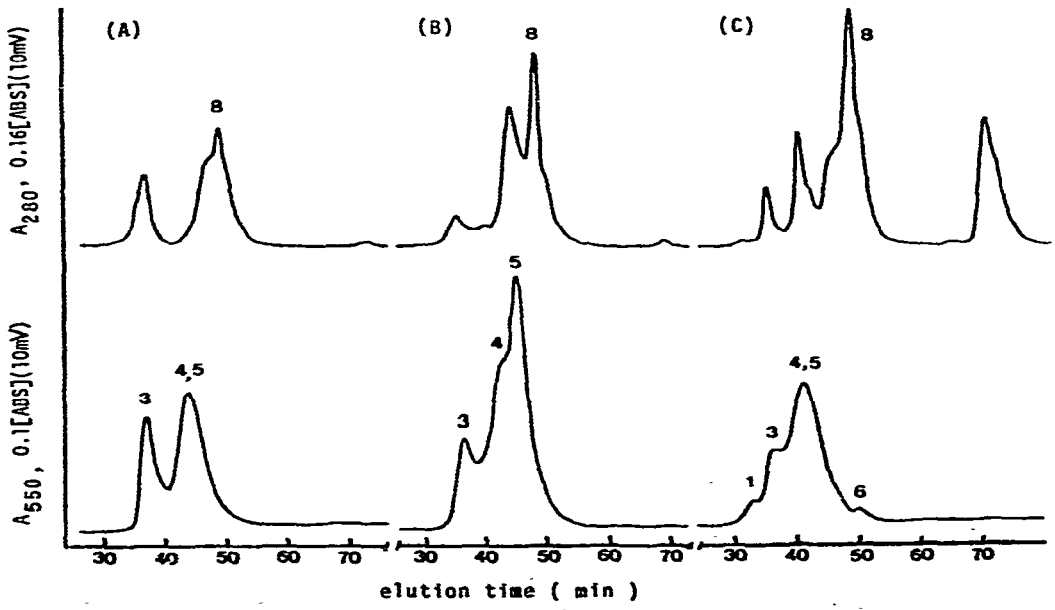

Fig. 9. Analyses of protein and cholesterol for animal sera. Samples: (A) 20 μl of whole serum (bovine); (B) 10 μl of whole serum (dog); (C) 50 μl of whole serum (rat). Peaks as in Fig. 8. Conditions as in Fig. 2.

of serum proteins are observed according to each species, the cholesterol peaks are separated into three major lipoprotein classes: VLDL, LDL, and HDL. With dog serum, HDL is separated into subfractions. In the case of rat serum, a small peak of cholesterol probably due to the VHDL fraction is always observed.

From these experiments, it is found that analyses of protein and lipoprotein of not only human but animal serum can be carried out with a very small amount of serum (10—50 μl) using the described flow system for high-performance GPC. The detection of cholesterol by enzymatic reaction is useful for studying the lipoprotein distribution in both human and animal sera. This analytical method is available for the investigation of lipoprotein metabolism and related diseases because of its short experimental time, high resolution and high sensitivity of detection. The quantitation of glyceride in serum lipoprotein is now being studied in our laboratory using this flow system for high-performance GPC.

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