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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF APOLIPOPROTEINS IN SERUM HIGH-DENSITY LIPOPROTEINS

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SUMMARY

A simple and rapid method for apolipoprotein analysis in serum high-density lipoproteins (HDL) has been developed using high-performance liquid chromatography (HPLC) with sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS) as eluent. In contrast to the use of urea solution as an eluent, apolipoproteins can be analysed by applying an incubation mixture of HDL and the eluent buffer. A TSK-GEL column of G3000SW was found to be more profitable than G2000SW or G4000SW for analysis of HDL apolipoproteins. Elution patterns monitored by absorbance at 280 nm using a G3000SW column can give precise quantitative as well as qualitative information about apolipoproteins of molecular weight between 10^4 and 10^5. HPLC patterns of HDL apolipoproteins were compared between individual human subjects with various diseases. Elution profiles for lipid components in an incubation mixture were also examined.

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INTRODUCTION

Serum apolipoproteins have become the centre of intense interest during recent years in the study on lipoproteins, because they have important roles in lipoprotein structure and metabolism. Serum lipoproteins are divided into several major classes according to density: chylomicrons, $d < 1.006$; very-low-density lipoproteins (VLDL), $d < 1.006$; low-density lipoproteins, $d = 1.006$—1.063; high-density lipoproteins (HDL), $d = 1.063$—1.210; very-high-density lipoproteins, $d > 1.210$. Individual lipoprotein classes contain a number of structurally different apolipoproteins.

In HDL, apolipoprotein (apo) A-I and apo A-II are major apolipoproteins, and apo C, apo E, apo D and apo A-IV are minor ones. Separation of these apolipoproteins has been performed by gel chromatography with urea solution as an eluent on the basis of differentiation of molecular weight [1, 2]. Recently, high-performance liquid chromatography (HPLC) has been applied to the analysis of HDL apolipoproteins using an aqueous gel permeation column (TSK-GEL, G3000SW) [3, 4]. In these cases, 6 *M* urea or 6 *M* guanidinium chloride solution is used as an eluent, and an apolipoprotein fraction, which is prepared by delipidation with organic solvents (ethanol—diethyl ether) from HDL, is applied to the HPLC system. More recently, we have developed a simple and rapid method for analysis of HDL apolipoproteins [5]. With our method, delipidation with organic solvents can be eliminated by incubation of a mixed solution of HDL and an eluent buffer: 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS). The good reproducibility and quantitation of this method have been reported previously [5].

In this paper, separation profiles of HDL apolipoproteins are compared between SDS and urea solutions on HPLC with a gel permeation column (G3000SW). Elution patterns of apolipoproteins under SDS solution are examined using TSK-GEL columns with different pore sizes. A few examples for analysis of HDL apolipoproteins from various subjects are reported. Elution profiles of lipid components in an incubation mixture of HDL and the eluent are also examined.

EXPERIMENTAL

Apparatus

HPLC was carried out using an HLC 803D (Toyo Soda, Tokyo, Japan) equipped with a variable-wavelength absorbance detector.

Ultracentrifugation for preparation of HDL from serum was performed using an RP 65 rotor in a Hitachi 55 P-2 ultracentrifuge (Hitachi Koki Co., Tokyo, Japan).

Lyophilization for preparation of apolipoproteins from HDL was carried out using a freeze drier (EYRA FD-5, Tokyo Rikakikai Co., Tokyo, Japan).

Materials and methods

Samples. Human serum and dog serum used in the experiments were obtained from humans and dogs after 12—16 h of fasting. HDL used for HPLC analysis was prepared from serum by the sequential flotation method by

Havel et al. [6]. The $d > 1.063$ fraction was obtained as the infranate layer after centrifugation (105,000 *g* at 15°C) for 24 h at a density of 1.063. After density adjustment to 1.210 with sodium bromide and centrifugation for 44 h at 105,000 *g*, the top layer was collected as HDL.

The apolipoprotein fraction of HDL (apo HDL) was prepared from HDL by delipidation with ethanol—diethyl ether as described by Scanu and Edelstein [2]. Standard apolipoproteins, apo A-I, apo A-II, apo E and apo C, were prepared as described previously [5]. Proteins used for column calibration were high-molecular-weight and low-molecular-weight electrophoresis calibration kits (Pharmacia Fine Chemicals, Uppsala, Sweden).

Reagents. Cholesterol and choline-containing phospholipids in the effluent from the gel permeation column were detected enzymatically by commercial reagent kits: Determiner TC"555" (Kyowa Medex Co., Tokyo, Japan) for cholesterol and PL kit K"f" (Nippon Shoji Co., Osaka, Japan) for choline-containing phospholipids. All chemical reagents were of the highest grade commercially available: sodium dodecyl sulphate (SDS), Wako; tris(hydroxymethyl)-aminomethane (Tris), Nakarai Chemical Ltd; urea (ultrapure), Schwartz/Mann (Spring Valley, NY, U.S.A.).

Sample preparation for HPLC analysis. HDL (d = 1.063—1.210), 10 μl containing 20—300 μg of protein, was added to 200 μl of 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS. The mixed solution was incubated at 60°C for 5 min and was used as a sample for HPLC analysis. Standard proteins, standard apolipoproteins and apo HDL were dissolved in the same buffer and incubated at 60°C for 5 min before HPLC analysis.

Analysis by HPLC. The separation of apolipoproteins in HDL was carried out by HPLC with aqueous gel permeation columns (TSK-GEL, Toyo Soda). HPLC conditions in this experiment were as follows. Columns: G2000SW, G3000SW, G3000SW + G3000SW, G4000SW (each column 600 × 7.5 mm I.D.). Eluents: 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS, 6 *M* urea at pH 3.15 (HCl), 6 *M* urea containing 0.05 *M* Tris—HCl buffer (pH 7.0). Flow-rate: 0.33 ml/min. Loaded volume: 200 μl. Eluted proteins from the column were detected by A_{280}. Cholesterol [7, 8] or choline-containing phospholipids [9, 10] in the effluent from the column was monitored by A_{550} or A_{500} after on-line enzymatic reaction as previously reported.

RESULTS AND DISCUSSION

The relation between elution volume and molecular weight in 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS was examined for various aqueous gel permeation columns (G2000SW, G3000SW and G4000SW) using standard proteins and apolipoproteins of known molecular weight. Molecular weights of these standard samples used for column calibration are listed in Table I. Other apolipoproteins contained in the HDL fraction from human serum are also presented in Table I. As shown in Fig. 1, the plot of elution volume against the logarithm of molecular weight is linear for all columns except for apo C. Several components with different molecular weights are contained in the apo C group (Table I). Since a mixture of these components is used as a standard for apo C in our experiment, the mean molecular weight of apo C is

TABLE I

MOLECULAR WEIGHTS OF STANDARD PROTEINS AND APOLIPOPROTEINS

Protein	Molecular weight	Apolipoprotein	Molecular weight
Thyroglobulin	669,000	Apo A-IV	46,000
Ferritin	440,000	Apo E	39,000
Catalase	232,000	Apo A-I	28,300
Lactate dehydrogenase	140,000	Apo D	20,000
Phosphorylase *b*	94,000	Apo A-II	17,000
Bovine serum albumin	67,000	Apo C-I	6500
Ovalbumin	43,000	Apo C-II	8800
Carbonic anhydrase	30,000	Apo $C\text{-}III_{0,1,2}$	8900
Soybean trypsin inhibitor	20,100		
α-Lactalbumin	14,400		
Cytochrome *c*	13,000		

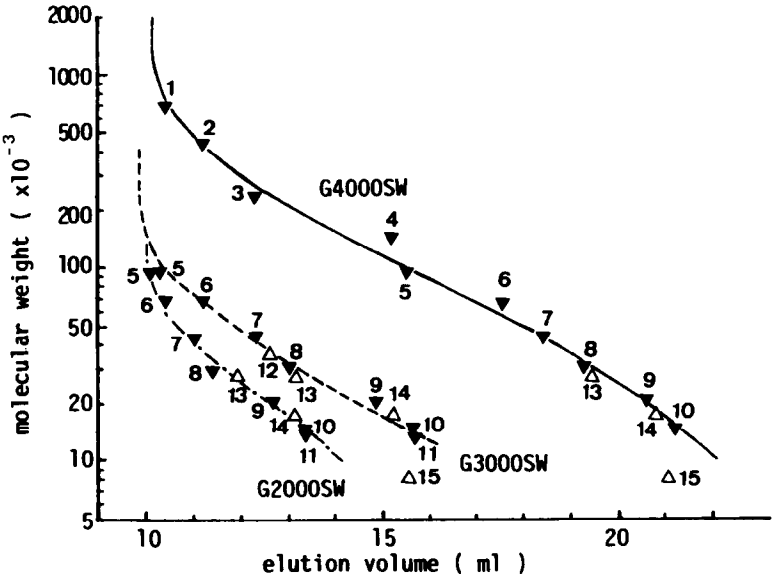

Fig. 1. Relation between elution volume and molecular weight for various gel permeation columns. Columns: G4000SW, G3000SW, G2000SW (each column 600 × 7.5 mm I.D.). Eluent: 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS. Flow-rate: 0.33 ml/min. Sample: 1, thyroglobulin; 2, ferritin; 3, catalase; 4, lactate dehydrogenase; 5, phosphorylase *b*; 6, bovine serum albumin; 7, ovalbumin; 8, carbonic anhydrase; 9, soybean trypsin inhibitor; 10, α-lactalbumin; 11, cytochrome *c*; 12, apo E; 13, apo A-I; 14, apo A-II; 15, apo C.

estimated to be about 8400. As reported by Fish et al. [11], the linear relation between Stokes' radius and molecular weight of proteins is broken at molecular weights of less than 15,000 for gel chromatography in SDS solution. Recently, the separation range of proteins by HPLC with gel permeation columns (G2000SW, G3000SW and G4000SW) under SDS solution has been reported [12, 13], and the lower limit of molecular weight is 10,000 or 15,000. The result of our experiment clearly shows that apo C is out in the separation range

under SDS solution for all three columns. Therefore, an eluent other than SDS solution, such as urea solution, is better for the separation of apo C as discussed later.

We have developed a simple and rapid method for the analysis of apolipoproteins in HDL without delipidation by organic solvents [5]. With this method, apolipoproteins separated on the basis of differentiation of molecular weight can be detected in terms of A_{280} only by injecting a mixed solution of HDL and eluent buffer (0.1 *M* sodium phosphate buffer containing 0.1% SDS) preincubated at 60°C for 5 min. In Fig. 2, elution patterns of an incubation mixture of HDL from a normal male subject and the eluent are shown for various columns. Elution positions of apo E, apo A-I, apo A-II and apo C determined by using the standard samples are shown as arrows with numbers. For all columns, elution positions of apo E and apo A-I, apo A-II and apo C are very close to each other. As previously reported, the separation of apo E from apo A-I can be improved by column elongation (G3000SW × 2) (see Fig. 4), but the separation of apo A-II and apo C can not be improved under SDS solution for the reason mentioned above. From the calibration curves in Fig. 1, molecular weights at the void volume of the column are estimated to be 1,000,000, 200,000 and 100,000 for G4000SW, G3000SW and G2000SW, respectively. From the elution pattern of Fig. 2, it is indicated that G3000SW is preferable for the analysis of HDL apolipoproteins than the other two columns.

It has been reported that urea or guanidinium chloride solution is better than SDS solution for the separation of proteins of molecular weight less than

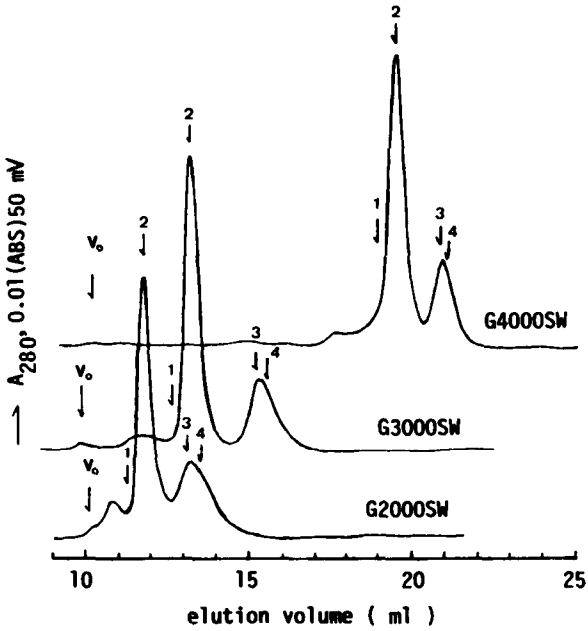

Fig. 2. Elution pattern of HDL apolipoproteins. Column: G3000SW (600 × 7.5 mm I.D.). Eluent: 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% SDS. Flow-rate: 0.33 ml/min. Sample: incubation mixture of HDL and the eluent. Loaded volume: 200 μl. Detector: A_{280}, 0.01 [ABS] 50 mV. Elution position: 1, apo E; 2, apo A-I; 3, apo A-II; 4, apo C.

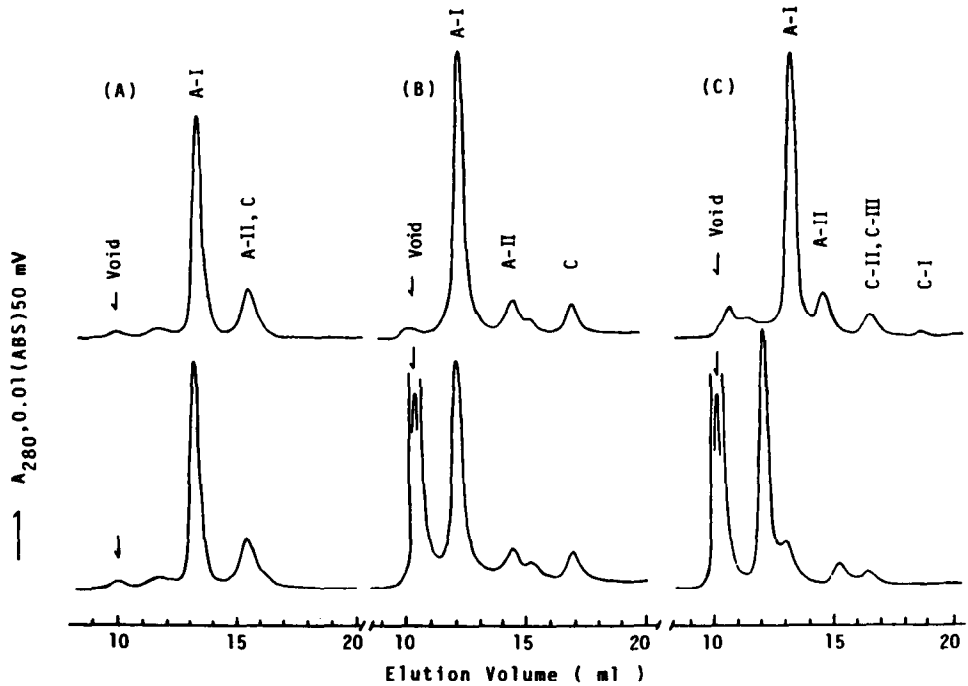

Fig. 3. Elution pattern of HDL apolipoproteins. Column: G3000SW (600 x 7.5 mm I.D.). Eluents: (A) 0.1 *M* sodium phosphate buffer (pH 7.0) + 0.1% SDS; (B) 6 *M* urea (pH 3.15 with HCl); (C) 6 *M* urea + 0.05 *M* Tris—HCl buffer (pH 7.0). Sample: apo HDL (upper traces) and an incubation mixture of HDL and the eluent (lower traces). Other HPLC conditions as in Fig. 2.

10,000 [13, 14]. In fact, apolipoproteins from HDL (apo HDL) have been analysed by HPLC using urea or guanidinium chloride solution [3, 4]. We compared the separation profiles of apo HDL using the G3000SW column between SDS and urea solutions for the same subject as in Fig. 2 (upper part of Fig. 3). In contrast to SDS solution, apo A-II and apo C can be separated by 6 *M* urea solution. Morever, apo C can be separated into large components (apo C-II + apo C-III) and a small one (apo C-I) by 6 *M* urea at pH 7.0. We also compared the elution patterns of an incubation mixture (60°C, 5 min) of HDL from the same subject and the eluent for three solvent systems: SDS, 6 *M* urea at pH 3.15, and 6 *M* urea at pH 7.0. The results are presented in the lower part of Fig. 3. In the case of SDS solution, the elution pattern as well as the elution position of each peak of the incubation mixture were very similar to those of apo HDL, and this suggests that HDL is delipidated completely by incubation with the eluent buffer. But the elution pattern of the incubation mixture was very different from that of apo HDL for the two 6 *M* urea solvent systems. In contrast to SDS solution, large peaks at the void volume were observed for the incubation mixture in both urea systems. In the case of 6 *M* urea at pH 3.15, the elution pattern except for the void peak of the incubation mixture was comparable to that of apo HDL. On the other hand, peak positions of the incubation mixture were quite different from those of apo HDL in the case of 6 *M* urea at pH 7.0. Therefore, urea solution can be used only for the

delipidated sample with organic solvents, apo HDL. This suggests that despite poor resolution of apo A-II and apo C, SDS solution is useful for apolipoprotein analysis because the experimental procedure is simplified without the delipidation by organic solvents. A detailed examination of the quantitation of apolipoproteins from the HPLC pattern monitored by A_{280} using an incubation mixture of HDL and SDS solution was reported previously [5].

Resolution of each apolipoprotein except for apo C can be improved by elongation of the G3000SW column [5]. HPLC patterns monitored by A_{280} using two G3000SW columns for an incubation mixture of HDL from various subjects and SDS solution are presented in Fig. 4. Among these subjects, hyper-

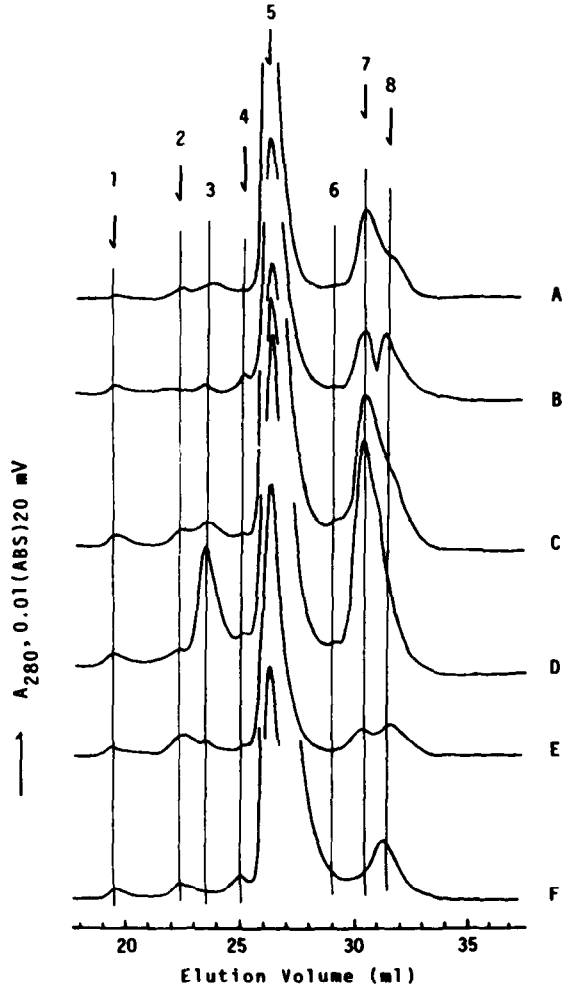

Fig. 4. Elution pattern of HDL apolipoproteins. Column: G3000SW + G3000SW (each 600 × 7.5 mm I.D.). Eluent: 0.1 *M* sodium phosphate buffer (pH 7.0) + 0.1% SDS. Sample: an incubation mixture of HDL from human serum (A—E) or dog serum (F) and the eluent. (A) normal male; (B) lecithin:cholesterol acyltransferase deficiency; (C) acute hepatitis; (D) hyper α-lipoproteinemia; (E) liver cirrhosis. Elution position: 1, void volume of the column; 2, bovine serum albumin; 3, apo A-IV (?); 4, apo E; 5, apo A-I; 6, apo D (?); 7, apo A-II; 8, apo C. Other HPLC conditions as in Fig. 2.

α-lipoproteinemia (Fig. 4D) has been confirmed to contain a large amount of apo A-IV by electrophoretic analysis on SDS-polyacrylamide gel [15]. Dog serum (Fig. 4F) is well known to lack apo A-II in HDL. A significant amount of apo E is known to be present in lecithin:cholesterol acyltransferase deficiency (Fig. 4B) compared to normal subjects. As shown in Fig. 4, as a small void peak, a large peak of apo A-I and a variable shaped peak depending on the composition of apo A-II and apo C were commonly observed for all subjects. For some subjects, a small peak of apo E was observed separately from apo A-I, and a small broad peak was observed at the elution volume of bovine serum albumin. Moreover, peaks at the elution volume corresponding to molecular weights of 46,000 and 20,000 for the calibration curve of Fig. 1 are assumed to be apo A-IV and apo D, respectively. These patterns obtained using our HPLC technique are found to give useful quantitative as well as qualitative information about apolipoproteins in HDL from a very small amount of HDL (10 μl) for a short experimental time.

Lastly, we examined the elution profiles of lipid components of HDL in our analytical system. Cholesterol or choline-containing phospholipids in the effluent from the G3000SW column was monitored by A_{550} or A_{500} by our established on-line technique using a commercial enzyme reaction kit [7—10]. Elution patterns monitored by A_{280}, cholesterol and choline-containing phospholipids for an incubation mixture of HDL and SDS solution are presented in Fig. 5. Elution patterns of both cholesterol and choline-containing phospholipids showed three peaks, the peak positions being consistent. This indicates that lipid components in HDL elute to form an aqueous mixed micelle with SDS and that there are three different sized particles depending on

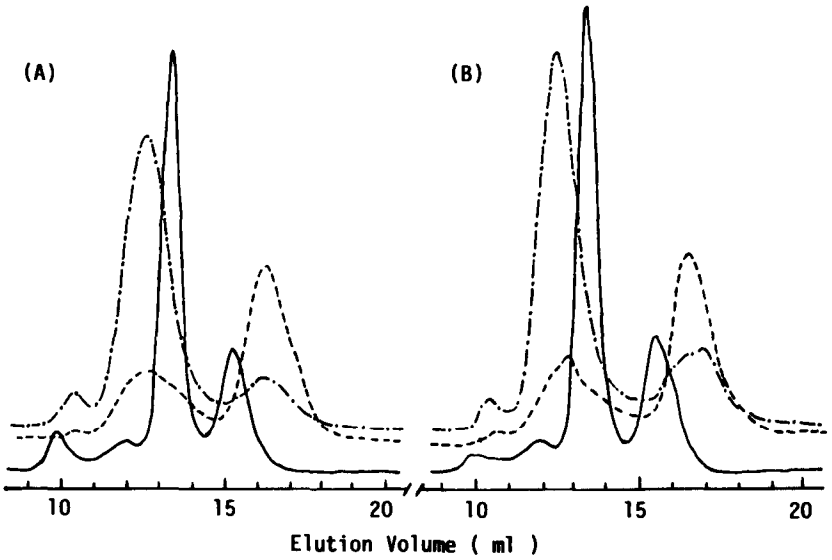

Fig. 5. Elution pattern of HDL apolipoproteins (——), cholesterol (—·—·—) and choline-containing phospholipids (— — —). Column: G3000SW (600 x 7.5 mm I.D.). Eluent: 0.1 *M* sodium phosphate buffer (pH 7.0) + 0.1% SDS. Sample (A and B): incubation mixture of human HDL and the eluent. Loaded volume: 200 μl. Detector: ——, A_{280}, 0.01 [ABS] 50 mV; —·—·—, A_{550}, 0.02 [ABS] 20 mV; — — —, A_{500}, 0.02 [ABS] 10 mV. Other HPLC conditions as in Fig. 2.

chemical composition. The size of the particles is assumed to increase with increase of the ratio of cholesterol to choline-containing phospholipids from the HPLC pattern of Fig. 5. Since these lipid micelles have no significant absorption at 280 nm, analysis of apolipoproteins by monitoring A_{280} may not be disturbed by the co-elution of lipid micelles. This is also confirmed by the similarity of HPLC patterns between the incubation mixture and apo HDL as presented in Fig. 3A.

Our analytical method for HDL apolipoproteins using SDS solution is very useful for clinical research because of the simple experimental procedure, short experimental time and small amount of sample. We are examining the application of this analytical method to other lipoprotein fractions such as VLDL.

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