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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN SERUM LIPOPROTEINS

SELECTIVE DETECTION OF CHOLINE-CONTAINING PHOSPHOLIPIDS BY ENZYMATIC REACTION

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SUMMARY

A convenient method for the quantitation of choline-containing phospholipids in each lipoprotein fraction has been developed by combining separation by high-performance liquid chromatography with gel permeation columns and selective detection by enzymatic reaction in the post-column effluent.

The elution patterns monitored by choline-containing phospholipids were compared with those monitored by cholesterol. The elution patterns of choline-containing phospholipids were found to give much more information about the distribution of lipoproteins according to their particle-size differentiation than analyses done by cholesterol.

This choline-containing phospholipid monitoring method not only resolves lipoprotein peaks of the major classes (chylomicron + VLDL, LDL, HDL_2 and HDL_3) quantitatively, but also detects the presence of abnormal lipoproteins containing a large amount of choline-containing phospholipids. We could detect these abnormal lipoproteins using a small amount of whole serum (10—20 μl) from patients with various liver diseases. Our examination of HDL subclasses using this technique showed that the HDL fraction was composed of several subfractions due to their particle-size differentiation.

INTRODUCTION

We have developed a new method for lipoprotein analysis using high-performance liquid chromatography (HPLC) with gel permeation columns (TSK-GEL, Toyo Soda, Tokyo, Japan) which achieves the separation of serum lipoproteins into their major classes due to particle-size differentiation: chylomicron, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoproteins (HDL_2 and HDL_3) [1, 2].

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It is well known that the particle size of serum lipoproteins depends on their chemical composition. Especially large-particle-size lipoproteins, such as chylomicrons and VLDL, contain a large amount of triglycerides. On the other hand, phospholipids are an essential component of the serum lipoprotein structure. All lipoproteins, not only normal lipoproteins, (i.e. chylomicrons, VLDL, LDL, HDL_2 and HDL_3), but abnormal lipoproteins which are found in the serum of the patients with dyslipoproteinemia, contain about 18—30% of phospholipids. Some abnormal lipoproteins contain a large amount of phospholipids, more than 60% [3—5]. This means that the detection of triglycerides is very suitable for the analysis of larger-particle-size lipoproteins and that of phospholipids for any lipoprotein, even abnormal ones.

A direct quantitation method for cholesterol in each serum lipoprotein class has been developed using a combination technique consisting of separation by HPLC with gel permeation columns and the selective detection of cholesterol using a commercial enzyme kit [6, 7]. This technique can be applied to the analysis of lipid components other than cholesterol in serum lipoproteins, such as triglycerides and phospholipids. In fact, we have developed a convenient and simple method for the detection and quantitation of triglycerides in serum lipoproteins using a gel permeation column (TSK-GEL, G5000PW) by this technique [8]. Moreover, we have established a quantitation method for choline-containing phospholipids in each lipoprotein class using a commercially available enzyme kit. The study of the optimum conditions for the enzymatic reaction in the flow diagram using a reaction-type high-speed chromatograph is reported in a separate paper [9].

In this paper, we examine the effect of the elongation of the reaction tube on the resolution of lipoproteins using the detection method of choline-containing phospholipids. Elution patterns monitored by choline-containing phospholipids were compared with those obtained by cholesterol monitoring using two different column systems. The size distribution of serum lipoproteins of patients with various liver diseases was examined by monitoring both cholesterol and choline-containing phospholipids.

EXPERIMENTALS

Apparatus

HPLC was carried out using a reaction-type high-speed liquid chromatograph (HLC 805, Toyo Soda) in the same schematic diagram as described in our previous papers [6, 7] except that the enzymatic reaction was performed using Teflon tubes of 0.5 mm or 1.0 mm I.D. with a length varying between 5000 and 30,000 mm in a thermostated water bath (Thermo Mini TM-100, Tokyo Rikakikai, Tokyo, Japan).

Ultracentrifugation for the separation of the standard lipoproteins from human serum was carried out using an RP 55 rotor in an Hitachi 55P-2 ultracentrifuge.

Materials and methods

Samples. Human sera used in these experiments were obtained from normal

men and women or from patients with hyperlipidemia and various liver diseases after 12—16 h of fasting. Chylomicron + VLDL ($d < 1.006$), LDL (d 1.006—1.063), HDL_2 (d 1.063—1.125) and HDL_3 (d 1.125—1.210) were isolated from human serum by the sequential flotation method [10]. After centrifugation (105,000 g, 8°C), lipoproteins in the top layer of the tube were collected using a tube slicer (Model TSU2, Hitachi). The lipoprotein fraction of $d < 1.210$ was prepared by the ultracentrifugation method in the same way as described in our previous paper [1].

Reagents. The concentration of cholesterol and choline-containing phospholipids in the samples applied to HPLC was determined enzymatically using commercial kits: Determiner TC"555" (Kyowa Medex, Tokyo, Japan), for cholesterol, and PL Kit K (Nippon Shoji, Osaka, Japan) for choline-containing phospholipids. The selective detection of choline-containing phospholipids was performed in terms of A_{500} using a commercial enzymatic reagent kit for autoanalyzers (PL Kit K"f", Nippon Shoji). This reagent was given in the form of a premixed lyophilized vial which contained 40 units of phospholipase D (from *Streptomyces* spp.), 130 units of choline oxidase (from *Arthrobacter* spp., EC 1.1.3.17), 110 units of peroxidase (EC 1.11.1.7), and 5.6 mg of 4-aminoantipyrine. For the detection of choline-containing phospholipids we used the enzyme solution which was prepared by dissolving one vial (PL Kit K"f") in 25 ml of a buffer solution for HPLC. This solution contained 44.4 mg of phenol and 93.8 mg of Triton X-100 per 100 ml of 0.05 M Tris—HCl buffer (pH 7.8). The details of the examination for the optimum preparation method of this buffer solution are reported in a separate paper [11].

Separation of lipoproteins by HPLC. The separation of lipoproteins was carried out by HPLC with gel permeation columns (TSK-GEL, G5000PW, G4000SW and G3000SW, Toyo Soda). Experimental conditions in this study were as follows. Column: G5000PW, G4000SW+G3000SW, G3000SW+G3000SW+G3000SW (each column 600 mm × 7.5 mm I.D.). Eluent: 0.15 M sodium chloride. Flow-rate: 0.50—0.60 ml/min.

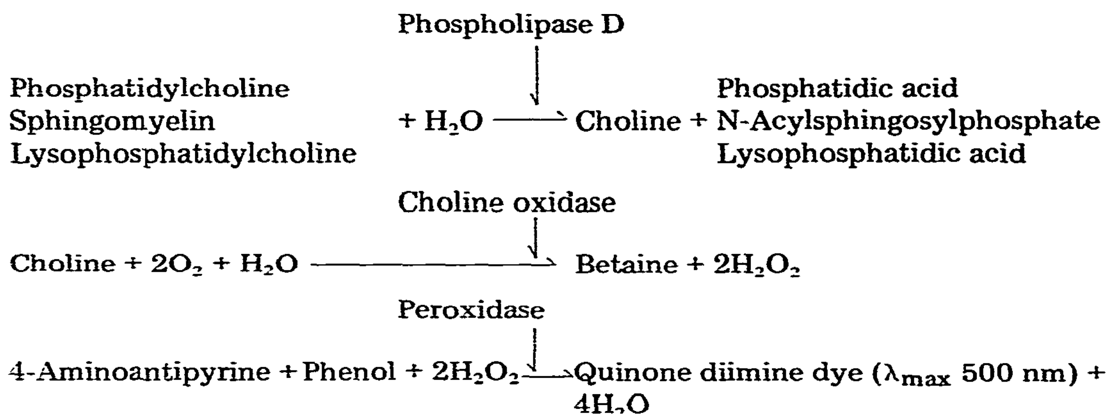
Detection of choline-containing phospholipids and cholesterol. Cholesterol was detected by measuring the A_{550} of the post-column effluent in the same way as described in our previous paper [6, 7].

Choline-containing phospholipids were monitored by the A_{500} of the mixed eluate and enzyme solution (PL Kit K"f") after passage through a reaction tube at constant temperature. Experimental conditions in this study are as follows: temperature of the reaction bath, 39°C; dimensions of the reaction tube, 20,000 mm × 0.5 mm I.D. (Teflon tube); flow-rate of the main path (i.e. the pathway of eluate from the column), 0.50—0.60 ml/min; flow-rate of the enzyme solution, 0.20—0.30 ml/min.

RESULTS AND DISCUSSION

We have already reported the direct quantitation method for cholesterol in each serum lipoprotein by combining separation by HPLC and the selective detection of cholesterol using the enzyme reaction kit [6, 7]. This method can be applied to other lipid components of serum lipoproteins if appropriate reagents for selective detection are obtained.

Takayama et al. [12] have developed a new enzymatic method for quantitation of choline-containing phospholipids. The quantitation of choline-containing phospholipids in a small amount of serum (20 μl) can be carried out in an aqueous system according to the following reaction schemes.



This enzymatic method is specific for the choline-containing phospholipids as shown above. Since these three choline-containing phospholipids comprise about 95% of the phospholipids in serum [13—15], this can be used for the selective detection of phospholipids in serum lipoproteins. For the detection of phospholipids we used a commercial kit (PL Kit K"f") utilizing the reaction schemes as described under Experimental. Using this reagent, choline-containing phospholipids can be detected by the absorbance at 500 nm after passage of the mixed eluate and enzyme solution through the reactor at constant temperature.

The optimum conditions for quantitation of choline-containing phospholipids in the flow diagram were determined as reported in a separate paper [9]. Since a sufficient reaction time, over 4.0 min, can not be given by using a reactor (20,000 mm × 0.25 mm I.D., stainless-steel tube) of the high-speed chemical derivatization chromatograph (HLC 805, Toyo Soda), a reaction tube of larger dimensions than a commerical one must be used.

In order to examine the relation between the dimensions of the reaction tube and the resolution of lipoprotein analysis, the enzymatic reaction was carried out using a Teflon tube (0.5 mm or 1.0 mm I.D.) of various lengths between 5000 and 30,000 mm keeping other experimental conditions optimal. The effect of the dimensions of the reaction tube on peak broadening was examined in the following way with use of the standard lipoprotein fractions LDL and HDL_2. The half band width of these lipoproteins monitored by A_{280} before passage through the reaction tube was compared with that monitored by A_{500} after the enzymatic reaction in the reaction tube. The elution patterns monitored by both A_{280} and A_{500} using reaction tubes of different diameters are presented in Fig. 1. For the reaction tube of 1.0 mm I.D. (Fig. 1c), an increase in band width of A_{500} for both lipoprotein fractions was observed in comparison with that of A_{280}. On the other hand, the increase of that of A_{500} was very small (less than 5%) in the case of the reaction tube of 0.5 mm I.D. (Fig. 1a and b).

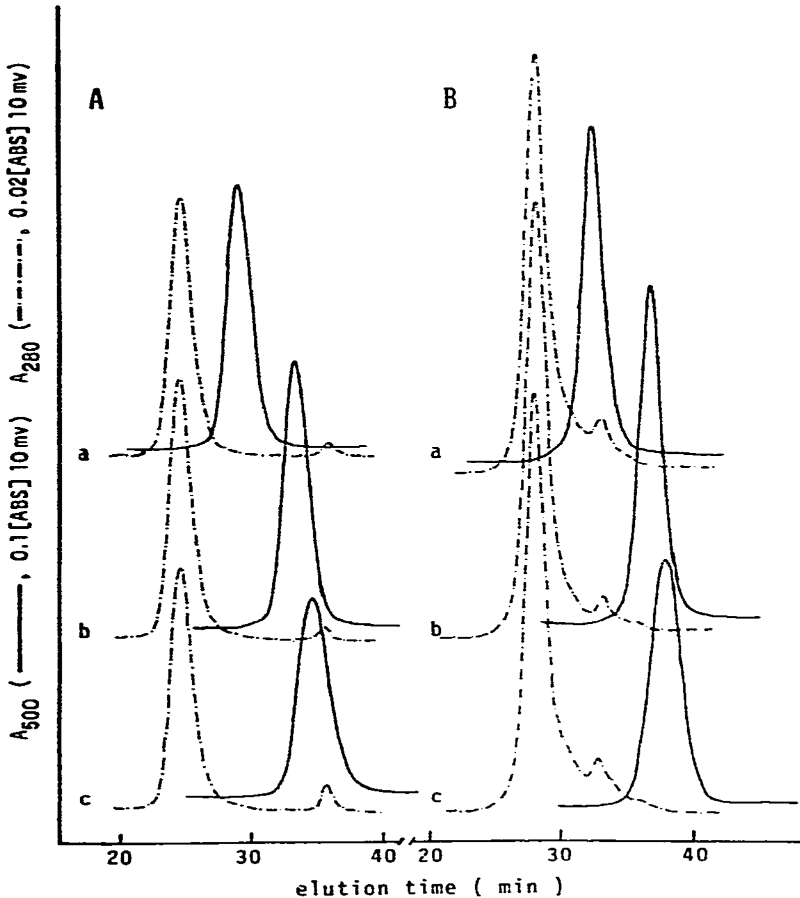


Fig. 1. Elution patterns monitored by A_{280} and A_{500} for standard lipoproteins. Column: G5000PW (600 mm × 7.5 mm I.D.). Eluent: 0.15 M sodium chloride. Flow-rate: 0.60 ml/min (main path), 0.30 ml/min (enzyme solution, PL Kit K"f"). Temperature of the reaction bath: 39°C. Dimensions of reaction tube (Teflon): (a) 15,000 mm × 0.5 mm I.D.; (b) 30,000 mm × 0.5 mm I.D.; (c) 11,000 mm × 1.0 mm I.D. Sample: A, LDL (d 1.006—1.063) fraction (220.5 mg/dl choline-containing phospholipids; B, HDL_2 (d 1.063—1.125) fraction (162.2 mg/dl choline-containing phospholipids). Loaded volume: A, 5 µl; B, 10 µl. Detector: A_{500} (——, 0.1 [ABS] 10 mV) and A_{280} (— · — · —, 0.02 [ABS] 10 mV).

It can be seen from Fig. 1 that the sensitivities for the detection of both LDL and HDL_2 fractions monitored by choline-containing phospholipids are several times higher than those obtained by the A_{280}.

Fig. 2 shows the relation between the half band width ($\omega_{1/2}$, ml) of these lipoprotein fractions monitored by the A_{500} and the length of the reaction tube of 0.5 mm I.D. The half band width of both fractions was constant when the reaction time was over 4.0 min. This reaction time can be obtained by using a reaction tube (0.5 mm I.D.) longer than 15,000 mm. We used a reaction tube of 20,000 mm × 0.5 mm I.D. (Teflon) for the detection of choline-containing phospholipids. Moreover, these examinations indicate that a longer reaction time of over 4.0 min can be obtained by elongation of the reaction tube

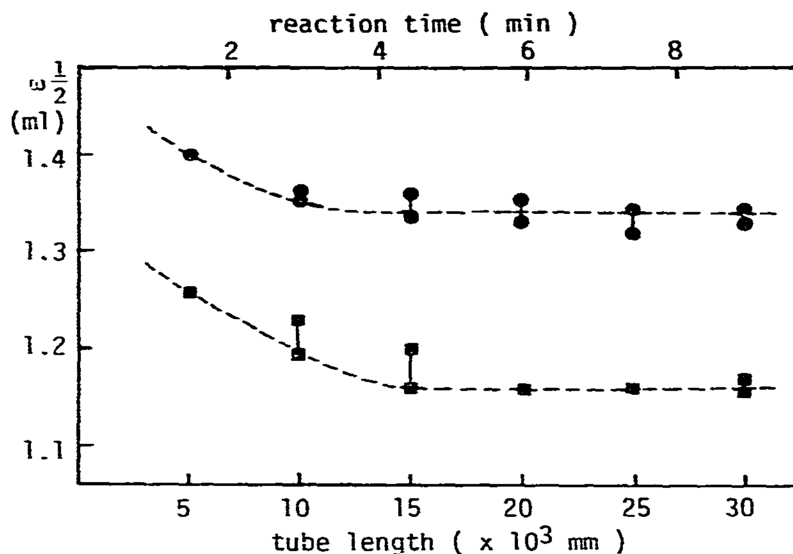


Fig. 2. Relation between half band width monitored by A_{500} and reaction tube length for standard lipoproteins. Sample: LDL (*d* 1.006—1.063) fraction, ●; HDL_2 (*d* 1.063—1.125) fraction, ■. Loaded volume: LDL, 5 µl; HDL_2, 10 µl. HPLC conditions are as in Fig. 1.

without peak broadening, provided the tubing is of appropriate diameter, less than 0.5 mm I.D.

Fig. 3 presents the elution patterns monitored by the A_{500} for the standard lipoprotein fractions (chylomicron+VLDL, LDL, HDL_2 and HDL_3) using the two combined column systems: G4000SW+G3000SW and G3000SW+G3000SW+G3000SW. These standard lipoprotein fractions were prepared from serum of normal males and females by the sequential flotation method [10]. It can be seen from these patterns that the G4000SW+G3000SW system achieves the separation of serum lipoproteins into their major classes: chylomicron+VLDL, LDL, HDL_2 and HDL_3 (Fig. 3A). On the other hand, the G3000SW+G3000SW+G3000SW system (Fig. 3B) was found to be very suitable for the analysis of HDL_2 (*d* 1.063—1.125) and HDL_3 (*d* 1.125—1.210) fractions. The elution patterns of HDL_2 and HDL_3 fractions using this column system suggest the heterogeneity of particle size within these two HDL subclasses.

The elution patterns of whole serum monitored by choline-containing phospholipids were compared between these two column systems (G4000SW+G3000SW and G3000SW+G3000SW+G3000SW) using the same subject. Three examples are shown in Fig. 4. The elution patterns of cholesterol and protein are also presented in the same figure. The arrows in Fig. 4 indicate the elution positions of the major lipoprotein classes and serum albumin. The elution position of each lipoprotein fraction was determined using the standard fractions as shown in Fig. 3.

The elution patterns of whole serum monitored by cholesterol and choline-containing phospholipids using the G4000SW+G3000SW column system gave clear peaks according to the major lipoprotein classes: chylomicron+VLDL, LDL and HDL subclasses. Since the high content of cholesterol in the LDL

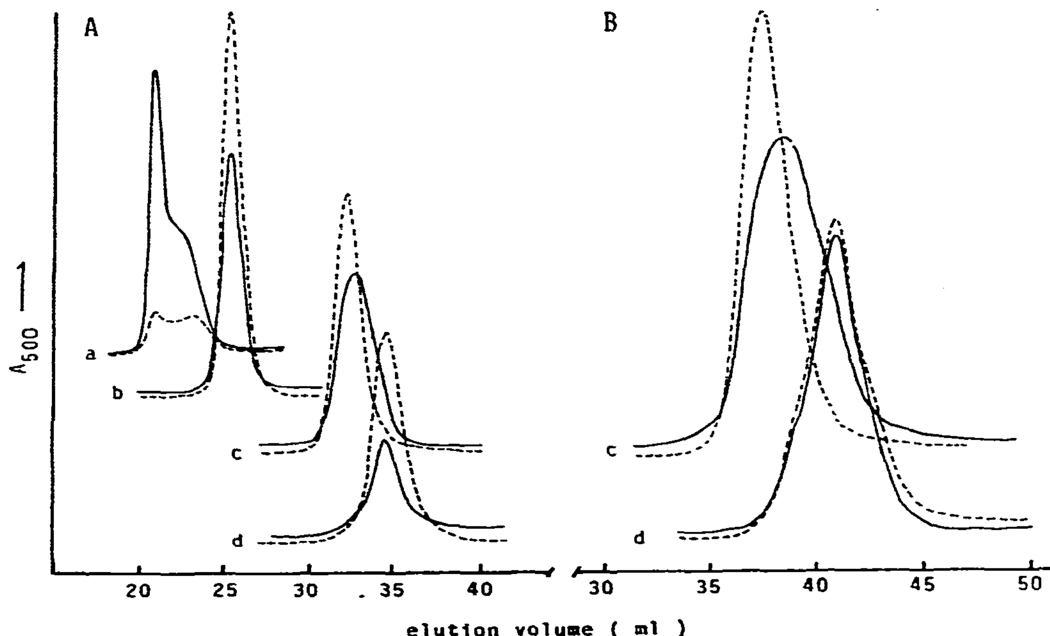


Fig. 3. Elution patterns monitored by A_{500} for standard lipoproteins using combined column systems. Column: A, G4000SW+G3000SW; B, G3000SW+G3000SW+G3000SW (each column size, 600 mm × 7.5 mm I.D.). Eluent: 0.15 *M* sodium chloride. Flow-rate: 0.60 ml/min (main path), 0.30 ml/min (enzyme solution, PL Kit M"f"). Temperature of the reactor (20,000 mm × 0.5 mm I.D. Teflon tube): 39°C. Sample: (a) chylomicron + VLDL fraction (d < 1.006); (b) LDL (d 1.006—1.063); (c) HDL_2 (d 1.063—1.125); (d) HDL_3 (d 1.125—1.210) from serum of normal male (——) and normal female (— — —). Loaded volume: 20—150 µl.

fraction interferes with the detection of peaks which appear near the LDL fraction, the resolution of peaks of lipoproteins monitored by choline-containing phospholipids is higher than that monitored by cholesterol.

As previously reported [1], the G3000SW column is suitable for the analysis of small-size lipoproteins such as HDL subclasses. As expected, the G3000SW+G3000SW+G3000SW system gave many peaks and shoulder peaks in the HDL fraction in comparison with the G4000SW+G3000SW system as presented in Fig. 4. Moreover, a shoulder peak of choline containing phospholipids at the elution position of serum albumin is completely separated in the G3000SW+G3000SW+G3000SW system. Elution patterns of the HDL fraction using this column system indicate that there exist many subclasses other than HDL_2 and HDL_3. Heterogeneity of the HDL fraction was examined by peak frequency analysis and rechromatography using this column system, and the results will be reported in a separate paper [16]. Our results for HDL subclasses according to particle-size differentiation using gel permeation columns are consistent with those reported by other investigators who used gradient gel electrophoresis [17] or rate zonal ultracentrifugation [18].

The elution patterns monitored by choline-containing phospholipids were compared between whole serum and its d < 1.21 fraction. The two examples

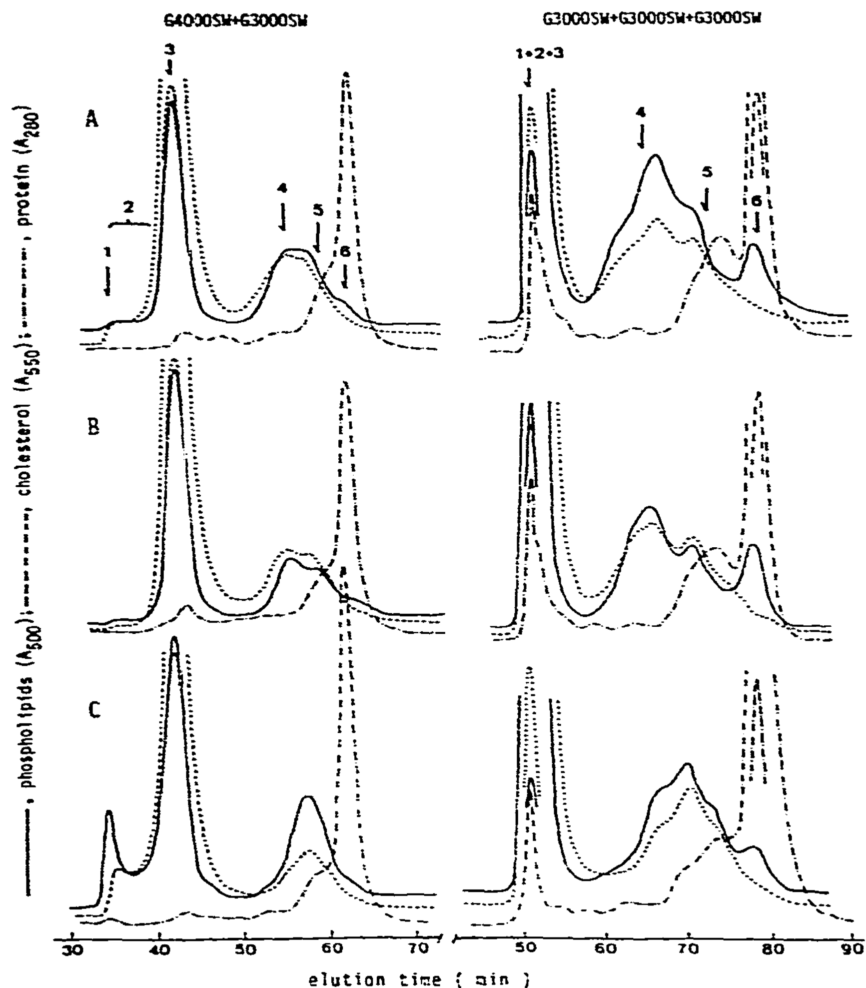


Fig. 4. Elution patterns of choline-containing phospholipids (———), cholesterol (— — —) and protein (— · — · —) for human whole serum using combined column systems. Column: G4000SW+G3000SW, G3000SW+G3000SW+G3000SW. Sample: whole serum of normal male (A), normal female (B) and hyperlipidemia patient (C). Loaded volume: 5 μl (G4000SW+ G3000SW) and 10 μl (G3000SW+G3000SW+G3000SW) for cholesterol and protein monitoring; 10 μl (G4000SW+G3000SW) and 20 or 30 μl (G3000SW+G3000SW+G3000SW) for choline-containing phospholipids monitoring. HPLC conditions for cholesterol monitoring: eluent, 0.15 M sodium chloride; flow-rate of main path, 0.60 ml/min; flow-rate of enzyme solution (TC"555"), 0.20 ml/min; temperature of the reactor (20,000 mm × 0.25 mm I.D. stainless-steel tube), 40°C. HPLC conditions for choline-containing phospholipids monitoring as in Fig. 3. Detector: choline-containing phospholipids (A_{500}, ———), 0.1 [ABS] 5 mV; cholesterol (A_{550}, — — —), 0.1 [ABS] 10 mV; protein (A_{280}, — · — · —), 0.32 [ABS] 5 mV. Elution position: 1, chylomicron; 2, VLDL; 3, LDL; 4, HDL_2; 5, HDL_3; 6, serum albumin.

are shown in Fig. 5. The elution patterns are in fairly good agreement except that peak No. 6 after the HDL_3 fraction disappears in the case of the $d < 1.21$ fraction. This peak might correspond to choline-containing phospholipids in the $d > 1.21$ fraction of serum, because there were considerable amounts of

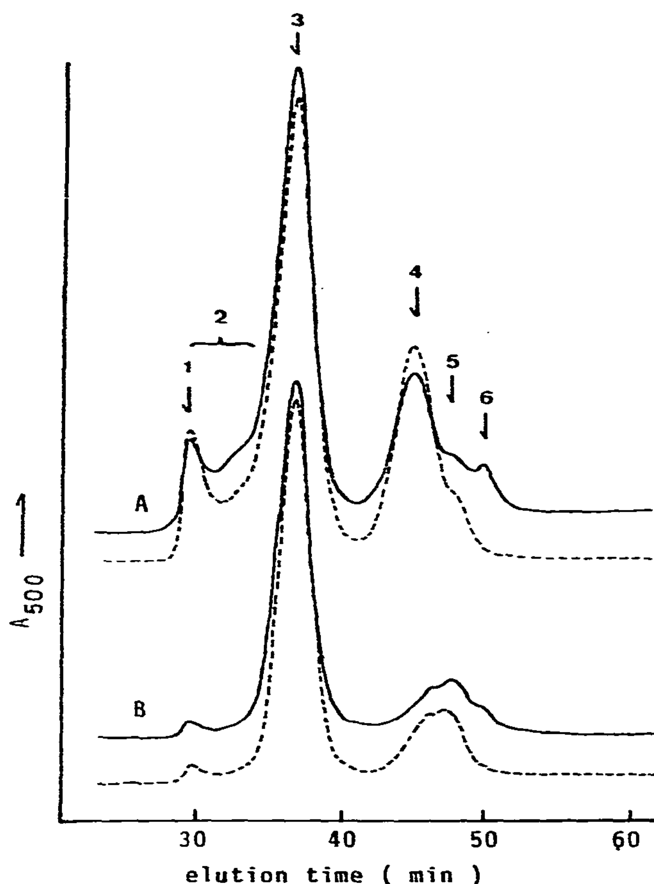


Fig. 5. Elution patterns monitored by A_{500} for whole serum and the $d < 1.21$ fraction. Column: G4000SW. Flow-rate: 0.50 ml/min (main path), 0.20 ml/min (enzyme solution, PL Kit K"f"). Sample: whole serum (——) and the $d < 1.21$ fraction (— — —) of a hyperthyroidemia patient (A) and a hyperlipidemia patient (B). Loaded volume: whole serum, 10 µl; the $d < 1.21$ fraction, 20 µl. Elution position and other HPLC conditions as in Fig. 4.

choline-containing phospholipids in the $d > 1.21$ fraction of serum, 17.6 ± 5 mg/dl (n = 23) in our ultracentrifugation examination. Similar results have been reported by other investigators [10, 19—22]. Therefore, peak No. 6 is identified as the choline-containing phospholipids in the $d > 1.21$ fraction which is designated as very high density lipoprotein (VHDL) [21].

The elution patterns of cholesterol and choline-containing phospholipids were examined for individual human serum samples (10—20 µl) from normal and pathological subjects using the G4000SW+G3000SW system. This system is the best combination for analysis of all major lipoprotein classes. A few examples obtained for patients with various liver diseases are shown in Fig. 6, where one example of a normal female is also shown as reference. Elution positions of major lipoproteins and serum albumin are shown in the figure in the same way as in Fig. 4. Elution patterns of liver diseases are different from those of normal subjects: disappearance of major lipoprotein classes and/or

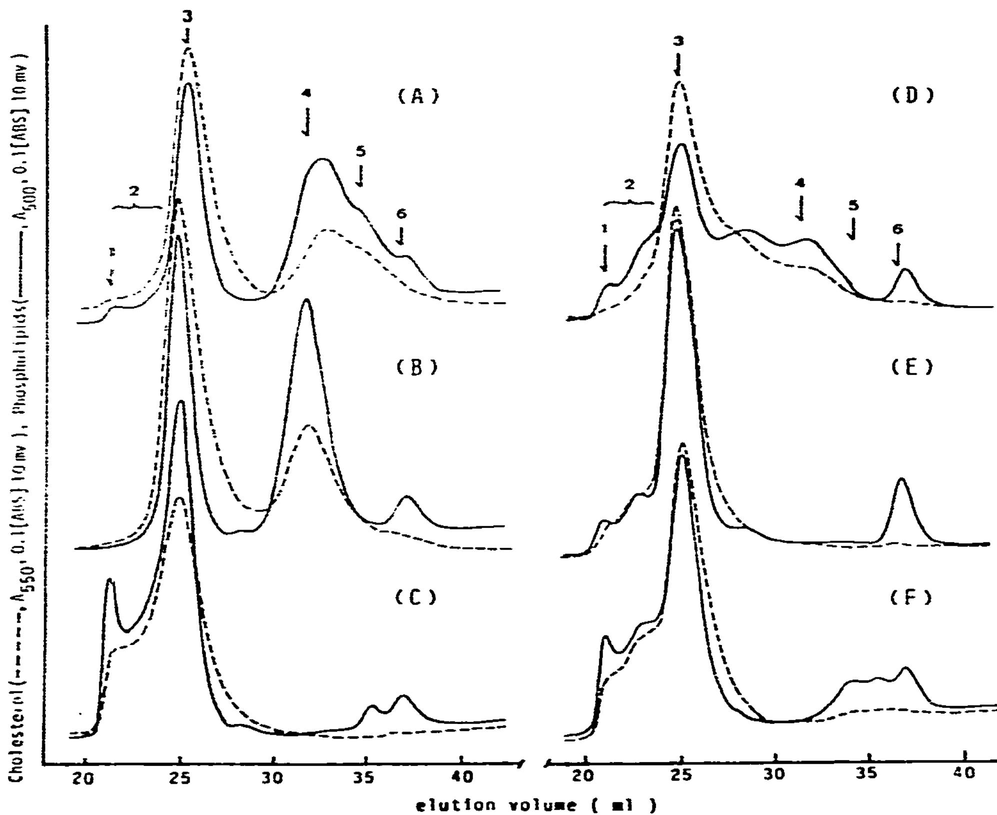


Fig. 6. Elution patterns of choline-containing phospholipids (———) and cholesterol (— — —) for whole serum from patients with various liver diseases. Column: G4000SW+ G3000SW. Sample: whole serum of normal female (A), and from patients with liver cirrhosis (B), acute hepatitis (C), primary biliary cirrhosis (D), intrahepatic cholestasis (E) and drug-induced liver injury (F). Loaded volume: 20 μl for choline-containing phospholipids monitoring, 10 μl for cholesterol monitoring. Detector: choline-containing phospholipids (A_{500}, ———) 0.1 [ABS] 10 mV, cholesterol (A_{550}, — — —) 0.1 [ABS] 10 mV. Elution positions and other HPLC conditions as in Fig. 4.

appearance of peaks other than major classes. It is well known that the liver plays a key role in the synthesis and secretion of lipoproteins and is involved in the uptake of intermediate and end-products of serum lipoprotein metabolism. Therefore, it is not surprising that lipoprotein abnormalities are observed in the elution patterns of liver diseases by our method as a result of hepatic injury. Since these abnormal lipoproteins contain a larger amount of choline-containing phospholipids than normal lipoproteins, the detection of these peaks could be successfully performed by choline-containing phospholipid monitoring as shown in Fig. 6.

Our method for lipoprotein analysis combining the two methods — separation by HPLC using gel permeation columns and selective detection of cholesterol and choline-containing phospholipids by enzymatic reaction — will make progress rapid in the study of lipoprotein metabolism and diagnosis of the various diseases.

We are now examining the characterization of HDL subclasses by combining this HPLC method and the electron-microscopic observations.

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REFERENCES

1 M. Okazaki, Y. Ohno and I. Hara, J. Chromatogr., 221 (1980) 257.
2 Y. Ohno, M. Okazaki and I. Hara, J. Biochem., 89 (1981) 1675.
3 G.M. Kostner, P. Laggner, H.I. Prexl and A. Holasek, Biochem. J., 157 (1976) 401.
4 J.B. Ragland, P.D. Bertram and S.M. Sabesin, Biochem. Biophys. Res. Commun., 80 (1978) 81.
5 D. Saidel, P. Alaupovic and R.H. Furman, J. Clin. Invest., 48 (1969) 1211.
6 M. Okazaki, Y. Ohno and I. Hara, J. Biochem., 89 (1981) 879.
7 M. Okazaki, K. Shiraishi, Y. Ohno and I. Hara, J. Chromatogr., 223 (1981) 285.
8 I. Hara, K. Shiraishi and M. Okazaki, J. Chromatogr., 239 (1982) 549.
9 M. Okazaki, N. Hagiwara and I. Hara, J. Biochem., 91 (1982) 1381.
10 R.J. Havel, H.A. Eder and J.H. Bragden, J. Clin. Invest., 34 (1955) 1345.
11 N. Hagiwara, M. Okazaki and I. Hara, J. Japan Oil Chem. Soc., 31 (1982) 262.
12 M. Takayama, S. Itoh, T. Nagasaki and I. Tanimizu, Clin. Chim. Acta, 79 (1977) 93.
13 E. Gjone and O.M. Orning, Scand. J. Clin. Lab. Invest., 18 (1966) 209.
14 E. Gjone, J.F. Berry and D.A. Turner, J. Lipid Res., 1 (1960) 66.
15 W.C. Vogel, L. Zieve and R.D. Carlton, J. Lab. Clin. Med., 59 (1962) 335.
16 M. Okazaki, N. Hagiwara and I. Hara, J. Biochem., 92 (1982) in press.
17 P.J. Blanche, E.L. Gong, T.M. Forte and A.V. Nichols, Biochim. Biophys. Acta, 665 (1981) 408.
18 W. Patsch, G. Schonfeld, A.M. Gotto and J.R. Patsch, J. Biol. Chem., 255 (1980) 3178.
19 L.A. Hillyard, C. Entenman, H. Feinberg and I.C. Chaikoff, J. Biol. Chem., 214 (1955) 79.
20 G.B. Phillips and L.E. Wille, Clin. Chim. Acta, 49 (1973) 153.
21 P. Alaupovic, S.S. Sanbar, R.H. Furman, M.L. Sullivan and S.L. Walranen, Biochemistry, 5 (1966) 4044.
22 J.R. Patsch, S. Sailer, G. Kostner, F. Sandhofer, A. Holasek and H. Brausteiner, J. Lipid Res., 6 (1974) 356.