Journal of Chromatography, 221 (1980) 257—264
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 688

HIGH-PERFORMANCE AQUEOUS GEL PERMEATION CHROMATOGRAPHY OF HUMAN SERUM LIPOPROTEINS

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(First received May 19th, 1980; revised manuscript received July 17th, 1980)

SUMMARY

A new application of high-performance aqueous gel permeation chromatography was developed for the analysis of human serum lipoproteins. A good combination of columns (TSK GEL, type PW and type SW) was found for the separation of serum lipoproteins: very low-density lipoprotein, low-density lipoprotein and high-density lipoproteins. Analyses of serum lipoproteins from individual normal subjects and pathological subjects were performed by this combination of columns. The effects of pH and salt concentration of the eluent on the separation of lipoproteins were also investigated.

INTRODUCTION

Gel permeation chromatography (GPC) is a type of liquid chromatography in which the separation is based only on the molecular size. With GPC the experimental procedure is simple and the interpretation of the results is easy, but it takes long time and the resolution is low.

Recently GPC columns packed with microspheres of hydrophilic polymer gels (TSK GEL, type PW; Toyo Soda, Tokyo, Japan) and a new chemically modified silica gel based aqueous support (TSK GEL, type SW; Toyo Soda) have become commercially available. These columns can be used under high pressure in aqueous systems and possess a large number of theoretical plates. Moreover, several grades of columns with different pore sizes are available.

High-performance liquid chromatography with these columns for the analysis of many biological substances, such as polypeptides, proteins, enzymes and polysaccharides, has been studied [1—8]. But these GPC columns have not yet been applied to the analysis of human serum lipoproteins. We were the

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0378-4347/80/0000—0000/$02.25

first to establish a method of separation of serum lipoproteins by high-performance GPC using these columns [9,10].

In this paper, the study of the combination of these columns for analysis of lipoproteins is described. The effects of pH and salt concentration of the eluent on the separation of lipoproteins are investigated. A few examples of analysis of normal and pathological sera are also reported. The new method of separating serum lipoproteins by high-performance GPC is useful for the study of lipoprotein metabolism and related diseases.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography was carried out using an HLC 805 (Toyo Soda) equipped with a variable-wavelength absorbance detector.

Ultracentrifugal isolation of lipoproteins from human serum was performed using an RP 505 rotor in an Hitachi 55 P-2 ultracentrifuge (Hitachi, Tokyo, Japan).

Materials and methods

Human sera used in these experiments were obtained from normal and pathological subjects after 12—16 h of fasting. Lipoprotein fractions for analysis by high-performance GPC were isolated from human serum by an ultracentrifugal method [11]. Samples of sera were adjusted to $d = 1.21$ with solid sodium bromide and diluted to five volumes with aqueous sodium bromide solution of $d = 1.21$. Aliquots of 5.0 ml of the solution were transferred to 6.0-ml centrifuge tubes and centrifuged at 105,000 *g* for 24 h at 8°C. After centrifugation, the top fractions of 0.5—1.0 ml containing lipoproteins were collected. The lipoprotein fractions prepared by this procedure did not contain the serum proteins except for a small amount of albumin as demonstrated by immunological assay.

Of these samples 20—40 μl were subjected to high-performance liquid chromatography on the HLC 805 equipped with GPC columns (TSK GEL, type PW and type SW) and eluted with 0.1 *M* Tris-HCl buffer (pH 7.4) at a flow-rate of 1.0 ml/min. The lipoprotein fractions were monitored by the absorbance at 280 nm. In the combined column system, the columns were connected by stainless-steel tubing with an internal diameter of 0.4 mm.

Ovalbumin dissolved in 0.15 *M* NaCl at a concentration of 1.0% was used to study the relationship between molecular weight and elution volume for the combination of GPC columns.

RESULTS AND DISCUSSION

Serum lipoproteins are classified according to their densities into very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoproteins (HDL_2 and HDL_3). The molecular weights and the chemical compositions of each lipoprotein class are summarized in Table I.

For analysis of serum lipoproteins by high-performance GPC we used the lipoprotein fractions ($d < 1.21$) of human serum prepared by the ultracen-

TABLE I

CHEMICAL COMPOSITION BY DRY WEIGHT OF HUMAN SERUM LIPOPROTEINS [12]

Particle*	Molecular weight ($\times 10^{-6}$)	Density (g/cm^3)	Protein (%)	Phospholipid (%)	Triglyceride (%)	Cholesterol (%)
VLDL	19.6	0.97	8	18	50	19
LDL	2.3	1.035	21	22	11	45
HDL_2	0.36	1.09	41	30	4.5	21.4
HDL_3	0.18	1.15	55	23	4.1	14.9

*VLDL = very low-density lipoprotein; LDL = low density lipoprotein; HDL = high-density lipoprotein.

trifugal method as described in the experimental part. These fractions did not contain the serum proteins having a molecular weight similar to those of lipoproteins. Therefore, the peaks of lipoproteins can be monitored by the absorbance at 280 nm. The small amount of albumin remaining in the lipoprotein fractions did not interfere with the detection of lipoproteins by absorbance at 280 nm.

The properties of the GPC columns used in this experiment are shown in Table II. Each column was 600 mm long with an internal diameter of 7.5 mm.

TABLE II

PROPERTIES OF TSK GEL COLUMNS

Grade	Particle size (μm)	Exclusion molecular weight		Theoretical plate number (plates/ft.)
		Protein	Dextran	
G6000PW	17 ± 2	—	3×10^7	3000
G5000PW	17 ± 2	—	7×10^6	3000
G4000SW	13 ± 2	1×10^6	6×10^5	> 5000
G3000SW	10 ± 2	3×10^5	1×10^5	> 5000

A mixture of lipoprotein fractions (sample 1) containing VLDL, LDL, HDL and albumin was examined using a single column (G3000SW, G4000SW, G5000PW and G6000PW), and their separation patterns monitored by absorbance at 280 nm are shown in Fig. 1 a–d, respectively. Though the G3000SW column gave very sharp peaks, only three peaks were observed and VLDL and LDL eluted as one peak at the G3000SW exclusion volume (Fig. 1a). In the case of the G4000SW column, four peaks were obtained (Fig. 1d). The G-5000PW column (Fig. 1c) and the G6000PW column (Fig. 1d) divided the high-molecular-weight fractions into two peaks and were useful for analysis of VLDL and LDL. But in both columns only three peaks were observed, and HDL and albumin eluted as one peak. From these experiments, it is concluded that a combination of GPC columns (G3000SW and G5000PW or G6000PW) was required for analysis of all lipoprotein fractions.

Examination of the analysis of the same sample as in Fig. 1 (sample 1) and the mixed lipoprotein fraction (sample 2) containing VLDL, LDL, HDL_2, HDL_3 and albumin, was carried out using combinations of columns composed

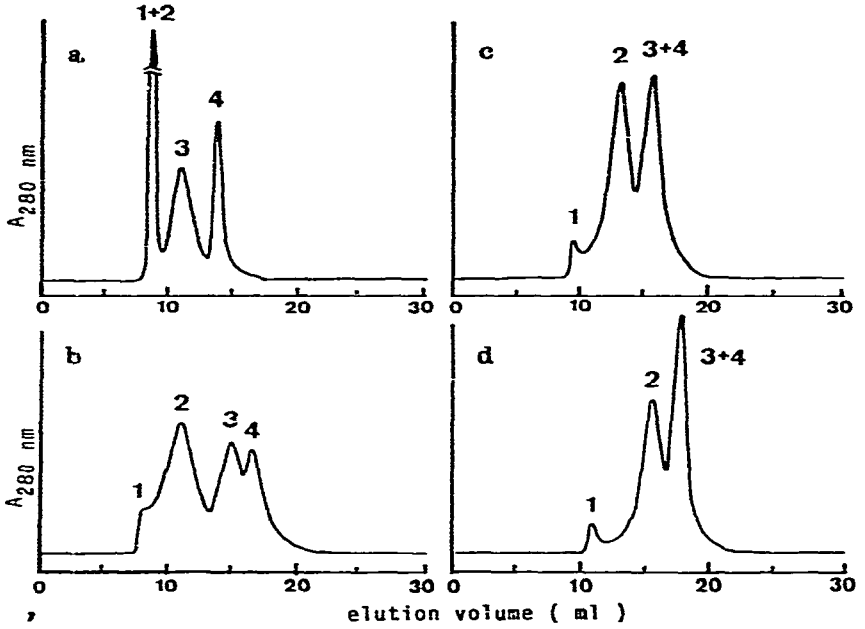

Fig. 1. Elution curves of a mixed lipoprotein fraction (sample 1) by a single column (600 mm × 7.5 mm I.D.). Column: (a) G3000SW; (b) G4000SW; (c) G5000PW; (d) G6000PW. Eluent: 0.1 *M* Tris-HCl buffer (pH 7.4). Flow-rate: 1.0 ml/min. Load volume: 20 µl. Temperature: room temperature. Peaks: 1 = VLDL; 2 = LDL; 3 = HDL; 4 = albumin.

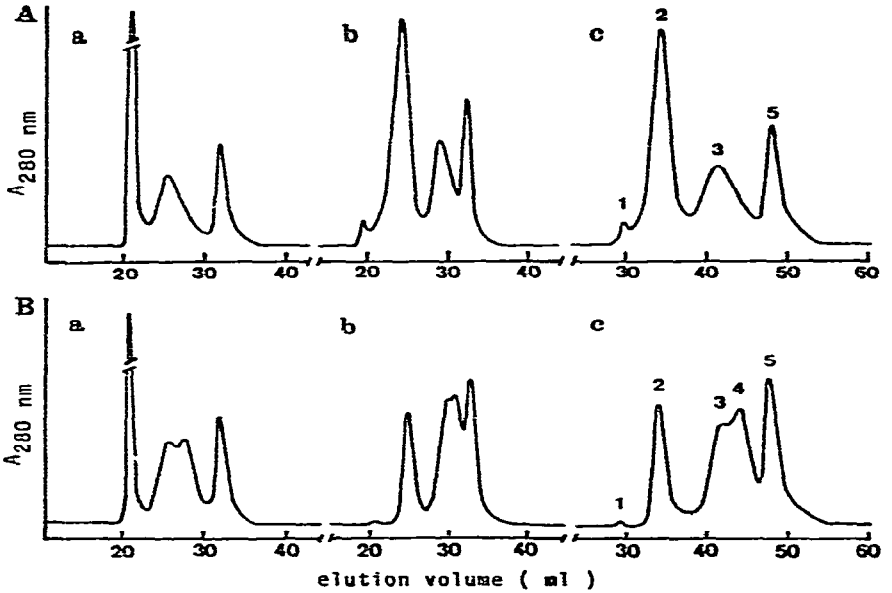

Fig. 2. Elution curves of mixed lipoprotein fractions (A, sample 1; B, sample 2) by combination columns of G3000SW and G5000PW. Column: (a) G3000SW × 2 (G3000SW + G3000SW, 1200 mm × 7.5 mm I.D.); (b) = G5000PW + G3000SW (1200 mm × 7.5 mm I.D.); (c) G5000PW + G3000SW × 2 (G5000PW + G3000SW + G3000SW, 1800 mm × 7.5 mm I.D.). Peaks: 1 = VLDL; 2 = LDL; 3 = HDL_2, 4 = HDL_3; 5 = albumin. Conditions as in Fig. 1.

of G5000PW and G3000SW. Their separating profiles are shown in Fig. 2. The combination columns, composed of G5000PW and G3000SW (G5000PW + G3000SW, G5000PW + G3000SW × 2), separated all lipoprotein fractions, i.e. VLDL, LDL, HDL_2 and HDL_3 (Fig. 2b and c).

The same experiments were performed using combinations of columns composed of G6000PW and G3000SW (G6000PW + G3000SW and G6000PW + G3000SW × 2), and similar elution patterns to those in Fig. 2 were obtained.

In Fig. 3 the results of the examination of GPC columns for the separation of lipoproteins in these experiments are summarized. Fig. 3 shows the relation

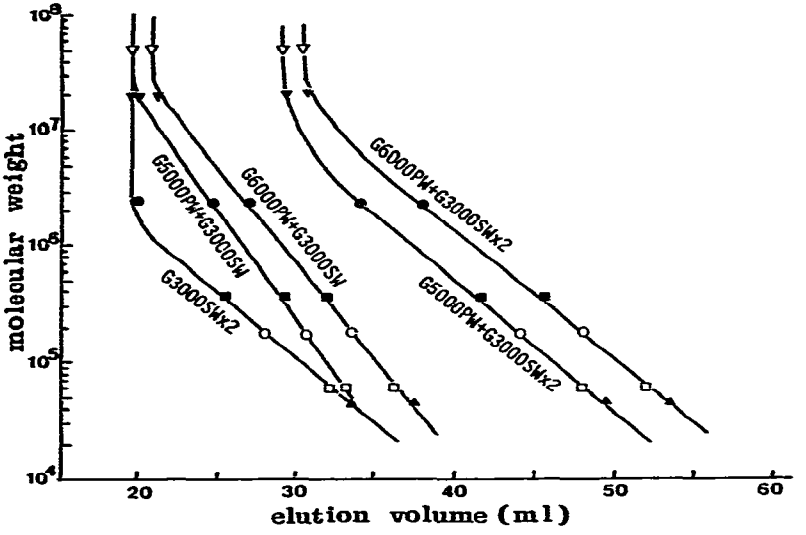

Fig. 3. The relation between molecular weight of lipoproteins and elution volume for the combination GPC columns. Conditions as in Fig. 1. ▽, chylomicron; ▼, VLDL; ●, LDL; ■, HDL_2; ○, HDL_3; □, albumin; ▲, ovalbumin.

between molecular weight of lipoproteins (VLDL, LDL, HDL_2 and HDL_3) and other samples (chylomicron, human serum albumin and ovalbumin) and their elution volume using 0.1 *M* Tris-HCl buffer solution (pH 7.4) as eluent at a flow-rate of 1.0 ml/min. The G3000SW column was useful for the separation of low-molecular-weight fractions less than 5×10^5; namely HDL_2, HDL_3 and albumin. This corresponds to the finding that the G3000SW column is optimum for the separation of most proteins of molecular weight between 4×10^4 and 4×10^5 [6]. The separation of high-molecular-weight fractions (VLDL and LDL) was carried out by the combination columns containing G5000PW or G6000PW. In the case of the combined columns (G5000PW + G3000SW and G6000PW + G3000SW), all of the plots for VLDL, LDL, HDL_2 and HDL_3 were linear and these column systems may be used to determine the molecular weights of lipoproteins.

It is found from Figs. 2 and 3 that the combined columns containing two G3000SW (G3000SW × 2, G5000PW + G3000SW × 2 and G6000PW + G3000SW × 2) are suitable for the separation of the fractions HDL_2 and HDL_3. Subsequently, the combination columns composed of G5000PW and two G3000SW or G6000PW and two G3000SW are good for analysis of all lipoprotein fractions.

The dependence of the separation of lipoproteins on pH and salt concentration of the eluent was examined using the combination of columns (G6000PW+ G3000SW × 2). The elution patterns of a mixed lipoprotein fraction (sample 3) containing VLDL, LDL, HDL_2, HDL_3 and albumin, with various eluents are shown in Fig. 4. The elution profiles with each eluent were similar, but the

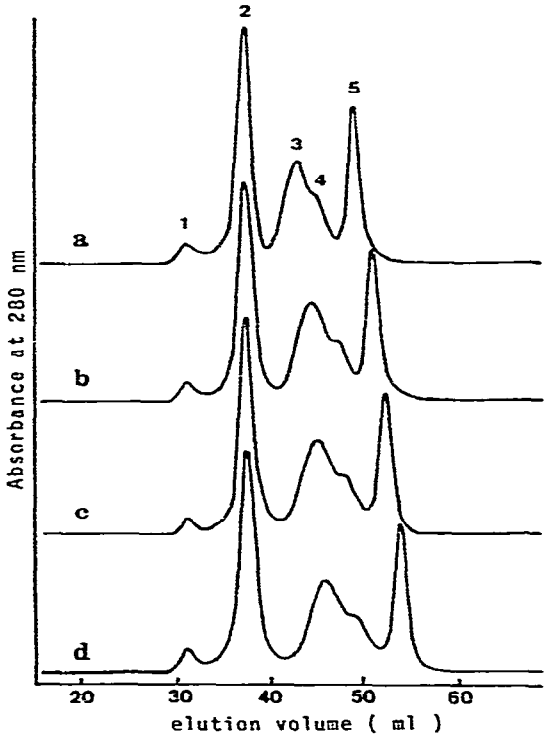

Fig. 4. Elution curves of a mixed lipoprotein fraction (sample 3). Column: G6000PW + G3000SW × 2. Eluent: (a) 0.1 *M* Tris-HCl buffer (pH 8.4); (b) 0.1 *M* Tris-HCl buffer (pH 8.4) containing 0.15 *M* NaCl; (c) 0.1 *M* Tris-HCl buffer (pH 7.4); (d) 0.15 *M* NaCl (pH 6—6.2). Flow-rate: 1.0 ml/min. Load volume: 40 µl. Peaks as in Fig. 2.

elution volumes of HDL fractions and albumin increased with a lower pH and addition of sodium chloride. On the other hand, the elution volumes of VLDL and LDL were not influenced by pH and salt concentration. Fig. 5 shows the relation between molecular weight and elution volume for LDL, HDL_2, HDL_3 and albumin using various kinds of eluents. It was found from Figs. 4 and 5 that each medium examined in this experiment could be used as eluent for analysis of lipoproteins and that lowering the pH and adding salts tended to increase the elution volumes of the fractions HDL_2 and HDL_3.

The analysis of the lipoprotein fractions prepared from the individual subjects by the ultracentrifugal method was achieved using the combination of columns (G5000PW + G3000SW × 2). Results of analysing two samples from normal subjects (subject 1 = young female; subject 2 = old male) and four examples of pathological subjects (subject 3 = coronary heart disease; subject 4 = liver cirrhosis; subjects 5 and 6 = hyperlipidemia) are shown in Fig. 6a—f,

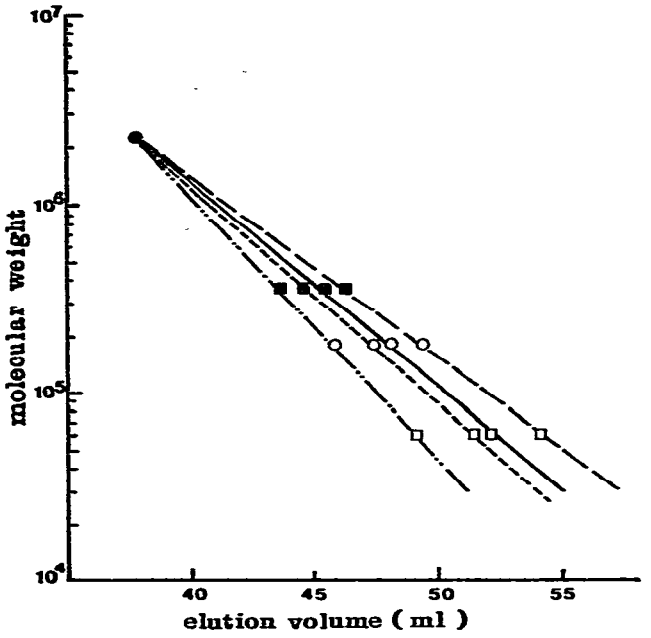

Fig. 5. Effect of pH and salt concentration of eluent on elution volume of each lipoprotein (— ·· — ··, 0.1 *M* Tris-HCl buffer (pH 8.4); - - - - - -, 0.1 *M* Tris-HCl buffer (pH 8.4) containing 0.15 *M* NaCl; ———, 0.1 *M* Tris-HCl buffer (pH 7.4); — — —, 0.15 *M* NaCl (pH 6—6.2). Conditions as in Fig. 4. •, LDL; ▪, HDL_2; ○, HDL_3; □, albumin.

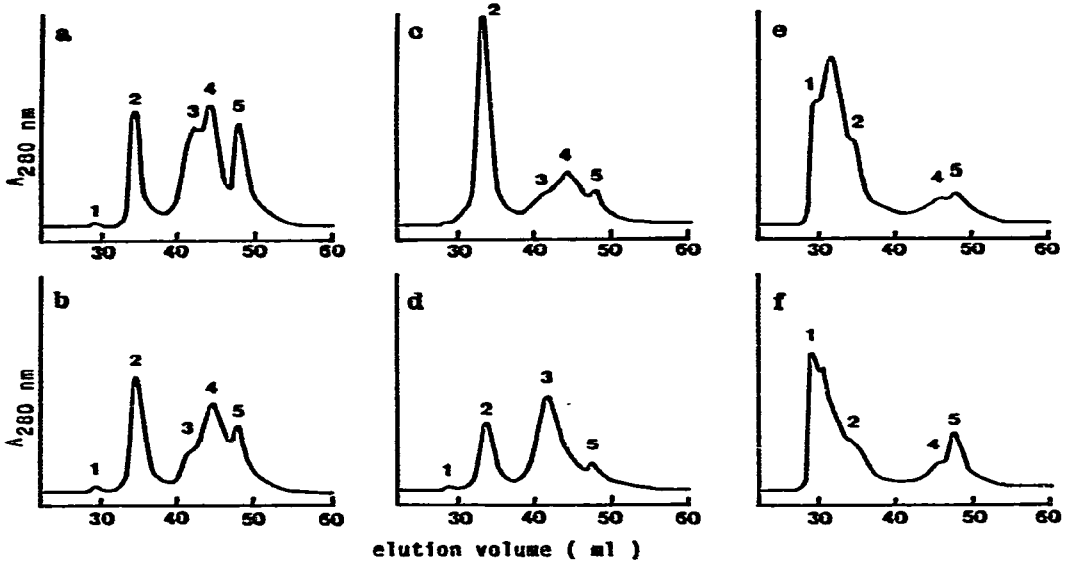

Fig. 6. Analysis of serum lipoproteins for individual subjects. (a) subject 1 (young female); (b) subject 2 (old male); (c) subject 3 (coronary heart disease); (d) subject 4 (liver cirrhosis); (e and f) subjects 5 and 6 (hyperlipidemia). Column: G5000PW + G3000SW × 2. Eluent: 0.1 *M* Tris-HCl buffer (pH 7.4). Flow-rate: 1.0 ml/min. Load volume: 20 μl. Peaks as in Fig. 2.

respectively. The level of each lipoprotein was found to vary with individual subjects. Especially in the case of pathological subjects, there were distinct patterns with respect to each disease. Coronary heart disease (Fig. 6c) had a higher LDL level and lower HDL level than those of normal subjects. In the case of liver cirrhosis, total lipoprotein levels were low and HDL fractions were mainly composed of HDL_2 (Fig. 6d). Hyperlipidemia (Fig. 6e and f) had characteristic patterns with a large amount of high-molecular lipoprotein fractions containing the fractions intermediate between VLDL and LDL.

From these experiments it was found that much information about each lipoprotein level in human serum could be obtained from the lipoprotein fraction ($d < 1.21$) by high-performance GPC.

This method of separation may be very convenient for the preparation and analysis of lipoproteins because of its short experimental time and high resolution. Moreover, analysis of cholesterol, triglyceride and phospholipid in each lipoprotein fraction can be performed by appropriate measurements after the separation by high-performance GPC. This analytical method is useful for the study of lipoprotein metabolism and related diseases.

In our laboratory, the micromethod of rapid analysis of cholesterol in each lipoprotein fraction was established using this method of separation [10], and studies of the relation between cholesterol content of each lipoprotein fraction and related diseases are being continued.

ACKNOWLEDGEMENTS

The authors deeply appreciate the great help of Mr. K. Sakane (Toyo Soda Manufacturing Co.) in these experiments and the serum collection of Dr. A. Tanaka, Tokyo Medical and Dental University.

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