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Anion-exchange high-performance liquid chromatographic assay of plasma lipoproteins of rabbits, rats and mice

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Abstract

A fast, accurate and precise high-performance liquid chromatographic assay method has been developed for plasma lipoproteins of experimental animals, rabbits, rats and mice. The method includes complete separation of high, low and very low density lipoproteins from one another within 20 min by a DEAE–glucomannan gel using stepwise elution, and determination by postcolumn reaction with an enzymatic cholesterol reagent as the total cholesterol level. The relative standard deviation of each lipoprotein assay was highly reproducible, being less than 2.0 and 2.4% for repeatability and intermediate precision, respectively. The method was successfully applied to the assays of plasma lipoproteins in three species of normolipidemic and hyperlipidemic animals.

Keywords: Hypercholesterolemia; Lipoproteins

1. Introduction

The major classes of lipoproteins in plasma are known as high, low and very low density lipoproteins (HDL, LDL and VLDL, respectively) and each lipoprotein plays a defined role in the transport of cholesterol and triglycerides. It is known that the initiation and progression of atherosclerosis and its related vascular diseases are facilitated by abnormal lipoprotein metabolism, including many kinds of modified lipoproteins [1–4]. Thus, it is of vital importance to develop a fast, accurate and precise method for the assay of lipoproteins in plasma and tissues.

The methods employed for separation and determination of these lipoproteins have included precipitation [5], electrophoresis [6], ultracentrifugation [7] and high-performance liquid chromatography (HPLC) [8–11]. Many reports refer to human samples, while only a few reports deal with the assay of the lipoprotein of experimental animals such as rabbit, rat and mouse, which are useful for studies of hypercholesterolemia, atherosclerosis and their related diseases. The ultracentrifugation method, which is mainly used for the assay of lipoprotein of experimental animals, requires a long time for analysis and has low reproducibility and poor resolution. Particularly, the characteristics of lipoproteins differ among experimental animals, and between normolipidemic and hyperlipidemic animals. Thus, it is difficult to separate completely the three lipoproteins in experimental animals by the ultracentrifugation method. HPLC methods previously reported for the assay of animal lipoproteins [12,13] could not attain

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complete separation of HDL, LDL and VLDL. Recently, we developed an anion-exchange HPLC assay method for human plasma lipoproteins [14], where the three human lipoproteins were completely separated by stepwise elution and determined fluorometrically based on the postcolumn reaction with an enzymatic cholesterol reagent as the total cholesterol (TC) level. This paper reports a modified HPLC assay method for the plasma lipoproteins of rabbits, rats and mice. Also, the optimized HPLC method was applied to the assay of the lipoproteins in three species of normolipidemic and hyperlipidemic animals.

2. Experimental

2.1. Materials and chemicals

Cholesterol oxidase (EC 1.1.3.6; 18 U/mg) from *Streptomyces* sp, peroxidase (EC 1.11.1.7; 134 U/mg) from horseradish and cholesteryl ester hydrolase (EC 3.1.1.13; 144 U/mg) from *Pseudomonas* sp were purchased from Toyobo (Osaka, Japan), homovanillic acid from Tokyo Kasei (Tokyo, Japan), and Triton X-100 and EDTA from Nacalai Tesque (Kyoto, Japan). Lipid Calibrator from Wako (Osaka, Japan) was used for the standard solution of TC. Other chemicals of analytical reagent grade were purchased from Nacalai Tesque and used without further purification. Water purified with a Milli Q Jr. (Millipore, Tokyo, Japan) was used for the preparation of the eluents.

2.2. Chromatography

The experimental setup used in this study is as reported previously [14]. Briefly, DEAE–glucomannan gel [15,16], which is not commercially available at present, was kindly donated by Kurita Industries (Tokyo, Japan). It was packed into a 50 mm×4.6 mm I.D. column using water as the slurry and packing solvent and used for the separation of the three lipoproteins. The eluents used were as follows: eluent A, 20 m*M* sodium phosphate buffer (pH 7.0) containing 1×10^{-3} *M* EDTA; eluent B, 500 m*M* sodium chloride containing 1×10^{-3} *M* EDTA. Although the optimal conditions were different for the different experimental animals, each lipoprotein in plasma was separated by stepwise elution using eluents A and B. The flow-rate was maintained at 1.0 ml/min. The separation was carried out at 25°C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan).

The same postcolumn reaction conditions reported previously [14] were used for the determination of TC levels of each lipoprotein. A cholesterol reagent, cholesteryl ester hydrolase (5 μg/ml, 0.7 U/ml), cholesterol oxidase (20 μg/ml, 0.4 U/ml), peroxidase (50 μg/ml, 7 U/ml) and homovanillic acid (500 μg/ml), was dissolved in 20 m*M* sodium phosphate buffer (pH 7.0) containing 0.2% Triton X-100, and delivered at a flow-rate of 0.5 ml/min. Further, a 0.1 *M* sodium hydroxide solution was delivered at a flow-rate of 0.5 ml/min to alkalize the effluent. After the postcolumn reaction with these reagents, lipoproteins were detected at an excitation wavelength of 325 nm and an emission wavelength of 420 nm using a RF-535 spectrofluorometer (Shimadzu, Kyoto, Japan). To determine the TC level of HDL, LDL and VLDL (HDL-TC, LDL-TC and VLDL-TC, respectively), the TC of a sample (the sum of HDL-TC, LDL-TC and VLDL-TC) was measured according to our previously reported method [17]. The peak area of each lipoprotein was proportional to the TC level of the lipoprotein. HDL-TC, LDL-TC and VLDL-TC were calculated from the relative peak areas of the three HPLC peaks and TC levels.

For size-exclusion chromatography, a TSK-gel Lipopropak column (300 mm×7.5 mm I.D.) and eluent for the analysis of lipoproteins, which were kindly donated by Tosoh (Tokyo, Japan), were used. The flow-rate was 1.0 ml/min. The same postcolumn reaction conditions described above were used for detection of lipoproteins.

2.3. Animals

Male Japanese white (JW) rabbits (about 2 kg), normolipidemic rabbits, were purchased from Shimizu Laboratory Animals (Kyoto, Japan) and both male and female Watanabe heritable hyperlipidemic (WHHL) rabbits (2–2.5 kg), hyperlipidemic rabbits, were obtained from our colony bred from mating pairs of homozygous WHHL rabbits kindly supplied

by Dr. T. Kita (Department of Geriatric Medicine, Faculty of Medicine, Kyoto University, Japan), originally obtained from Dr. Y. Watanabe (Institute for Experimental Animals, School of Medicine, Kobe University, Japan). Both rabbits were fed standard rabbit chow (RC-4, Oriental Yeast, Tokyo, Japan).

Six-week-old male Sprague–Dawley (SD) rats and ICR mice (both animals from Japan SLC, Hamamatsu, Japan) were maintained for 8 weeks on a basal diet or a high-cholesterol diet. The basal diet contained 20% casein, 63.2% sucrose, 10% corn oil, 2% agar, 0.8% vitamin mixture and 4% salt mixture. The high-cholesterol diet consisted of the basal diet including 1.5% cholesterol and 0.5% cholic acid. All animals were housed in an air-conditioned room (23±1°C, 60±10% humidity) under an artificial 12-h light/dark cycle (7:00 a.m. to 7:00 p.m.). Each animal was allowed free access to their diets and water.

2.4. Preparation of blood samples

Blood samples were obtained from various normolipidemic and hyperlipidemic animals. Blood was drawn into tubes containing EDTA at a final concentration of 1 mg/ml. Plasma was separated by centrifugation (1500 *g* for 10 min) from blood, stored at 4°C and used for HPLC assay within 7 days.

2.5. Ultracentrifugation method

The VLDL fractions ($d<1.006$ g/ml) of experimental animals were separated by ultracentrifugation (400 000*g* for 250 min at 4°C) from plasma samples using a Beckman TL-100E ultracentrifuge with a TLA 100.1 fixed-angle rotor (both apparatus from Beckman Instruments, Palo Alto, CA, USA) [7].

2.6. Precipitation method

The HDL fraction was separated from a portion of the plasma samples by the heparin–manganese precipitation procedure [18]. In order to prevent the inhibitory action of Mn^{2+} on the enzymatic reaction, a 50 ml aliquot of the HDL fraction was mixed with 50 μl 0.15 *M* EDTA solution.

2.7. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of HDL, LDL and VLDL fractions separated by the HPLC method was performed as described by Laemmlis [19]. Aliquots of the HDL, LDL and VLDL fractions were loaded on SDS-PAGE and stained with Coomassie Brillian Blue.

2.8. Method validation: repeatability, intermediate precision and calibration graph

The repeatability and intermediate precision data were obtained from the assay of a plasma sample from normolipidemic animals. The samples were stored at 4°C until assay. The repeatability and intermediate precision tests were performed for five and three replicates, respectively. The calibration graph was checked as described previously [14], and was linear with a correlation coefficient of 0.998.

3. Results and discussion

3.1. Separation of HDL, LDL and VLDL

Previously, we attained separation of lipoproteins in human plasma by the HPLC method using a DEAE–glucomannan gel and stepwise elution [14]. HDL, LDL and VLDL were eluted in this order by increasing the salt concentration. The characterization of HDL, LDL and VLDL was performed using the β-lipoprotein fraction obtained by the precipitation procedure and the VLDL fraction obtained by the ultracentrifugation procedure: the β-lipoprotein precipitation fraction included only LDL and VLDL, and the ultracentrifugation VLDL fraction included VLDL. The TC level of each lipoprotein obtained with the optimized HPLC method agreed well with that obtained with the ultracentrifugation or precipitation method.

In the present study we tried to separate each lipoprotein of the experimental animals based on the HPLC method used for the determination of human

Table 1
Stepwise elution conditions for each animal[a]

Time (min)	Eluent B (%)		
	Rabbit	Rat	Mouse
0–2	0	0	0
2–7	35	55	50
7–12	55	70	40
12–22	100	100	100

[a]Eluent A, 20 m*M* sodium phosphate buffer (pH 7.0) containing 1×10^{-3} *M* EDTA; eluent B, 500 m*M* sodium chloride containing 1×10^{-3} *M* EDTA. The initial condition is 100% eluent A.

plasma lipoproteins with a slight modification. Two eluents, A and B, which were 20 m*M* sodium phosphate buffer (pH 7.0) and 500 m*M* sodium chloride (both including 10^{-3} *M* EDTA), respectively, were used for stepwise elution. As shown in Table 1, the HDL, LDL and VLDL of rabbits were eluted with 25, 55 and 100% eluent B, respectively; 60, 75 and 100% eluent B for rats and 50, 60 and 100% eluent B for mice were used. The HDL, LDL and VLDL were separated completely within 20 min, as shown in Fig. 1. As shown in Fig. 2, the HDL precipitation and ultracentrifugation VLDL fractions of a mouse plasma sample included only HDL and VLDL, respectively. Further, apolipoproteins included in the HDL, LDL and VLDL fractions were examined by SDS-PAGE to characterize the separated lipoprotein fractions, as shown in Fig. 3. This shows that the VLDL fraction includes apolipoproteins B, C and E, the LDL fraction only apolipoprotein B, and the HDL fraction apolipoproteins A, C and E. The HDL fraction included a large amount of albumin. However, this did not interfere with the HDL assay by the stepwise HPLC method, because the HDL was detected as the total cholesterol level.

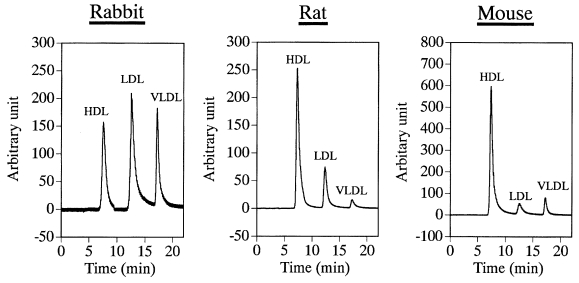

Fig. 1. Chromatograms of normolipidemic plasma samples from rabbit, rat and mouse. A 10-μl aliquot of each plasma sample was injected. HPLC conditions: column, a 50 mm×4.6 mm I.D. stainless column packed with a DEAE–glucomannan gel; eluents, 20 m*M* sodium phosphate buffer (pH 7.0) containing 1×10^{-3} *M* EDTA for eluent A and 500 m*M* sodium chloride containing 1×10^{-3} *M* EDTA for eluent B; flow-rate, 1.0 ml/min; detection, excitation wavelength at 325 nm and emission wavelength at 420 nm. For the stepwise elution pattern for each animal, see Table 1. For the postcolumn reaction conditions, see Experimental section.

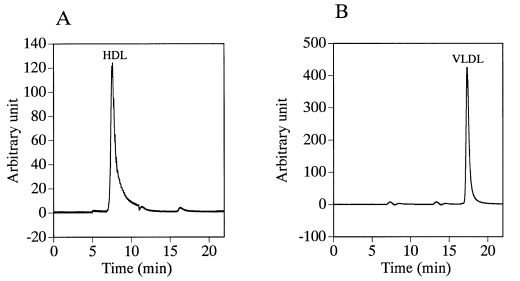

Fig. 2. HPLC separation of the HDL fraction obtained by the heparin–manganese precipitation procedure (A) and the VLDL fraction obtained by ultracentrifugation (B) in mice. HPLC conditions as in Fig. 1.

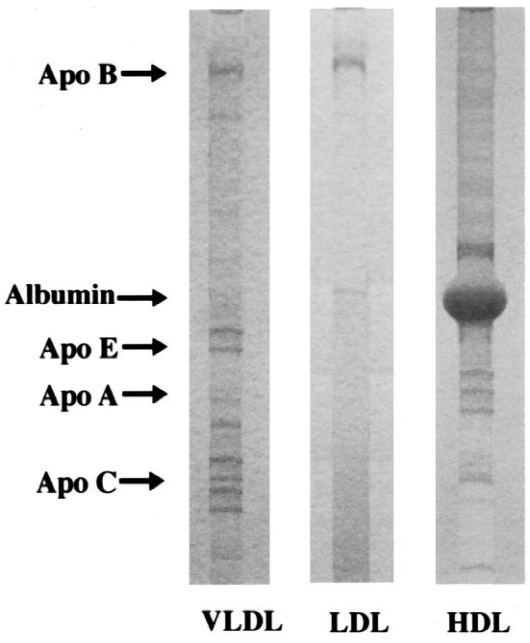

Fig. 3. SDS-PAGE of VLDL, LDL and HDL fractions separated by the optimized HPLC method. Aliquots of lipoproteins were loaded on SDS-PAGE and stained with Coomassie Brillian Blue. The position of each apolipoprotein is indicated by an arrow.

Table 2
Lipid compositions of HDL, LDL and VLDL fractions separated by HPLC[a] in a normolipidemic rabbit

Lipoprotein	Total cholesterol (%)	Triglycerides (%)	Phospholipids (%)
HDL	37.1	14.4	48.5
LDL	44.2	28.7	27.1
VLDL	22.3	19.1	58.6

[a]Experimental conditions given in the text.

Table 2 shows the lipid compositions of the HDL, LDL and VLDL fractions separated by the optimized HPLC method in normolipidemic rabbit. The lipid compositions of the three lipoproteins in rabbit plasma samples obtained by the present authors were consistent with the results reported by Pescador [20]. These results reveal that the three HPLC peaks in Fig. 1 should correspond to HDL, LDL and VLDL. Similar results were obtained for other animal samples.

The concentration of sodium chloride required for elution of HDL and LDL varied with the animal species. Alexander and Day [21] reported that the migration patterns of lipoproteins in agarose gel electrophoresis were very different for animals of several species. Our results agreed with theirs. These results reveal that the HPLC conditions are required to be optimized individually in order to separate and determine each lipoprotein of the experimental animals.

3.2. Cross-validation with the optimized HPLC and size-exclusion HPLC methods

Table 3 shows cross-validation data between the optimized HPLC method and a size-exclusion HPLC method using a TSK-gel Lipopropak column. Good correlation was obtained for the HDL and LDL+VLDL values of normolipidemic plasma samples between the two methods. As shown in Fig. 4, HDL was separated from LDL and VLDL, while LDL overlapped with VLDL with the size-exclusion HPLC method. Further, the HDL in WHHL rabbit plasma is observed with a very small shoulder. Thus,

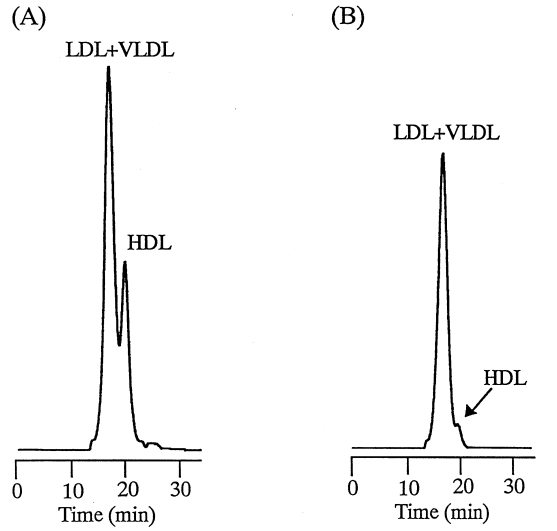

Fig. 4. Chromatograms of plasma samples from JW (A) and WHHL (B) rabbits. A 10-μl aliquot of each plasma sample was injected. HPLC conditions: column, TSK-gel Lipopropak (300 mm×7.5 mm I.D.); eluent, eluent for analysis of lipoprotein (Tosoh); flow-rate, 1.0 ml/min. For the postcolumn reaction conditions, see Experimental section.

Table 3
Cross-validation data for the optimized anion-exchange HPLC and size-exclusion HPLC methods on normolipidemic plasma of rabbits and mice [each lipoprotein-TC level/plasma-TC level (%)]

	Rabbit plasma		Mouse plasma	
HPLC method:	Anion-exchange	Size-exclusion	Anion-exchange	Size-exclusion
HDL	23.9±1.5	25.9±3.5	83.0±0.3	85.6±0.4
LDL+VLDL	76.1±1.7	74.1±4.3	17.0±0.4	14.4±0.3

Each value represents mean±SE. The number of animals used was three.

Table 4
Repeatability and intermediate precision data for assay of normolipidemic plasma[a]

Lipoprotein	Repeatability[b]			Intermediate precision[c]		
	Mean peak area ($\times$mV·s$\times10^5$)	Estimated TC level[e] (mg/100 ml)	RSD[d] (%)	Mean peak area ($\times$mV·s$\times10^5$)	Estimated TC level[e] (mg/100 ml)	RSD[d] (%)
HDL	1.372	13.9	1.4	1.394	14.1	2.3
LDL	3.284	33.3	1.5	3.249	32.9	2.4
VLDL	0.321	3.3	2.0	0.326	3.4	1.7

[a]Experimental conditions are given in the text.
[b]Five replicates.
[c]Three replicates.
[d]Relative standard deviation.
[e]Total cholesterol.

the HDL and LDL+VLDL values of normolipidemic plasma samples were compared for the two methods.

3.3. Repeatability and intermediate precision

Table 4 shows the repeatability and intermediate precision of HDL, LDL and VLDL assays in the plasma sample of a normolipidemic rabbit. The relative standard deviation of each lipoprotein assay was highly reproducible, being less than 2.0 and 2.4% for repeatability and intermediate precision, respectively.

3.4. Application to determination of plasma lipoproteins

Table 5 shows the HDL-TC, LDL-TC, VLDL-TC and TC levels of plasma samples from three species of normolipidemic and hyperlipidemic animals. The main lipoprotein in normolipidemic rabbit plasma was LDL. The plasma TC level of hyperlipidemic WHHL rabbits was markedly higher than that of normolipidemic JW rabbits. The main lipoprotein increase was LDL in WHHL rabbit plasma. This result is consistent with a previous report [22] that the LDL-TC level of WHHL rabbits increases markedly.

The main lipoprotein in the plasma from normolipidemic rats and mice was HDL. The plasma TC levels of rats and mice were increased by feeding the high-cholesterol diet. The main lipoprotein increased was LDL in rat plasma and VLDL in mice plasma. Increased LDL-TC and VLDL-TC levels in hyperlipidemic rats and mice, respectively, have been reported previously [23,24]. Typical chromatograms of normolipidemic plasma and hyperlipidemic plasma from rabbit, rat and mouse are shown in Fig. 5. The LDL-TC level of WHHL rabbits was 86-fold higher than the HDL-TC level, as shown in Fig. 5. The present method can determine the TC level of each lipoprotein even if the differences are very large. This is ascribable to the complete separation of

Table 5
Quantitation of total cholesterol level for each lipoprotein in normolipidemic and hyperlipidemic plasma from rabbits, rats and mice (mg/100 ml)

	Rabbit		Rat		Mouse	
	Normolipidemic	Hyperlipidemic[a]	Normolipidemic	Hyperlipidemic[b]	Normolipidemic	Hyperlipidemic[b]
Plasma TC	46.4±2.5	505±14	67.0±1.3	169.2±1.7	181±2	358±19
HDL-TC	11.1±1.0	5±1	45.6±1.5	58.1±0.7	150±2	102±7
LDL-TC	32.0±1.6	430±13	18.8±0.2	107.2±1.2	15±1	126±7
VLDL-TC	3.4±0.3	70±7	2.6±0.1	3.9±0.2	16±1	130±7

Each value represents mean±SE.
[a] WHHL rabbits.
[b] Animals fed the high-cholesterol diet.

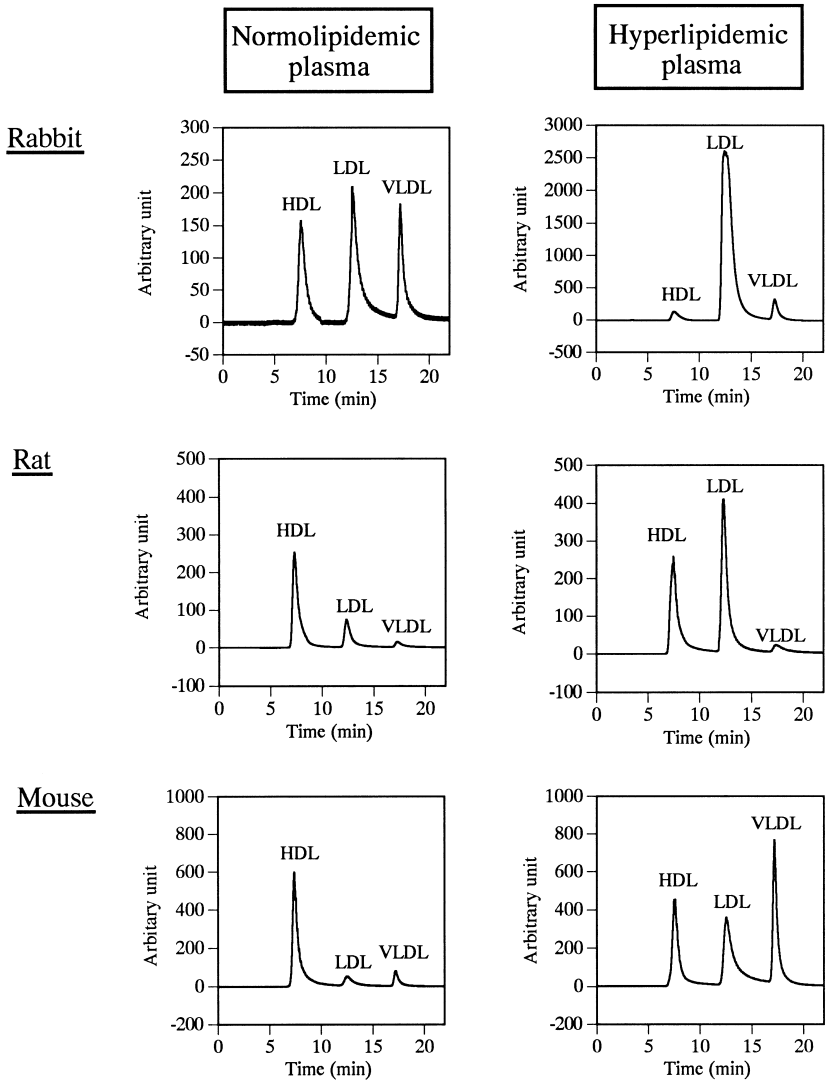

Fig. 5. Chromatograms of normolipidemic and hyperlipidemic plasma samples from rabbit, rat and mouse. A 10-μl aliquot of each plasma sample was injected. The HPLC conditions are as in Fig. 1.

each lipoprotein. The TC levels of the three lipoproteins in rabbit and rat plasma samples obtained by the present authors are very similar to the results reported by Kieft et al. [13]. These results reveal that the optimized HPLC method is suitable for the determination of the lipoproteins of experimental animals.

It has been reported that oxidatively modified LDL and β-VLDL play an important role in the initiation of atherosclerosis [25,26]. Recently, the existence of modified lipoproteins in plasma and serum has been confirmed by many investigators [27–30]. The broad peak of the LDL of a WHHL rabbit plasma sample (Fig. 5) may be due to the concomitant elution of a modified LDL. On the other hand, the β-VLDL might be eluted with VLDL in hyperlipidemic mice fed a high-cholesterol diet (Fig. 5). Further study is required to clarify the presence of oxidatively modified LDL and β-VLDL in plasma.

We conclude that the present optimized HPLC

method is fast, accurate and precise. Also, it can completely separate each lipoprotein and determine the TC level of each lipoprotein in normolipidemic and hyperlipidemic plasma from experimental animals.

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