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Note

Fractionation of baboon chylomicrons and very-low-density lipoproteins by high-performance liquid chromatography

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Chylomicrons are secreted by the intestine in response to dietary lipids and contain apoprotein (apo) B-48, which differentiates them from other lipoproteins [1,2]. Very-low-density lipoproteins (VLDLs), on the other hand, are secreted by the liver in humans and in most animals and contain apo B-100 as their major protein [3]. Triglycerides of chylomicrons are hydrolyzed by lipoprotein lipase (EC 3.1.1.34) situated at the endothelial surfaces of extrahepatic tissues [4]. The chylomicrons then exchange lipids and apoproteins with other plasma lipoproteins and are converted into remnants, which are rapidly and quantitatively removed by the liver [5,6]. Due to rapid removal of chylomicron remnants from circulation, the VLDLs isolated from fasting plasma do not contain any appreciable amount of chylomicrons or chylomicron remnants. However, the VLDLs isolated from postprandial plasma or from human subjects with chylomicronemia are contaminated with substantial amounts of chylomicrons when separated by ultracentrifugation [7]. The metabolic studies of chylomicrons, therefore, have been fraught with problems [7]. The present studies were conducted to achieve a superior method for the separation of chylomicrons and VLDLs by high-performance chromatography (HPLC). Furthermore, we wished to determine whether VLDLs could be subfractionated at the same time because no suitable rapid method is available for measuring VLDL heterogeneity.

EXPERIMENTAL

Animals and diets

Adult baboons (*Papio* sp.) fed a diet rich in cholesterol and saturated fat were used for these studies. The composition of this diet has been described previously

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[8]. The baboons were kept in gang cages and were moved into individual cages for a short time to obtain blood samples. A few animals consuming a chow diet were used for comparisons of lipoprotein patterns.

Isolation of lipoproteins

For the isolation of fasting lipoproteins ($d < 1.006$ g/ml), blood was drawn from fasting (14–16 h) animals into tubes containing EDTA (1 mg/ml) by immobilizing the animals with ketamine hydrochloride (8–10 mg/kg). For the isolation of postprandial lipoproteins ($d < 1.006$ g/ml), baboons were bled 4–6 h after eating the cholesterol- and saturated fat-rich diet [8]. Plasma was obtained by low-speed centrifugation (1900 *g*). Chylomicrons were isolated by ultracentrifuging 5 ml of plasma, overlayered with saline, using an SW 41 Ti rotor at 200 000 *g* for 30 min at 6 °C in a Beckman ultracentrifuge Model L8-70 (Beckman, Palo Alto, CA, U.S.A.). The top 1-ml chylomicron layer was aspirated and washed again using ultracentrifugation under similar conditions. VLDLs were isolated from the infranatant by transferring them into 50 Ti rotor tubes and overlayering them with saline [9]. These tubes were ultracentrifuged at a speed of 145 000 *g* for 22 h at 6 °C. After ultracentrifugation, the top 3.5-ml layer of VLDLs was sliced with the aid of a tube slicer and further washed by ultracentrifugation under similar conditions.

In another experiment, both fasting and postprandial plasma were ultracentrifuged in a 50 Ti rotor to separate lipoproteins of $d < 1.006$ g/ml as described above for VLDLs without removing chylomicrons. These samples were obtained from animals fed a chow diet and from those fed the diet enriched with cholesterol and saturated fat.

Isolation and characterization of lymph chylomicrons

Lymph was isolated by cannulating the thoracic duct of a baboon fed the cholesterol- and saturated fat-enriched diet. The lymph was kindly provided by Dr. K. Dee Carey. The lymph was collected into tubes containing EDTA, centrifuged in a low-speed centrifuge, transferred to SW 41 Ti rotor tubes, overlayered with saline, and ultracentrifuged in a Beckman ultracentrifuge Model L8-70 (Beckman) at a speed of 200 000 *g* for 30 min at 6 °C. The chylomicron pellet floating on the top was removed by spatula, resuspended in saline, and washed by ultracentrifugation under similar conditions for six to eight times. The final pellet was suspended in saline and was applied to the column. To characterize apo-B of lymph chylomicrons, a small aliquot was lyophilized and delipidated by chloroform–methanol (2:1) followed by diethyl ether. Delipidated fractions were separated by slab gel electrophoresis (3.5% acrylamide) using the Laemmli system [10]. The lymph chylomicrons thus isolated contained apo B-48 but not apo B-100 [3].

Chromatographic (HPLC) separation of chylomicrons and VLDLs

A number of columns were tested for separation of chylomicrons from VLDLs. These columns included TSK 4000SW (600 mm × 7.5 mm, Kratos, Westwood, NJ, U.S.A.), a combination of TSK 4000PW followed by TSK 3000PW (600

mm×7.5 mm, Kratos), Fractogel 65-F (330 mm×30 mm, Bodman Chemicals, Aston, PA, U.S.A.), and GF-450 (250 mm×9.4 mm, DuPont, Roswell, GA, U.S.A.). The HPLC apparatus consisted of a Waters Assoc. Model 204 liquid chromatograph (Waters Assoc, Milford, MA, U.S.A.), a Model 6000A pump, a Model 440 UV detector, a Rheodyne injector (Rainin, Emeryville, CA, U.S.A.) with a 500-μl loop and a 0.2-μm filter. This was coupled with a Waters data module. Samples (0.5 ml) were injected and eluted with 0.15 *M* sodium chloride containing sodium azide (0.05%), which was filtered using a 0.45-μm Millipore filter (Waters Assoc.) and degassed. Prior to the injection of lipoproteins, the column was equilibrated with sodium chloride (0.15 *M*). The fractions were collected with the aid of an ISCO (Foxy) fraction collector (ISCO, Lincoln, NE, U.S.A.) kept in a refrigerated chromatography chamber (Powers, Hatboro, PA, U.S.A.) maintained at 6°C.

Chemical measurements

Fractions corresponding to three major peaks (chylomicrons and two subfractions of VLDLs) were pooled and dialyzed against ammonium bicarbonate (0.05 *M*) buffer (pH 8.0). Cholesterol, triglycerides, and proteins were measured in these pooled fractions. Cholesterol and triglycerides were measured by using kits (Sigma, St. Louis, MO, U.S.A.). Proteins were measured by the method of Lowry et al. [11].

Electron microscopy

The size of lipoprotein particles separated by HPLC was measured by electron microscopy. Electron microscopy was performed on lipoprotein samples dialyzed against EDTA (0.1%) after mixing with an equal volume of 2% sodium phosphotungstate pH 7.4 as suggested by Forte et al. [12].

Recovery of lipoproteins

To determine the recovery of lipoproteins in VLDLs [^{3}H]triglyceride-labeled VLDLs obtained from de novo synthesis after injecting [^{3}H]glycerol (ICN Chemicals and Radioisotope Division, Irvine, CA, U.S.A.) were fractionated by the GF-450 column. Radioactivity in the VLDL region was counted using a scintillation counter (Searle, Nuclear, Chicago, IL, U.S.A.). To determine the recovery of chylomicrons, ultracentrifugally prepared chylomicrons were separated by the GF-450 column. The chylomicron fraction was collected and the cholesterol and triglycerides were measured. Since there were no VLDLs in this sample, the recovered cholesterol and triglycerides were considered as the recovery of chylomicron fraction.

RESULTS

Comparison of the separation of chylomicrons and VLDLs by different columns

Among all the columns used, only Fractogel 65-F and GF-450 separated chylomicrons from VLDLs. The GF-450 column was selected because of its superior resolution over that of the Fractogel column. It not only separated chylomicrons

and VLDLs, but resolved VLDLs into two main peaks (see below). All the validations were done using this column.

Separation of lymph chylomicrons and plasma VLDLs

Lymph chylomicrons separated by the GF-450 column had a major peak at a retention time of 14.0 min (elution volume, 7.0 ml). There was a minor peak corresponding to a retention time of 24 min (Fig. 1A). The lipoproteins of $d < 1.006$ g/ml from postprandial plasma isolated by ultracentrifugation were also separated by the GF-450 column. These lipoproteins were separated into three to five peaks (Fig. 1B). One of these major peaks had a retention time of 14.18 ± 0.29 min ($n = 41$) corresponding to an elution volume of 7.09 ml. This peak corresponded to the major peak from the lymph chylomicrons as described above. The other peaks had retention times corresponding to 23.85 ± 0.26 min ($n = 32$), 24.66 ± 0.40 min ($n = 21$), 26.52 ± 0.31 min ($n = 37$), and 27.44 ± 0.44 min ($n = 9$). Of the latter four peaks, two were minor. However, the relative proportions of these peaks varied with the individual animal. Chylomicrons from postprandial plasma isolated by ultracentrifugation using the SW 41 Ti rotor, however, had only one peak corresponding to a retention time of 14.29 ± 0.36 min ($n = 8$) and an elution volume of 7.15 ml (Fig. 1C). Thus, the major peak for chylomicrons from both the lymph and postprandial plasma had a similar retention time (Fig.

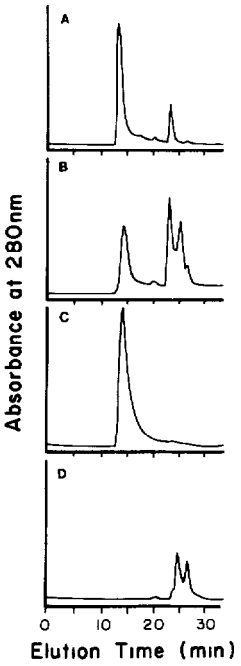

Fig. 1. Separation of triglyceride-rich lipoproteins by HPLC using GF-450 column. (A) Lymph chylomicrons; (B) lipoproteins of $d < 1.006$ g/ml from postprandial plasma; (C) postprandial chylomicrons; (D) lipoproteins of $d < 1.006$ g/ml from fasting plasma. Chylomicrons and VLDLs were isolated by ultracentrifugation as described in Experimental. VLDLs obtained from 200–500 μl of plasma were injected.

1A and C). When the plasma VLDLs ($d<1.006$ g/ml) from fasting baboons maintained on a high cholesterol, high saturated fat diet were separated by the GF-450 column, two major peaks were obtained (Fig. 1D). One of these peaks had a retention time of 25.30 ± 0.10 min ($n=11$) and the other had a retention time of 27.27 ± 0.10 min ($n=11$). Sometimes one or two minor peaks were also seen. VLDLs ($d<1.006$ g/ml) from fasting animals maintained on a chow diet were also separated into two peaks with retention times similar to those from high cholesterol and high fat diet fed animals. The second peak was often very small compared to the first peak.

Composition of lipoproteins separated by HPLC

Fractions collected for chylomicrons and VLDLs were analyzed for cholesterol, triglycerides, and proteins. The chylomicron fraction was extremely rich in triglycerides. Triglyceride, cholesterol, and protein contents of this fraction were 3.6 ± 2.9, 0.45 ± 0.39, and 0.15 ± 0.11 mg/dl (mean $\pm$S.D., $n=3$), respectively. VLDL fractions were relatively poor in triglyceride content but were comparatively richer in protein and cholesterol. Triglyceride, cholesterol, and protein contents for $VLDL_1$ were 0.80 ± 0.35, 0.16 ± 0.10, and 0.20 ± 0.09 mg/dl, respectively. Similarly, triglyceride, cholesterol, and protein contents of $VLDL_2$ were 0.50 ± 0.10, 0.16 ± 0.07, and 0.13 ± 0.03 mg/dl, respectively. $VLDL_2$ had a slightly higher cholesterol/protein ratio. The level of these fractions in each animal varied considerably.

Particle size of these fractions was measured by electron microscopy. Chylomicrons were the largest particles with an average size of 27.7 ± 19.1 nm. $VLDL_1$ and $VLDL_2$ were smaller with average sizes of 15.6 ± 2.9 and 17.1 ± 3.6 nm, respectively. Chylomicrons were very heterogenous whereas both VLDL subfractions were relatively homogeneous.

Heterogeneity of VLDLs

As described above, chylomicrons, whether from postprandial plasma or from lymph, gave only one peak. However, VLDLs from plasma samples of different animals gave two to four peaks, depending upon treatment conditions. Thus, the heterogeneity of VLDLs was affected by diet and the fasting or fed state of the animal (Fig. 1). To determine if these VLDL subfractions differed in particle size and/or composition, we separated a few proteins of known molecular sizes. Among these, baboon serum albumin was eluted at 20 min, suggesting that separation is not based on the particle size alone.

To determine differences in composition, VLDL subfractions were analyzed for triglyceride and cholesterol composition. Four animals maintained on the high cholesterol, high saturated diet were bled 6 h after consuming the feed. Plasma lipoproteins of $d<1.006$ g/ml were isolated by ultracentrifugation and were separated by HPLC using the GF-450 column. Cholesterol and triglyceride concentrations in each fraction are given in Table I. Among these fractions, chylomicrons were richest in triglyceride (cholesterol/triglyceride ratio 0.14 ± 0.11, $n=4$). $VLDL_2$ had a significantly higher cholesterol/triglyceride ratio than $VLDL_1$ (0.17 ± 0.07 for $VLDL_1$ versus 0.26 ± 0.13 for $VLDL_2$).

TABLE I

CHOLESTEROL/TRIGLYCERIDE RATIO IN TRIGLYCERIDE-RICH POSTPRANDIAL LIPOPROTEIN SUBFRACTIONS SEPARATED BY ULTRACENTRIFUGATION AND HPLC

Animal No.	$d < 1.006$ g/ml*	Chylomicrons**	$VLDL_1$**	$VLDL_2$**
X-2170	0.21	0.04	0.20	0.30
X-3077	0.18	0.13	0.20	0.24
X-3086	0.10	0.08	0.06	0.10
X-3470	0.10	0.30	0.20	0.40
Mean ± S.D.	0.15 ± 0.06	0.14 ± 0.11	0.17 ± 0.07***	0.26 ± 0.13***

*Lipoproteins of $d < 1.006$ g/ml were isolated by ultracentrifugation from postprandial plasma samples from baboons maintained on a high cholesterol, high saturated fat diet.
**Lipoproteins of $d < 1.006$ g/ml were subfractionated by HPLC.
***Values differ significantly ($p < 0.05$).

Recovery of lipoproteins

The recovery of VLDL fractions as measured by [^{3}H]triglycerides in VLDL fractions was 81.9 ± 7.54% ($n = 3$). The recovery of chylomicrons as measured by triglyceride and cholesterol content was 94.87 ± 25.67% ($n = 4$) and 80.25 ± 22.05%, respectively. Thus the recovery of both chylomicrons and VLDLs was > 80%.

DISCUSSION

These studies demonstrate that plasma triglyceride-rich lipoproteins can be easily separated by HPLC using a GF-450 column. Chylomicrons are eluted first, followed by the subfractions of VLDLs. Subfractions of VLDLs differ in composition. The second peak is richer in cholesterol and poorer in triglycerides. Thus, the subfractionation of VLDLs by HPLC is very similar to that obtained by heparin–Sepharose chromatography [13], but the HPLC method is faster and requires less blood.

Characterization of plasma chylomicron metabolism has been difficult because of problems in separating chylomicrons from VLDLs, which overlaps chylomicrons in particle size and density. The present study is a significant step in solving this problem. With a GF-450 column, the chylomicrons are well separated from VLDLs. This technique, however, did not separate chylomicrons from chylomicron remnants. Recently, chylomicrons and their remnants have been separated by column chromatography with DEAE-Sephacel or protamine-Affigel 10 [14].

It is now clearly established that most lipoproteins are heterogeneous [15–19]. Non-denaturing gradient gels have been used to detect this heterogeneity for most lipoproteins [20]. VLDLs being larger particles, cannot be separated on these gels and, therefore, have been subfractionated using heparin–Sepharose [13,21] and immunoaffinity chromatography [22]. These techniques are time-consuming and recovery of lipoproteins is often poor. The present study overcomes these problems. First, VLDLs are resolved into subfractions which differ in composi-

tion. Second, the chylomicrons are well separated from VLDLs without contamination. Finally, the recovery of lipoproteins as measured by radioactivity in triglycerides was $>80\%$.

There was 10 min difference between the elution of chylomicrons, which eluted first, and VLDLs. Because chylomicrons are larger than VLDLs, the separation between these two may be due to particle size. However, since albumin was eluted at 20 min, it appears that the separation is not entirely due to particle size and that column GF-450 separates these lipoproteins on the basis of both size and affinity or charge. The subfractions of VLDLs separated by this column also differ in composition. This technique will be useful in studying the metabolic heterogeneity of VLDLs in the baboon.

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