papers on methodology

Protocol for the study of the metabolism of retinyl esters in plasma lipoproteins during postprandial lipemia

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Abstract An efficient protocol is described for the study of the kinetics of retinyl esters in whole plasma and several lipoprotein fractions following the consumption of an oral fat load containing vitamin A (retinol). To allow for a more complete characterization of the kinetics of retinyl esters in different lipoprotein fractions, a simplified two-step ultracentrifugation procedure is reported for the efficient and reproducible isolation of triglyceride-rich chylomicrons from nonfasting subjects, VLDL-sized lipoprotein particles, and the triglyceride-poor lipoprotein fraction. The present method for the determination of retinyl esters is based on the direct application of the lipid fraction onto a normal phase HPLC column without requiring the lipid extract to be desiccated and resolubilized. All of the commonly occurring esters of retinol elute as a single peak with a retention time of 1.6–1.8 min followed by retinyl acetate (serving as the internal standard) and retinol with retention times of 2–2.5 min and 5–5.5 min, respectively. With this system, a new sample can be processed every 10 min and a complete set of 60 samples from a typical oral fat load can analyzed in one working day with minimal technical interaction. By normalizing to the area under the internal standard to correct for variability in the injected volume, the coefficient of variability for the concentration retinyl esters within a single run is less than 5% and less than 10% between runs.—**Ruotolo, G., H. Zhang, V. Bentsianov, and N-A. Le.** Protocol for the study of the metabolism of retinyl esters in plasma lipoproteins during postprandial lipemia. *J. Lipid Res.* 1992. **33:** 1541–1549.

Supplementary key words vitamin A • oral fat load • chylomicrons • very low density lipoproteins

There has been renewed interest in the potential association between intestinally derived lipoproteins during the post-absorptive state and atherosclerosis (1–5). Abnormal clearance of intestinally derived lipoproteins has been suggested to be an independent risk factor for coronary artery disease (CAD) (4, 5). While apoB-48 may be the marker of choice for intestinally derived lipoproteins (5, 6), methods available for the measurement of apoB-48 are tedious and time-consuming and the ability to perform kinetic studies with multiple time samples is still very limited.

Oral vitamin A (retinol) has been shown by many investigators to be a good alternate marker for the intestinally derived chylomicron particles (7–11). The absorbed retinol is esterified by the intestinal cells and secreted into the bloodstream in the core of chylomicrons carrying newly absorbed cholesterol and triglycerides (12). In the circulation, triglycerides (TG) are hydrolyzed leaving the retinyl esters (RE) with the chylomicron particles. The clearance of RE has been reported to be associated with the uptake of the chylomicron particles via a receptor-mediated pathway (13–15). Hazzard and Bierman (7) demonstrated this distinction between chylomicron-TG and chylomicron-RE when they reported that the defect in type III hyperlipoproteinemia is associated with the delayed clearance of the RE core of the chylomicron remnant and not with the catabolism of triglycerides. Abnormal clearance of retinyl esters after the consumption of an oral fat load containing vitamin A has also been reported in patients with hypertriglyceridemia (3, 10, 16), with renal disease (17), and with coronary artery disease (5, 18).

A number of high performance liquid chromatography (HPLC) methods are available for the direct measurement of RE in plasma without requiring the use of radioactive material (19–22). Many of the earlier reports used a reverse-phase column (3, 9, 10, 16–18) and required the lipid fractions to be extracted, desiccated, and resolubilized in the mobile phase buffer in preparation for appli-

Abbreviations: HPLC, high performance liquid chromatography; VLDL, very low density lipoproteins; RA, retinyl acetate; RE, retinyl esters; RP, retinyl palmitate; FSD, fractional standard deviation; CAD, coronary artery disease; TG, triglyceride.

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cation onto the column. The elution time for the retinyl palmitate peak ranged anywhere from 14–15 min (10) to 40 min in one of the original reports (20). While one of the advantages of the reverse-phase column is the ability to resolve different ester forms of retinol (20), most of the studies that used this HPLC method reported data only on the concentration of the major peak corresponding to retinyl palmitate (RP). Cohn et al. (6) and Krasinski et al. (11) have thus simplified the method by using a normal phase HPLC column with a two-solvent gradient system (22) to elute all of the esters of retinol into a single peak.

Another limitation in investigating the kinetics of retinyl esters during the post-prandial state is the lack of a reproducible method for the efficient recovery of different lipoprotein fractions from a large number of plasma samples. As a result, only one group examined the metabolism of RP in the $S_f > 400$ fraction and its conversion to the VLDL-sized particles and to the TG-poor lipoprotein fraction ($d > 1.006$ g/ml) (10, 23). Most studies limited their analyses to the concentrations of RP in the TG-rich lipoproteins defined either as $S_f > 1000$ (3, 24), $d < 1.006$ g/ml (6, 11), or $d < 1.019$ g/ml (5). Data from Weintraub, Eisenberg, and Breslow (24) that demonstrated that the area under the RP curve corresponding to the $S_f < 1000$ nonchylomicron fraction was significantly smaller in subjects with E 4/3 than in subjects with E 2/3 phenotypes would highlight the imporance of examining the concentrations of RP in different classes of lipoproteins. This is of particular interest since the kinetic curves of RP associated with the $S_f > 1000$ chylomicron fraction did not appear to vary with the different apoE isoforms.

The objective of this work is twofold: *1)* to describe an efficient ultracentrifugal procedure for the reproducible isolation of three lipoprotein fractions that reflects changes in the kinetics of intestinally derived retinyl esters during postprandial lipemia; and *2)* to describe an efficient automated HPLC procedure for the measurement of retinyl esters in a large number of lipoprotein samples.

MATERIALS AND METHODS

Reagents and materials

HPLC-grade hexane, n-butyl chloride, acetonitrile, methanol, and acetic acid were obtained from J. T. Baker Inc. (Phillipsburg, NJ). Pure retinyl palmitate (RP), retinyl acetate (RA), and retinol (R) standards were obtained from Sigma Chemicals Co. (St. Louis, MO). Autosampler vials (1.1 ml) equipped with a Teflon septum were from Shimadzu Corporation (Kyoto, Japan). Ultra-Clear (13 × 64 mm) open-top ultracentrifuge tubes and clear 3-ml polycarbonate (13 × 51 mm) tubes were available from Beckman Instruments (Palo Alto, CA). Agarose electrophoresis was carried out using pre-made plates from Ciba-Corning Diagnostics Corp (Palo Alto, CA).

The basic HPLC system included a Model 510 pump system and a Model 740 data module (Waters Associates, Milford, MA), a Model 1706 variable wavelength UV detector (Bio-Rad Co., Richmond, CA), and a Model SIL-9A autoinjector (Shimadzu Corporation, Japan) equipped with refrigeration and a darkened glass door. A custom pre-packed silica column SupelcoSil LC-SI (5 μm, 25 cm × 4.6 mm ID) was provided by Supelco Inc.

The fat-containing meal consisted of an 8-oz fruitshake prepared with frozen orange juice, plain low-fat yogurt, sugar, and approximately 17 g of fat in the form of Lipomul (24 cc, Upjohn Laboratories). The fruitshake was maintained frozen and allowed to thaw in the refrigerator 24 h prior to the study. On the morning of the study, 1.2 ml of Aquasol A (60,000 USP, Armour Pharmaceutical Company, Kanakee, IL) was thoroughly mixed into the fruitshake, and given to the participant along with two yolks from freshly prepared hard-boiled eggs as the source of dietary cholesterol. The total fat contents of the test meal was 26.53 g.

Lipoprotein fractionation

After the ingestion of an oral fat load containing vitamin A, blood samples (10 ml) were collected in tubes containing EDTA (0.1% final concentration) at frequent intervals. From each sample, three lipoprotein fractions were isolated by ultracentrifugation including, chylomicrons ($S_f > 400$), VLDL-sized lipoproteins (S_f 20–400, $d < 1.006$ g/ml), and the triglyceride-poor lipoprotein fraction ($d > 1.006$ g/ml). Plasma samples (2–3 ml each) were first subjected to ultracentrifugation at density 1.006 g/ml using the 50.4 Ti rotor (40,000 rpm for 16 h at 20°C). The infranate from the 50.4 Ti spin was adjusted to 5 ml and defined as the triglyceride-poor fraction ($d > 1.006$ g/ml) for the determination of lipid and retinyl ester concentration.

A total of 1 ml of the supernate that contained both newly secreted TG-rich intestinal chylomicrons and VLDL-sized lipoprotein particles was removed by aspiration, and placed directly into individual tubes for the TLA-100.3 rotor. A small volume (100 μl) of a d 1.020 g/ml solution was added to each tube to allow proper layering with distilled water. The tubes were then ultracentrifuged in the TL-100 Beckman tabletop centrifuge. Chylomicrons of $S_f > 400$ were recovered in the supernate by aspiration after a 5-min spin at 50,000 rpm (20°C). The supernate (0.5–0.75 ml) was adjusted to 1 ml using a d 1.006 g/ml saline solution containing 0.1% EDTA prior to any measurement. Approximately 0.5 ml of the clear middle layer was removed and discarded, the infranate was collected, and adjusted to 2 ml with saline. This is defined as the VLDL fraction (corresponding to S_f 20–400).

Chromatography

Different volumes of the various fractions (100 μl for plasma and chylomicrons, 100–500 μl for VLDL and the d > 1.006 g/ml fraction) were placed in 13 × 100 mm glass tubes. The total volume in each tube was adjusted, as necessary, to 500 μl using normal saline. Retinyl acetate (40 ng in 200 μl of mobile phase buffer) was added to each tube as internal standard. Five hundred μl of methanol was added followed by the addition of 500 μl of the mobile phase buffer for a total volume of 1.7 ml. The mobile phase buffer was prepared fresh on a daily basis by combining 90 ml of hexane, 15 ml n-butyl chloride, 5 ml acetonitrile, and 0.01 ml acetic acid (82:13:5 by volume with 0.01 ml of acetic acid). The tubes were thoroughly mixed after each step. The final mixture was centrifuged at 350 *g* for 15 min (room temperature) and the upper layer was carefully removed by aspiration and placed into individual autosampler vials (1.1 ml capacity). The autoinjector was programmed to deliver 100 μl per injection and a new sample every 10 min. The flow was maintained at a constant rate of 2 ml/min and the peaks were detected at 330 nm. A total of 48 samples corresponding to the various lipoprotein fractions from a single study were loaded in the autosampler (100-sample capacity), kept under refrigeration with minimal exposure to ambient light and analyzed in one run. In order to correct for any variability in the delivery of 100 μl of the lipid extract by the automatic sampler as well as any evaporation that may have occurred as the samples were waiting to be injected into the column (up to 10 h), the concentration of RE in each sample was expressed in terms of the ratio of the area under the RE peak to the area under the RA peak (19) instead of the peak height.

The RE concentration (in RA equivalent units) present in the sample can be calculated from the RE/RA ratio and the known amount of the RA internal standard using the formula:

$$\text{RE (ng RA/ml)} = (\text{RE/RA}) \times (1/\text{volume of sample used}) \times 40 \text{ ng RA}.$$

An appropriate correction factor must be applied in order to express the RE concentration in the lipoprotein sample in terms of actual concentration in whole plasma. For instance, if 3 ml of plasma was used to recover 1 ml of VLDL by ultracentrifugation and 100 μl of this VLDL fraction was extracted and applied to the HPLC column, the [volume of sample used] in the above formula would be 0.1. This concentration of RE in the sample will have to be divided by 3 to obtain the VLDL-RE concentration per milliliter of plasma. In some instances, only 1 ml of plasma was available for lipoprotein fractionation and this correction factor would not be required. It should be noted that the RE concentration is expressed in terms of RA mass unit. The correction factor that can take into account the difference in molecular weights between RA and the mixture of retinyl esters included in the RE peak is not available to convert this concentration into molar concentration.

Subjects

Patients with type IV and/or type V hyperlipoproteinemia with fasting triglyceride in the range of 500–1500 mg/dl were recruited for a double-blind placebo-controlled multicenter trial designed to investigate the effectiveness of the modified release form of gemfibrozil (LOPID-SR) in reducing plasma triglycerides. Participants were maintained for 6 weeks on a phase I dietary regimen prior to being randomized into the study which consisted of two placebo periods and two treatment periods. Preliminary data on the oral fat load studies during the two placebo periods are presented here only to illustrate the reproducibility of the lipoprotein isolation and HPLC procedures.

RESULTS

Lipoprotein fractionation

The chylomicron fractions isolated by the tabletop ultracentrifuge were first screened for the presence of pre-beta-lipoproteins by agarose electrophoresis prior to lipid extraction. All samples were shown to be devoid of pre-beta-migrating material by agarose electrophoresis and no stained material could be visualized at the point of application when the VLDL-sized fraction was submitted to agarose electrophoresis (data not shown). When the samples were severely hypertriglyceridemic, a second 5-min spin was required and the two supernates were combined as the chylomicron fraction. Reproducibility of this isolation scheme was demonstrated by less than 6.5% variability in TG and CHOL in either the d 1.006 g/ml top or the d 1.006 g/ml bottom fraction of the same fasting plasma sample subjected to ultracentrifugation in six separate tubes in the same run and in six separate ultracentrifugal runs on 3 consecutive days (data not shown). In the processing of actual samples from an actual oral fat load study, the change in TG levels in the plasma samples collected at different time points postprandially may affect the recovery. Reproducibility was also demonstrated in the constant concentration of the VLDL-cholesterol isolated from plasma samples that contained varying concentrations of chylomicron-TG collected at different times after the oral fat load (**Table 1**). The recovery data were similar in these individuals when their triglyceride levels were low (VLDL-TG of 96 mg/dl) as well as when their triglycerides were high (VLDL-TG of 760 mg/dl).

Quantitation of retinyl palmitate

Fig. 1 illustrates the typical chromatograms obtained with this HPLC system. The chromatogram from a mixture of the three pure standards RP, RA, and retinol is

TABLE 1. Recovery of VLDL-cholesterol during postprandial lipemia

Group	Fasting VLDL Lipids[a]		Postprandial VLDL Cholesterol Normalized Mean ± FSD[b]				
	VLDL-TG	VLDL-CHOL	1 h	2 h	3 h	6 h	12 h
	mg/dl						
A (n = 20)	176.0 (± 27.66%) min = 96 max = 244	49.7 (± 49.84%) min = 18 max = 111	0.984 (9.03%)	1.009 (9.58%)	1.060 (12.30%)	1.017 (11.35%)	0.915 (12.25%)
B (n=20)	444.4 (± 30.74%) min = 285 max = 769	100.7 (± 30.74%) min = 58 max = 204	0.992 (8.94%)	0.999 (8.75%)	1.017 (10.54%)	1.006 (10.53%)	0.969 (7.76%)

[a]The subjects were separated into two groups depending on whether the fasting VLDL-TG level was less than or equal to 250 mg/dl or greater than 250 mg/dl. The mean concentrations of triglyceride and cholesterol in the VLDL density fraction are presented with the fractional standard deviation (FSD) as well as the minimum and maximum concentrations. Subjects in Group A are the same individuals in Group B who were studied after 6 weeks on treatment with a triglyceride-lowering drug.

[b]The VLDL-cholesterol concentrations at each specified time point (hour after the administration of the oral fat load) were normalized to the individual's baseline concentration and were averaged for all the individuals in the group. The mean normalized values are presented with the fractional standard deviation from all subjects.

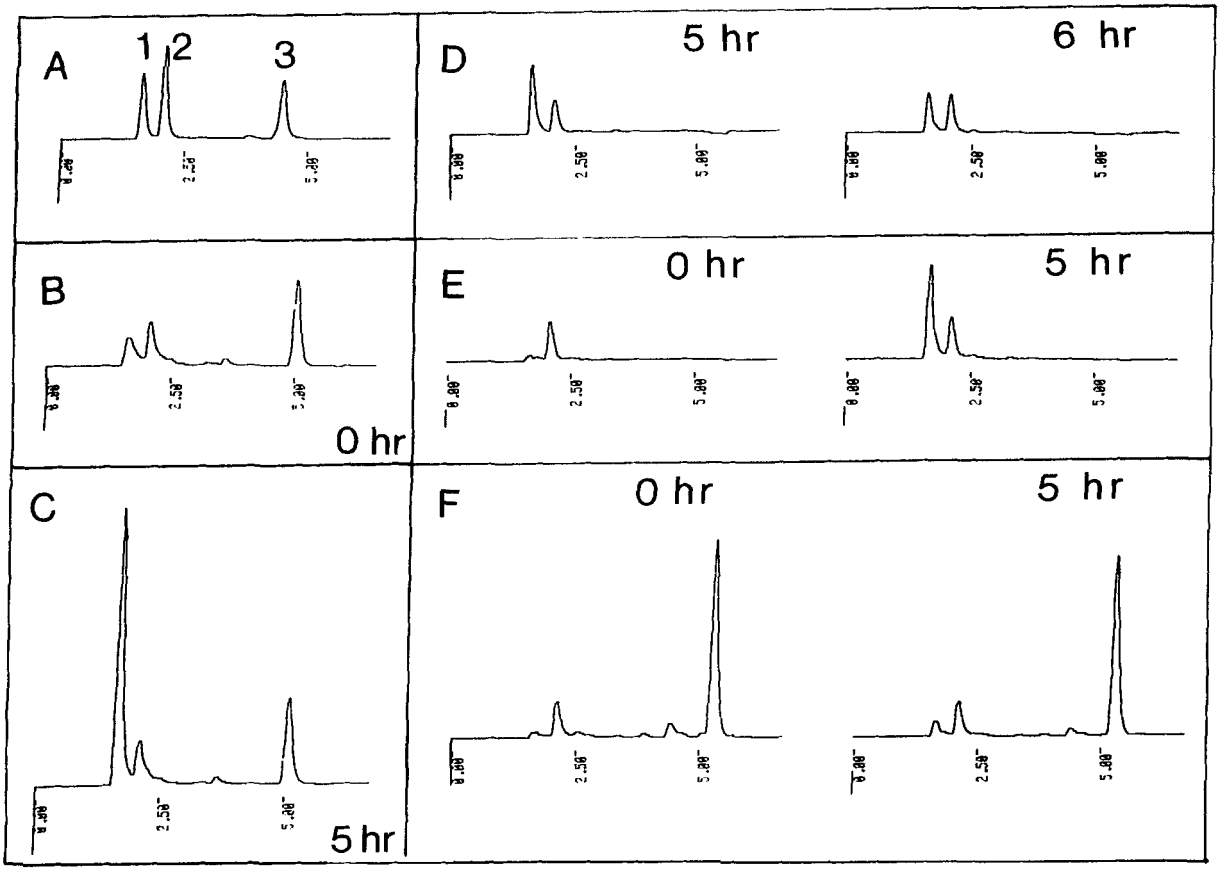

Fig. 1. HPLC chromatograms of pure standards and of lipoprotein fractions. Panel A: Chromatogram of a preparation containing a mixture of pure standards is shown to illustrate the elution times obtained for the three pure standards, including 1) retinyl palmitate, 2) retinyl acetate, and 3) retinol. Panels B and C: Chromatograms of fasting plasma (panel B) and of a plasma sample taken at 5 h after the administration of the oral fat load. The RA peak corresponds to 40 ng of internal standard in the plasma sample prior to extraction. Minimal change in the retinol peak was noted during the postprandial state. Panel D: Chromatograms of the $S_f > 400$ lipoprotein fraction corresponding to particles from nonfasting subjects isolated from plasma samples collected at 5 and 6 h after the administration of the oral fat load. Note that there is no retinol peak in this fraction. Panel E: Chromatograms of the S_f 20–400 lipoprotein fraction corresponding to the VLDL density fraction isolated from plasma samples collected at baseline after an overnight fast and at 5 h after the fat load. Again there is no retinol peak in this fraction. Panel F: Chromatograms of the $S_f < 20$ lipoprotein fraction corresponding to the $d > 1.006$ g/ml density fraction which includes LDL and HDL isolated from plasma samples collected at baseline and at 5 h after the fat load. The peak corresponding to free retinol is entirely (> 95%) recovered in this fraction.

shown in panel A. The mean retention times (± FSD, fractional standard deviation) from the analysis of a series of 25 consecutive samples were 1.706 ± 0.11%, 2.182 ± 0.30%, and 5.15 ± 1.76% for RP, RA, and free retinol, respectively. Panels B–F illustrate typical chromatograms obtained from plasma, chylomicrons, VLDL, and the TG-poor fraction collected at different times after the oral fat load in one subject.

Fig. 2 illustrates the linearity of the assay for concentration of pure RP ranging from 10 to 150 ng/ml (Fig. 2A, r = 0.999). The linearity for RA and RP using pure standards for the lower range of the assay (1–20 ng) is presented in Fig. 2B (r = 0.999 for both). To examine the linearity of the RE/RA ratio as an estimate of RE contents, a plasma sample was collected from a normolipidemic volunteer 3 h after the ingestion of an oral fat load containing vitamin A and used for the preparation of six separate sets of serial dilutions with d 1.006 g/ml saline. A total of 30 samples were processed as independent samples and demonstrated a linear range from 0.75 to 10 for the RE/RA ratio (Fig. 2C, r = 0.986). The slope of the regression was 1.026 between the actual RE/RA ratio and the expected RE/RA ratio based on serial dilution of a plasma sample with an RE/RA ratio of 9.77 ± 0.096. The RE/RA ratios in lipoprotein samples collected during a typical oral fat load study ranged from 0.25 for the fasting plasma sample to 8.0 for the chylomicron fraction at the 5-h sample.

The mean area (± FSD) under the RA peak for a constant amount of internal standard was 21.965 ± 8.51% from a typical series of 12 consecutive injections of 100 μl of sample containing 40 ng of pure RA standard. This variability, which may reflect the ability of the autosampler to pick-up and inject 100 μl of lipid extract, can be corrected for by using the ratio RE/RA of the areas (19). The range of the FSD for the actual area under the RA peak from 80 separate runs each consisting of 60 consecutive injections can be as low as 4% on some days to as high as 14% on other days.

The reproducibility of the RE/RA ratios was assessed by analyzing a single plasma sample on 3 different days over a 3-week period. Each day six separate aliquots of the plasma samples were spiked with 40 ng of RA, extracted, and placed in the autosampler for injection onto the

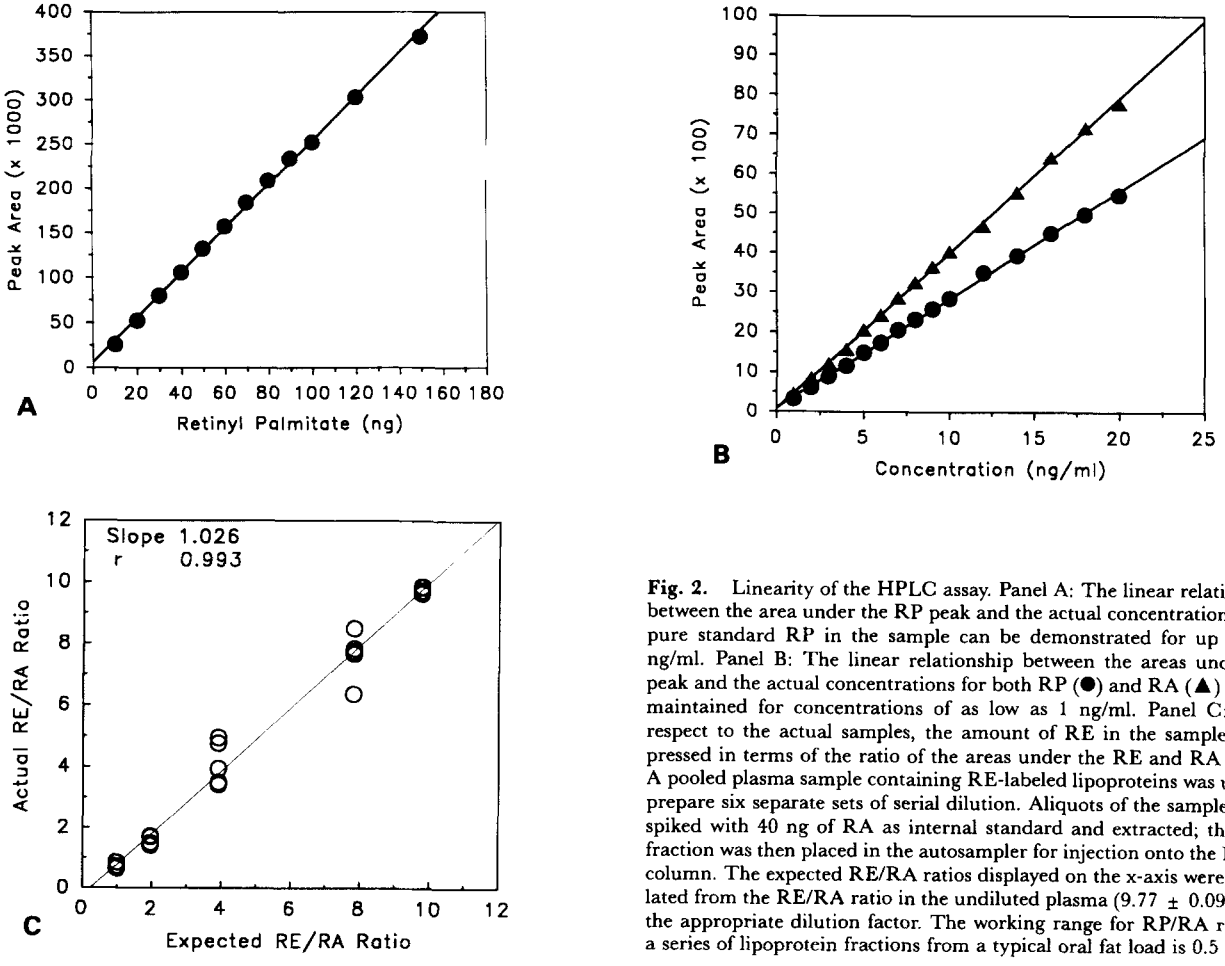

Fig. 2. Linearity of the HPLC assay. Panel A: The linear relationship between the area under the RP peak and the actual concentration of the pure standard RP in the sample can be demonstrated for up to 150 ng/ml. Panel B: The linear relationship between the areas under the peak and the actual concentrations for both RP (●) and RA (▲) can be maintained for concentrations of as low as 1 ng/ml. Panel C: With respect to the actual samples, the amount of RE in the sample is expressed in terms of the ratio of the areas under the RE and RA peaks. A pooled plasma sample containing RE-labeled lipoproteins was used to prepare six separate sets of serial dilution. Aliquots of the samples were spiked with 40 ng of RA as internal standard and extracted; the lipid fraction was then placed in the autosampler for injection onto the HPLC column. The expected RE/RA ratios displayed on the x-axis were calculated from the RE/RA ratio in the undiluted plasma (9.77 ± 0.095) and the appropriate dilution factor. The working range for RP/RA ratio in a series of lipoprotein fractions from a typical oral fat load is 0.5 to 8.0.

HPLC column. The mean (± FSD) values for the RE/RA ratio obtained were 3.373 ± 3.37%, 3.565 ± 4.26%, and 3.580 ± 4.07% for the 3 separate days. The intra-assay coefficient of variation was less than 4.5% based on these series of six replicates.

For the estimation of the inter-assay coefficient of variation, two plasma samples containing different concentrations of RE were analyzed at least once every day over a 1-month period. For this special purpose, an appropriate amount of RA internal standard (final concentration of 400 ng RA/ml of plasma) was added to the plasma samples before the aliquots (100 μl containing 40 ng of RA) were prepared and kept frozen at −20°C until the day of assay. The goal of this series of samples was to eliminate variability in the amount of internal standard added to each sample prior to organic extraction. The mean RE/RA ratios (± FSD) for these two control samples based on 72 separate injections over a 4-week period were 6.754 ± 2.43% and 2.84 ± 3.87%, corresponding to a coefficient of variation of less than 4%. When 40 ng of the internal standard was independently added each day to the control plasma samples, the coefficient of variation was slightly higher at 10% (see below).

Quality control

For each oral fat load study a total of 64 samples were analyzed in a single HPLC run. They included 12 samples each of plasma, chylomicrons, VLDL, and d > 1.006 g/ml fraction. Typically, all 12 samples from one lipoprotein fraction were assayed as a set which also included four quality control samples. These latter samples include: *1)* the newly prepared mobile phase buffer, *2)* a mixture of pure standards, and *3)* two pooled plasma control samples containing different levels of RE. RA was added to these pooled plasma controls and to the 12 lipoprotein samples at the same time and all 14 tubes were extracted as a set.

The quality of each set of 16 injections corresponding to a complete 24-h RE concentration curve was determined from three criteria. First, a constant zero baseline must be demonstrated with the injection of the mobile phase buffer. The column would have to be extensively washed or replaced if baseline could not be reproduced. Second, the retention times and areas under the peak of the pure standards must be reproduced within the range corresponding to the mean ± 2 SD. Third, the RE/RA in the two quality control samples must be within the defined acceptable range. Poor recovery of the RE/RA ratios from these two quality control samples would suggest problems during the extraction procedure and the entire set of 12 samples would be re-extracted and re-analyzed. Poor recovery of the ratios of areas in these quality control samples could also arise when an incorrect amount of retinyl acetate was inadvertently used. This could be assessed by examining the actual areas under the RA peaks. In some instances, it may be concluded that a constant amount of RA was used in all the samples of the set. A different amount of RA could then be used in the formula (see Methods) without requiring all of the samples to be reanalyzed.

Retinyl esters in lipoprotein fractions

Fig. 3 illustrates the reproducibility of the methods with the data from one subject being studied for two separate placebo periods separated by 12 weeks. As shown, even when the baseline fasting triglyceride concentration was different (panel 3A), by normalizing the postprandial triglyceride response to the fasting triglyceride, the two responses became more comparable (panel 3B). The RE curves in whole plasma (panel 3C), in S_f > 400 (panel 3D) as well as in S_f 20–400 (panel 3E) were indistinguishable between the two placebo periods.

DISCUSSION

There has been renewed interest in the potential role of postprandial lipoproteins in the development of premature coronary artery disease (4, 5). The question of whether the concentration of apoB-48 should be used as a more reliable marker of intestinal lipoproteins than retinyl palmitate is still under debate. Many studies have shown that, in spite of the absence of detectable apoB-48 by polyacrylamide gel electrophoresis, retinyl palmitate can still be detected several hours after the consumption of a single fat meal containing retinol (10). Some investigators interpreted this finding to suggest that there is exchange of retinyl esters from triglyceride-rich lipoproteins to apoB-100-containing lipoproteins. In vitro and in vivo data, however, have indicated that such exchange is minimal and could not account for the observed concentration of retinyl esters in apoB-100 lipoproteins (9, 10). Data from Tall, Sammett, and Granot (25) suggested that any transfer of cholesteryl esters that may occur during postprandial lipemia would more likely be from the high density lipoproteins to the apoB-containing lipoproteins. Whenever RP data in the d > 1.006 g/ml fraction were available for analysis, the RP curve was consistent with the conversion of 2–5% of the RP flux through the VLDL density fraction according to our compartmental model (10). Another explanation would be that RE may be immediately incorporated by the liver in the core of newly formed apoB-100-containing lipoproteins without being degraded. This hypothesis has yet to be examined.

While the pathway underlying the association of postprandial RE—at later time points—to apoB-100-containing lipoproteins (11) is still not understood, the metabolism of RE during the first 6–8 h postprandially is believed to reflect the metabolism of intestinal lipoproteins. Furthermore, in view of the contribution of several different lipoprotein fractions in the metabolism of RE, data on the

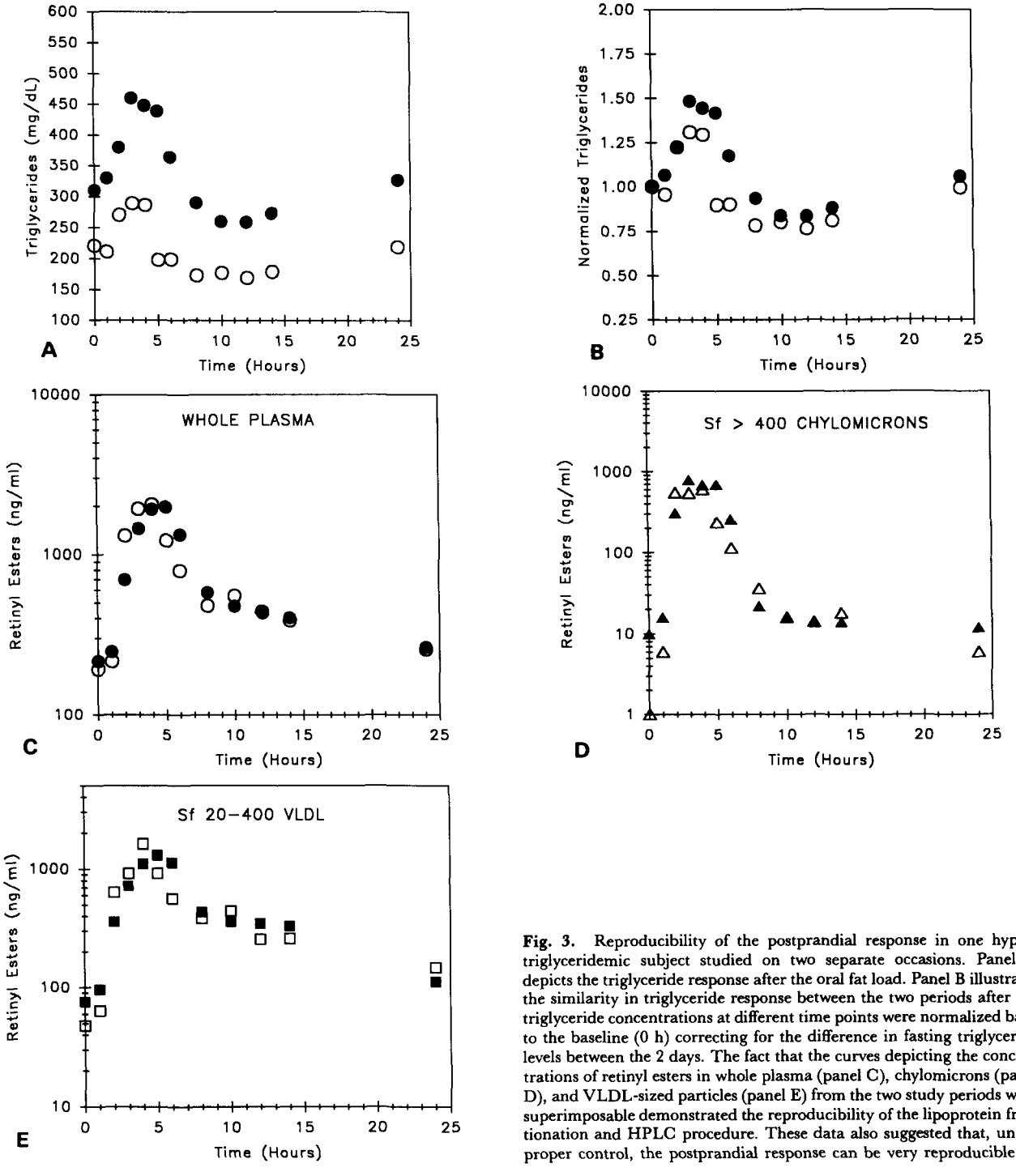

Fig. 3. Reproducibility of the postprandial response in one hypertriglyceridemic subject studied on two separate occasions. Panel A depicts the triglyceride response after the oral fat load. Panel B illustrates the similarity in triglyceride response between the two periods after the triglyceride concentrations at different time points were normalized back to the baseline (0 h) correcting for the difference in fasting triglyceride levels between the 2 days. The fact that the curves depicting the concentrations of retinyl esters in whole plasma (panel C), chylomicrons (panel D), and VLDL-sized particles (panel E) from the two study periods were superimposable demonstrated the reproducibility of the lipoprotein fractionation and HPLC procedure. These data also suggested that, under proper control, the postprandial response can be very reproducible.

kinetics of RE in whole plasma alone clearly are not sufficient to appreciate these complex pathways (24). It is crucial that the kinetics of RE in chylomicrons, i.e., TG-rich particles that are found in plasma only after the oral fat load, be differentiated from those of RE associated with VLDL-sized particles and in TG-poor particles of d > 1.006 g/ml. Earlier data from our group (10) as well as from others (7) has suggested that by varying the fat content of the test meal, newly absorbed retinyl esters may be associated with S_f > 400 or S_f 20-400 lipoprotein fractions. All these lipoprotein classes correspond to a simplistic and arbitrary separation of a continuous spectrum of particles varying in size and composition. A number of other separation schemes, each with its own advantages

and disadvantages, have been reported (3, 5, 11). In this work, our goal was to develop a simple procedure that can reproducibly and efficiently separate plasma lipoproteins into particles that are found in fasting plasma, VLDL and non-VLDL ($d > 1.006$ g/ml fraction) and particles that contain primarily newly absorbed dietary fat. Using compartmental analysis, the contribution of direct input and conversion from one lipoprotein fraction to another can potentially be dissected (10, 23). Direct experimental data on the concentrations of retinyl esters in these lipoprotein fractions would be required for the validation of these models.

We have described a simple procedure that first separates the $d < 1.006$ g/ml TG-rich fraction from the $d > 1.006$ g/ml CHOL-rich fraction in an overnight ultracentrifugation step followed by a 5-min spin for the separation of chylomicrons of $S_f > 400$ from the VLDL fraction using the tabletop ultracentrifuge. As TG and CHOL contents in the chylomicrons change in different samples postprandially, the concentrations of VLDL-CHOL recovered from these samples remain constant (Table 1). This suggests that varying degrees of lipemia in the samples did not interfere with the isolation procedure.

The second part of this report addresses the measurement of retinyl esters by HPLC. Most of the available HPLC methods, either reverse-phase (19, 20) or normal phase (21), require extensive sample processing including lipid extraction, desiccation, and resolubilization before the sample can be applied to the HPLC column. In addition to the extensive sample preparation time, the retention time for RP has typically ranged from 10–15 min (10) to 40 min (19), thus allowing the analysis of only a few samples at a time. The normal phase HPLC used by Cohn et al. (6) significantly reduced the run time per sample but required a dual pump HPLC system and still required extensive processing time. By eliminating the need to desiccate the lipid extract prior to resolubilization in the mobile phase buffer and the utilization of a shorter HPLC column, a complete set of samples from a typical oral fat load study (approximately 48 samples and the appropriate quality control samples) can be analyzed within 10 h. With the inclusion of an appropriate timer that automatically powers-down the system after a preset wash period has been completed, the HPLC system is ready for operation by the next morning. Most importantly, with the autosampler and the quality control program described herein, the entire procedure can be completed with minimal technical supervision.

One of the major advantages of vitamin A as a marker of intestinal lipoproteins during postprandial lipemia is the fact that it does not involve radioactive isotopes and that it is economical when compared to alternative tracer methods using stable isotopes. With the simplified centrifugation and HPLC protocol presented here, this procedure may be more widely used to assess the metabolism of postprandial lipoproteins in a large number of individuals with a variety of metabolic abnormalities.

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