A time-consuming sample preparation and measuring procedure is required for the quantitation of retinyl palmitate by HPLC. We developed a fluorometric method for the determination of total retinyl esters in chylomicrons, chylomicron remnants, and VLDL. This method is precise, sensitive, rapid, simple, and particularly useful for large-scale studies of postprandial lipid metabolism. Because the turbidity of postprandial lipemic samples interferes with the fluorescence measurement, all samples were incubated for 10 min with a clearing buffer containing esterase and detergents. This buffer eliminates the turbidity and hydrolyzes all retinyl esters to retinol. The fluorescence signal (excitation wavelength, 330 nm; emission wavelength, 490 nm) was linear from 0.1 mg/L up to 4 mg/L retinyl palmitate, and the CVs were 3.6% within-run and 5.1% within-series. A first application studied postprandial lipoproteins, which were first separated by ultracentrifugation and then subjected to size exclusion chromatography. Fluorescence analysis revealed that the chylomicron density fraction contains large amounts of chylomicron remnants.

Several observations indicate that chylomicron remnants can cause and sustain atherogenesis. In cell culture, chylomicron remnants are taken up by smooth muscle cells (1) and induce the transformation of macrophages to foam cells (2, 3). In cholesterol-fed rabbits, increased remnant lipoproteins cause marked hypercholesterolemia associated with rapidly growing atherosclerosis (4–6), and in humans, the hereditary disease “type III hyperlipoproteinemia”, an accumulation of remnant lipoproteins, is accompanied by severe atherosclerosis (7). Hence, it may be important for the assessment of atherosclerotic risk to test individuals for the presence of increased concentrations of postprandial triglyceride-rich lipoproteins, in particular of chylomicron remnants (8, 9).

The plasma concentrations of chylomicron remnants cannot be assessed by the plasma triglyceride concentration alone, because this value is composed of triglycerides transported in several lipoprotein classes secreted from different organs. Exogenous triglycerides are transported in chylomicrons and chylomicron remnants, whereas the bulk of endogenous triglycerides are transported in VLDL. To differentiate between triglyceride-rich lipoproteins of intestinal origin (chylomicrons and their remnants) and lipoproteins of hepatic origin (VLDL), only two methods are currently available. One method is the specific determination of apolipoprotein B48 (a marker for the intestinal origin of the lipoprotein), and the alternative method is the labeling of lipoproteins of intestinal origin with retinyl palmitate (10). Because the former determination is difficult to obtain by immunological techniques due to the identity of apolipoprotein B48 with the amino terminus of apolipoprotein B100 (a marker for lipoproteins secreted by the liver), several investigators have decided to determine retinyl esters after a fatty meal containing high doses of retinyl palmitate as a marker of chylomicrons and chylomicron remnants (10–15). This is possible because retinyl esters are incorporated into chylomicrons during intestinal assembly, and they remain an integral part of the lipoprotein particle during the intraplasmatic degradation of chylomicrons to chylomicron remnants until the chylomicron remnants are cleared by liver and bone marrow. Retinyl ester concentrations are very low under physiological conditions, and the liver does not secrete retinyl esters. Therefore, the plasma concentration of retinyl esters after the ingestion of a fatty meal containing high doses of retinyl palmitate is a good estimate of the plasma concentration of chylomicrons and chylomicron remnants (16).
The conventional method for the determination of retinyl esters in plasma is HPLC. This method is specific and precise but requires time-consuming sample preparation and measuring procedures, and is therefore not appropriate for the determination of retinyl esters in large sample numbers. We therefore strove to develop an alternative procedure that allows easier and more rapid determination of retinyl esters in triglyceride-rich lipoproteins. For this purpose, we took advantage of the fluorescent properties of retinol (17). If used with an appropriate clearing buffer, this new procedure turned out to be precise, sensitive, rapid, and very easy to perform.

### Materials and Methods

The protocol for all studies involving human subjects was approved by the local ethics committee.

#### Oral Fat Loads

After an overnight fast, male volunteers received a liquid fatty meal consisting of fresh dairy cream and vitamin A (AROVIT®, Hoffmann-La Roche). The fatty meal (1 g fat per kg body weight and 60,000 IU vitamin A (retinyl palmitate) per m² body surface area) was consumed within 10 min (18). Similar fatty meals without retinyl palmitate were administered to obtain lipoproteins that are not labeled with retinyl esters.

#### Clearing Buffer

Samples were mixed 1:1 (by volume) with a clearing buffer [1.2 g/L polyoxyethylene 9-lauryl ether (Boehringer Mannheim), 220 mmol/L magnesium aspartate (Fluka), 40 mmol/L sodium cholate (Merck), 2000 U/L cholesterolesterase (E.C. 3.1.1.13; Boehringer Mannheim), and 100 mmol/L Tris buffer, pH 7.7] and incubated for 10 min at room temperature before being submitted to the fluorescence measurement. The clearing buffer was stable at 4 °C for up to 1 week.

#### Fluorescence Measurement

Fluorescence was determined in a Hitachi spectrofluorometer with a flow-through cuvette (Model F 1050, Merck) or with a LS-50 spectrofluorometer with a standard cuvette (Perkin–Elmer). Wavelengths were 330 nm for excitation and 490 nm for emission. Because the substance producing the fluorescence signal in the sample was retinol, the method was calibrated with retinol dissolved in ethanol (20 μL of retinol diluted into 500 μL with saline + 500 μL of clearing buffer). The concentration of retinol can be measured by spectrophotometry. The calibration with chylomicron samples containing known concentrations of retinyl palmitate to rule out the possibility that differences in background signal and quenching of the calibrators interfere with the calibration. (The retinyl palmitate concentrations of these chylomicron preparations from volunteers obtained 4 h after a retinyl palmitate-containing fatty meal had been measured before by HPLC.)

Because dilution curves show linear signals over a wide fluorescence range, calibration was carried out as a single-point calibration.

#### HPLC of Retinyl Esters

All solvents were of HPLC quality and purchased from Merck. All assays were carried out in subdued light to prevent chemical alterations. Retinyl palmitate (0.05–1.2 mg/L; Sigma Chemical Co.) dissolved in sample solution [acetonitrile/ethanol; 6:4 (by volume)] served as a standard. The concentrations of retinyl palmitate and the internal standard retinyl margarinate (Hoffmann-La Roche) dissolved in ethanol were measured by spectrophotometry.

Lipoproteins (200 μL) were first extracted with four volumes of ethanol containing the internal standard (0.25 mg/L retinyl margarinate) and then with 20 volumes of hexane. The hexane layer was evaporated under nitrogen, and the residue was dissolved in 200 μL of acetonitrile/ethanol 6:4 (by volume) containing 0.5 mL/L butylated hydroxytoluene. The sample (50 μL) was injected into a Merck HPLC system, and retinyl esters were separated by reversed-phase HPLC using an analytical C_{18}-bonded silica Lichrospher RP 8-100 HPLC column (Bischoff) with RP18 Lichrocart 4-4 guard columns (Merck) and a mobile phase of 940 mL/L acetonitrile–50 mL/L tetrahydrofuran–10 mL/L water at a flow rate of 1.5 mL/min. The absorbance of the retinyl esters (oleate, margarinate, and stearate) and of retinol was recorded at 325 nm. The detection limit for retinyl palmitate was 10 μg/L. Extraction recovery was 90.7% ± 5.5% for retinyl palmitate and 89.0% ± 6.4% for retinyl margarinate (mean ± SD).

#### Isolation of Lipoproteins from Plasma

With the subjects in a standardized sitting position, EDTA blood samples were collected by clean venipuncture 4 h after the fatty meal, when chylomicrons and chylomicron remnants reach their maximum concentrations. Chylomicrons (S<sub>r</sub> >1000) were isolated by preparative ultracentrifugation of postprandial plasma, using an L5 ultracentrifuge (Beckman) and a Ti 50.3 rotor (1.6 × 10<sup>6</sup> g, 10 °C, 30 min). VLDL, intermediate density lipoprotein (IDL), and LDL were prepared by sequential ultracentrifugation of the chylomicon infranatant at densities of 1.006, 1.019, and 1.063 kg/L, respectively, for 18 h each.

The investigation of unspecific fluorescence was car-
ried out with lipoproteins obtained after gel filtration of postprandial plasma. This separation procedure was chosen because it allows the continuous visualization of the entire lipoprotein size range. Because the separating properties of a single Sepharose product do not cover the entire size range of human lipoproteins, a combination of two different Sepharose media was used. The lower 53 cm of glass columns (1000 mm length, 10 mm i.d.) were filled with Sepharose CL-6B, the upper 43 cm with Sepharose CL-2B (Pharmacia). Plasma (2.5 mL) was applied at a flow rate of 100 μL/min (filtration buffer: 145 mmol/L NaCl, 0.21 mmol/L Na₂EDTA, and 25 mmol/L Tris, pH 7.5). To avoid decay of retinyl esters by light, the separation was carried out in the dark. Eluates were collected in 2-mL fractions and were analyzed for triglyceride and cholesterol concentrations by enzymatic tests (Boehringer Mannheim) with an automatic analyzer (Hitachi 747, Boehringer Mannheim) and for fluorescence by fluorometry (excitation, 330 nm; emission, 490 nm) after incubation with the clearing buffer. For fluorometry, 200 μl of each fraction was mixed with 200 μl of clearing buffer. To compare lipoprotein separation by this method with results obtained after separation by ultracentrifugation, isolated lipoprotein fractions after ultracentrifugation (chylomicrons, VLDL, IDL, and LDL) were separated over the same column, and triglyceride and cholesterol concentrations and fluorescence were measured in the eluate fractions.

Results
Because the turbidity of lipemic samples causes considerable unspecific fluorescence, which makes the analysis of specific fluorescence impossible, a clearing buffer must be used. This clearing buffer eliminates unspecific fluorescence in chylomicron samples within 2–6 min, depending on the sample triglyceride concentration (Fig. 1). Therefore, an incubation period of 10 min was chosen for all following analyses. The clearing buffer reliably cleared lipemic samples with triglyceride concentrations up to 11.3 mmol/L (10 g/L).

The clearing buffer hydrolyzes ester bonds not only in triglycerides and cholesterol esters but also in retinyl esters. This was demonstrated by HPLC analyses of retinyl ester-containing chylomicrons before and after incubation with the clearing buffer (Fig. 2). Without clearing buffer, the major peak was retinyl palmitate; after incubation with the clearing buffer, this peak virtually disappeared, whereas a corresponding retinol peak appeared.

The correlation of the results of the new fluorometric method and the HPLC method was examined with 20 chylomicron samples obtained from different individuals after the ingestion of a fatty meal containing retinyl palmitate that have been isolated by gel chromatography (Fig. 3, left panel). Results from both methods correlate well (r = 0.989), and as is evident from the Bland-Altman plot (19), both agree well at low and high concentrations of retinyl palmitate (Fig. 3, right panel).

To test for linearity, dilutions of a retinyl ester-labeled...
chylomicron preparation (which had been isolated by gel chromatography) showed a good linearity up to 4 mg/L ($r = 0.989$) (Fig. 4). If higher concentrations of retinyl ester were present, i.e., especially in samples of hypertriglyceridemic patients, the samples were diluted with saline.

The CVs were tested by measuring aliquots of one chylomicron sample labeled with retinyl palmitate 10 times in series and on 10 consecutive days. The CVs within-run and between-days were 3.6% and 5.1%, respectively.

To examine whether plasma lipoproteins yield unspecific fluorescence, two plasma samples obtained from the same individual were separated by gel filtration, and the fluorescence and the triglyceride concentrations in the eluates were compared (Fig. 5). Both samples were obtained 4 h after a fatty meal; however, retinyl palmitate was added to the second fatty meal only. In the sample without retinyl palmitate, fluorescence was not detected in the eluate in the size range of chylomicrons and chylomicron remnants, and little autofluorescence was detected in the range of small VLDL and IDL (Fig. 5). High autofluorescence was present in the LDL size region and particularly in the size range of HDL and of plasma proteins. Upon addition of retinyl palmitate to the fatty meal (which specifically labels chylomicrons and chylomicron remnants), two large fluorescent peaks in the size range.
range of large lipoproteins appeared, indicating that chy-
lomicrons and chylomicron remnants had now been la-
beled by retinyl esters.

The addition of retinyl palmitate to the fatty meal did
not alter the elution profile of triglycerides in lipoproteins
(Fig. 5). Both triglyceride elution profiles overlapped well,
indicating that the triglyceride response after repeated
standardized fatty meals is very reproducible.

The first application of this method was to study the
distribution of the fluorescence label among lipoprotein
density fractions in plasma obtained 4 h after a fatty meal
containing retinyl palmitate. These fractions had been first
isolated by sequential ultracentrifugation and were then
further separated by gel filtration (Fig. 6). The bulk of
the fluorescence was found in the chylomicron fraction
($S_f >1000$), which contained two lipoprotein peaks of
different sizes. In the first peak, which contained large
lipoproteins, the distribution of fluorescence and triglyc-
erides corresponded well. In the second peak, however,
the maxima of fluorescence and triglycerides did not
correspond. The fluorescence maximum was eluting be-
fore the triglycerides maximum. Apolipoprotein B48
could be demonstrated in both fluorescence peaks by
sodium dodecyl sulfate-polyacrylamide gel electrophore-
sis and Coomassie® staining (data not shown). Size and
composition (lipids, fluorescence, and protein) of the
second peak imply that this peak is composed of chylo-
micron remnants. Less fluorescence than in the chylo-
micron fraction was found in VLDL, and only minute
amounts were detectable in IDL. Fluorescence in LDL was
pronounced and was probably caused by retinoids (Fig.
5). Similar results were obtained when lipoprotein frac-
tions were analyzed by fluorometry directly without
additional fractionation by gel filtration [chylomicron
fraction: 27.7 arbitrary units (AU); VLDL fraction, 5.8 AU;
IDL fraction, 0.9 AU; LDL fraction, 32 AU].

**Discussion**

This procedure was developed to measure retinyl esters
in chylomicrons and chylomicron remnants by a rapid
and easy fluorometric method (17) as an alternative to
HPLC. Before retinyl esters in postprandial plasma can be
measured by fluorometry, two problems had to be solved:
the interference of lipemic turbidity with fluorometry and
the interference of other fluorescing substances in plasma
with the fluorescence of retinyl palmitate. The problem
with the turbidity was resolved by the use of a clearing
buffer, which was derived from a reagent mixture used in
a commercial test kit for cholesterol determination (cat.
no. 290319, Boehringer Mannheim). The cholesterol ester-
ase contained in this kit serves two purposes. First, it
liberates free cholesterol from cholesterol esters for sub-
sequent reactions, and second, it also cleaves other ester
bonds, in particular those in triglycerides, because this
enzyme is rather unspecific. Thus, this cleavage of tri-
glycerides, as well as the detergents contained in the
buffer, clears the lipemic sample and allows cholesterol
determination even in samples in which turbidity is
caused by lipemia. In our procedure, this enzyme again
serves two purposes. First, it clears the turbidity of the
sample by cleaving esters bonds in triglycerides, and
second, it hydrolyzes all retinyl esters completely to
retinol. Therefore, this procedure is highly sensitive, with
a detection limit of 0.1 mg/L retinyl palmitate. Because the substance producing the fluorescence signal is retinol, the method can be calibrated easily with retinol. The results from the new procedure agree well with the results obtained by HPLC. The method is linear up to 4 mg/L retinyl palmitate and shows satisfactory CVs.

The problem with interference by other fluorescing substances in plasma was resolved by separation of triglyceride-rich lipoproteins from other plasma proteins. Two methods may be used for this separation. One method, separation by ultracentrifugation, is easy to carry out but does not separate chylomicrons and chylomicron remnants well, as we could show in a first application of this method (Fig. 6). A large proportion of chylomicron remnants appeared in the chylomicron fraction (S<sub>r</sub> > 1000) and only a small proportion in the chylomicron infranatant. These data indicated that the analysis of retinyl esters in samples that have been separated by ultracentrifugation might underestimate the concentration of chylomicron remnants and overestimate the concentration of chylomicrons. Another method for the separation of lipoproteins, gel filtration, can separate chylomicrons and chylomicron remnants well (Figs. 5 and 6). Because gel filtration generates a large number of samples, the procedure might be further simplified by using a continuous flow system. If the eluate of a fast protein liquid chromatography column or a gel filtration column and a continuous supply of clearing buffer would be connected with the flow cell of a spectrofluorometer, both the retinyl ester concentration and the size of retinyl ester-labeled lipoproteins could be obtained simultaneously.

One limitation of the method is its applicability only to lipoproteins with a density of VLDL and lower, i.e., chylomicrons and chylomicron remnants. The high unspecific fluorescence of LDL (Figs. 5 and 6) is probably caused by both the protein moiety and by retinoids in LDL. HDL and, of course, unfractoned plasma samples cannot be measured by this procedure because the unspecific fluorescence of proteins and other retinoids far exceeds the fluorescence signal of retinyl esters in the sample (17). This limitation, however, does not diminish the usefulness of the procedure. Retinyl esters that have been added to a fatty meal are transported almost exclusively in chylomicrons and chylomicron remnants (16), and both lipoproteins have only a negligible unspecific fluorescence (Fig. 5). As long as these lipoproteins are separated from other plasma proteins, the determination of their retinyl ester label by fluorescence is reliable (Fig. 3) and specific for retinyl esters (Fig. 5).

It should be kept in mind that the fluorescence signal is caused by retinol derived from all retinyl esters present in a given sample and not only from retinyl palmitate. Most investigators of postprandial lipid metabolism determine only retinyl palmitate (11, 15, 20, 21), which normally makes up about 80% of all retinyl esters after a fatty meal containing retinyl palmitate. Because this percentage is assumed to be fairly constant, retinyl palmitate is regarded as representative for all retinyl esters in the sample (16). Because the new method determines all retinyl esters, no assumption of a constant reesterification to palmitate must be made. This might be advantageous, especially when the effects of fatty meals containing different fatty acids are to be compared. When fatty acids other than palmitate are present in high concentrations, intestinal reesterification of retinol derived from retinyl palmitate may occur with these fatty acids, and a higher proportion of retinol may be found in retinyl esters other than retinyl palmitate.

The procedure is easy to carry out. Sample preparation and measurement are rapid because all that must be done is to add clearing buffer to the isolated sample and mix briefly, wait 10 min, and transfer the mixture into a spectrofluorometer, either by pipetting into a cuvette or by pumping through a flow cell. The alternative method, HPLC, requires considerably more time for sample preparation (extraction with organic solvents, phase separation, drying, and resuspension) and the measuring process; this limits the use of HPLC to studies with small sample numbers. Therefore, the new method will be particularly suitable whenever large sample numbers of retinyl ester-containing lipoproteins must be analyzed or for laboratories without HPLC equipment. Applications of this method, for example, are large-scale investigations of postprandial lipoprotein metabolism in which lipoproteins have been isolated by ultracentrifugation or studies involving the separation of lipoproteins by gel filtration.

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References


