Determination of Apolipoprotein B-48 in Plasma by a Competitive ELISA

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Background: Apolipoprotein B-48 (apoB-48) is produced by the small intestine, as part of chylomicrons, and appears to be a suitable marker for clinical studies of postprandial lipoproteins and related cardiovascular risk. Our aim was to develop, for routine analysis, an assay to quantify apoB-48 in plasma samples.

Methods: A microtiter plate was coated with a C-terminal apoB-48-specific heptapeptide. Plasma samples were incubated with appropriate detergent to allow competition between immobilized antigen and plasma apoB-48. Appropriate calibration curves were obtained in the ELISA, using calibrated lymph and chylomicrons.

Results: Treatment of plasma samples with the mild detergent Triton X-100 allowed an efficient competition between immobilized antigen and plasma apoB-48. No cross-reactivity was found with apoB-100, as checked by ELISA and Western blot analysis. Intra- and interassay CVs were 5.4% and 5.5%, respectively. In healthy subjects, apoB-48 concentrations markedly increased in the postprandial state, in parallel with triglycerides.

Conclusions: This new ELISA allows determination of the concentration of apoB-48 in normolipidemic plasma.

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Apolipoprotein B-48 (apoB-48)4 is an excellent marker of postprandial lipoproteins because it is associated exclusively in humans with intestinally derived chylomicrons (CMs) and their remnants, circulating particles that have been described as atherogenic (1).

Because of the very high homology of hepatic apoB-100 with apoB-48, quantification of apoB-48 by immunotechnology has been difficult. Furthermore, in the fasting state, the plasma apoB-48 concentration is ~100-fold lower than the concentration of apoB-100.

Most attempts to quantify apoB-48 have used analytical electrophoresis [sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] with densitometric scanning of Coomassie Brilliant Blue-stained gels (2–4). Cohn et al. (5) developed an affinity chromatography technique to separate apoB-48- and apoB-100-containing particles, the latter being retained with monoclonal antibody. More recently, Smith et al. (6) described an SDS-PAGE electrophoresis method, followed by Western blot with anti-apoB antibodies and enhanced chemiluminescence. The visualization of proteins was ~10 times more sensitive than Coomassie staining.

In 1993, Peel et al. (7) described a specific antiserum raised against apoB-48 that was used by Lovegrove et al. (8) in 1996 to develop an ELISA to quantify apoB-48 in human triglyceride-rich lipoproteins (TRLs). All of these techniques are time-consuming and require separation of TRLs by ultracentrifugation. In addition, detection after SDS-PAGE separation shows only moderate sensitivity.

We have developed a competitive ELISA assay that uses polyclonal antibodies raised against the C-terminal part of the protein. In addition, the use of Triton X-100 allowed us to optimize conditions and enabled accurate quantification of apoB-48 in plasma samples without isolation of TRLs, which had been necessary in all previously described methods. The method is simple and reliable for measuring apoB-48 in normolipidemic plasma and is suitable for routine analysis.

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4 Nonstandard abbreviations: apoB, apolipoprotein B; CM, chylomicron; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TRL, triglyceride-rich lipoprotein; TG, triglyceride; BSA, bovine serum albumin; and PBS, phosphate-buffered saline.
Materials and Methods
PREPARATION OF CM CALIBRATORS
Human CMs (d < 1.006 kg/L) were isolated from fresh plasma of healthy donors in the postprandial state after intake of a mixed meal (~800 kcal) containing 40 g of fat (9) (49% energy). Lipids were composed mainly of saturated and monounsaturated fatty acids provided by butter, meat sauce, and cottage cheese.

Pooled plasma (1 mL) was overlayed with 2 mL of a saline solution (9 g/L NaCl) and ultracentrifuged in a 100.3 rotor in a Beckman TLX 100 ultracentrifuge at 540,000 g for 6 min (10); 1.5 mL of the upper layer (TRL fraction) was then carefully removed, mixed, aliquoted, and frozen at −20 °C for later analysis.

Human LDL was isolated from fasting plasma samples by the density gradient ultracentrifugation procedure (10).

Triglycerides (TGs) were determined by colorimetry after enzymatic hydrolysis and quantification of liberated glycerol (Roche Diagnostics).

QUANTIFICATION OF APOB-48 IN THE CM CALIBRATOR
A sample of human thoracic duct lymph that was obtained from a patient undergoing thoracic surgery was kindly supplied by C. Williams (University of Reading, Reading, United Kingdom). The amount of apoB-48 in this sample was determined by the SDS-PAGE method of Karpe and Hamsten (2). The apoB-48 concentration in the prepared CM calibrator was determined by ELISA using the lymph sample as the primary calibrator. The apoB-48 content was further checked by the SDS-PAGE method.

PREPARATION OF SPECIFIC IMMUNOGLOBULINS
The C-terminal hexapeptide (Cys-Leu-Gln-Thr-Tyr-Met-Ile) described by Peel et al. (7) was synthesized by CIML (Marseille, France). The addition of an N-terminal cysteiny1 residue allowed us to conjugate the peptide to ovalbumin, using maleimidocaproic acid N-hydroxysuccinimide ester (11), for injection into rabbits (12). The peptide was reacted with activated ovalbumin in a 3:1 molar ratio, and the reaction was stopped by dialysis. We measured the capacity of ovalbumin to bind the SH-group on the cysteine before and after the reaction to determine the amount of peptide coupled to ovalbumin.

Two animals were immunized with five injections at 2-week intervals. For the first immunization, rabbits received intradermal injections of 200 µg of the peptide in complete Freund’s adjuvant (Sigma-Aldrich); for the boost, they received intramuscular injections of 100 µg of the peptide in incomplete Freund’s adjuvant.

Because in the assay no ovalbumin is introduced, there was no need to adsorb the antibodies to ovalbumin. We used preimmune serum obtained from rabbits before immunization as control and bovine serum albumin (BSA) as negative control.

Immunoglobulins were purified from rabbit antisera by gel affinity chromatography on protein A-Sepharose (Pharmacia-Biotech) (13).

SUBJECTS
Twenty-eight healthy male volunteers (age range, 20–35 years) were enrolled for quantification of apoB-48 concentrations. Blood was drawn at fasting and 2, 3, 4, and 7 h after intake of a standard fat-containing meal (9). Blood samples were collected into EDTA tubes and centrifuged at 700 g; plasma was separated and frozen at −20 °C for later analysis. The protocol has been accepted by the Local Medical Ethics Committee (CCPRRB, Marseille, France).

apoB-48 ELISA PROTOCOL
A 96-well microtiter plate (Nunc Polylabo) was coated by incubation overnight at 4 °C with conjugated heptapeptide (10 mg/L) in 0.1 mol/L carbonate buffer, pH 9.6. The heptapeptide was conjugated to thyroglobulin, using N-succinimidyl 3-(2-pyridyldithio)propionate (14).

After the microtiter plate were washed with phosphate-buffered saline (PBS; 100 mmol/L phosphate, pH 7.4), wells were saturated with 200 µL of PBS containing 10 g/L BSA (PBS-BSA).

For detergent treatment of CM calibrators and plasma, samples were incubated with 70 mL/L Triton X-100 in PBS (final concentration, 2 mL/L) for 1 h at 37 °C.

A five-point calibration curve was prepared by serial dilution of CMs in PBS-BSA. Competition was started in tubes with an equivalent volume of immunoglobulin diluted in PBS-BSA and competitor (diluted calibrator or plasma sample). Aliquots (100-µL) from each tube were then added, in duplicate, to the wells, and the plate was incubated at 37 °C for 2 h. After the plate was washed

Fig. 1. Improvement of competition in apoB-48 ELISA by treatment with Triton X-100 at final concentrations of 0 (□), 1 (+), 2 (△), and 5 (+) mL/L.
three times, 100 µL of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Tebu) diluted in PBS-BSA was added to each well and incubated at 37 °C for 2 h. After the plate was washed as before, bound peroxidase was determined using 3,3′,5,5′-tetramethylbenzide as substrate. The color was developed for 30 min, and the reaction was stopped by the addition of 50 µL of 1 mol/L HCl to each well. The plate was read at 450 nm using a Labsystem plate reader. The percentage of competition was calculated from the absorbances without (A₀) or with (Aₐ) competitor added and was: \( \frac{1}{2} \left( \frac{A_a}{A_0} \right) \times 100.

**SDS-PAGE AND IMMUNOBLOTTING**

Control of CM was performed by SDS-PAGE using the Phast System (Pharmacia-Biotech). We used Phastgel 8–25% gradient gels (thickness, 0.45 mm), polyacrylamide gradient gels designed for separation of high-molecular mass proteins (apoB-48 = 260 kDa; apoB-100 = 550 kDa). CM and lymph samples were loaded onto SDS-PAGE gels and electrophoresed at 90V-h, 250 mV, 15 °C. Gels were stained by Coomassie Blue.

After electrophoresis, proteins were electrotransferred directly onto nitrocellulose sheets (0.45 µm), using the Phast System. The nitrocellulose membrane was incubated with our specific rabbit antibody, washed, and incubated with labeled peroxidase anti-rabbit immunoglobulins. Apolipoprotein blots were visualized by the addition of peroxidase substrate (tetramethylbenzide).

**Results**

**DETERGENT TREATMENT**

In our hands, apoB-48 in plasma samples was not fully and/or reproducibly recognized by the raised antibodies. Thus, to improve recognition of apoB-48 by anti-peptide antibodies, we tested several delipidating agents, such as mixtures of alcohol/ethyl ether, ionic or nonionic detergents (SDS, Tween 20, CHAPS, octylglucoside), and enzymes (phospholipase, triglyceride lipase).

Treatment of samples with Triton X-100 improved recognition of plasma apoB-48 with antibodies. As shown in Fig. 1, several concentrations of Triton X-100 (1–5 mL/L) were tested, and 2 mL/L was selected for accurate routine analysis. This detergent treatment was further applied systematically to plasma samples as well as the calibrators.
CALIBRATION CURVE

Appropriate calibration curves were obtained with human lymph and CMs. The curve was linear from 0.045 to 3.0 mg/L apoB-48. Lymph, CM, and postprandial plasma samples produced comparable displacement (Fig. 2) without significantly different slopes (P < 0.05). Moreover, parallel curves were obtained with fasting plasma samples (not shown). The apoB-48 concentration in the CM calibrator was 7.45 mg/L in the ELISA and 7.0 mg/L as determined by analytical SDS-PAGE as a reference method.

SPECIFICITY AND TEST CHARACTERISTICS

The specificity of the immune serum was checked by ELISA as well as by SDS-PAGE. Molecular mass markers were myosin (200 kDa) for apoB-48, and thyroglobulin (660 kDa) for apoB-100. SDS-PAGE of CM and lymph samples revealed the presence of apoB-48 and apoB-100, but after immunoblotting, only one apoB-48 band for either CMs or lymph was revealed by antipeptide antibodies. In the ELISA, we tested apoB-100-containing LDL at concentrations up to 10-fold higher than the concentration of apoB-48 usually detected (Fig. 3), and no competition was observed.

The lower limit of detection (mean absorbance + 3 SD) was 0.06 μg/L. The intraassay CVs for plasmas with high and low concentrations were 5.4% and 7.9%, respectively, based on 15 measurements. The interassay CVs were 5.5% and 8.3%, respectively (n = 12).

apoB-48 CONCENTRATIONS IN PLASMA SAMPLES FROM HEALTHY SUBJECTS

Mean fasting concentrations in normolipidemic subjects were 0.080 ± 0.01 mg/L, and mean postprandial peak values were 0.34 ± 0.04 mg/L (Fig. 4). Data indicated that apoB-48 concentrations in serum increased in the postprandial state up to 3 h and returned to baseline concentrations after 7 h. apoB-48 ran parallel to TG concentrations postprandially.

Discussion

Non-cross-reactive rabbit antibodies can be raised against human apolipoproteins, using synthetic peptide as immunogens (7, 15). The specificity of anti-apoB-48 antibodies obtained herein was demonstrated by immunoblotting (no detection of apoB-100) and by competitive ELISA (no competition with apoB-100-containing LDL).

The reliable quantification of apoB-48 by ELISA is particularly difficult. We aimed to define new assay conditions to make quantification of apoB-48 readily feasible in any laboratory using a classically obtained antiseraum.

It appeared that not all apoB-48 antigenic sites are accessible in native plasma. This led us to mildly delipidate calibrators and samples for the assay. Most delipidating agents tested did not improve apoB-48 recognition by the anti-peptide antibodies. Some of them were too drastic (ethanol/ether mixtures) or inefficient (lipases) or induced alterations in the tertiary structure of the protein (and hence its immunoreactivity) (16).

Treatment of plasma with Triton X-100 significantly increased apoB-48 recognition by anti-peptide antibodies, consistent with data obtained by Fisher and Schumaker (17) and Brasaemle et al. (18). An optimal concentration (2 mL/L) was selected for the initial treatment of calibrators and samples for further routine measurements.

Uchida et al. (19) recently used a monoclonal antibody to develop a sandwich ELISA for measurement of apoB-48, but the authors did not report whether any kind of sample treatment was needed; therefore, a possible optimization of this method remains to be developed for full validation. We were aware of the use of a readily available calibrator (human CMs) to replace human lymph, and it was thus necessary to check that raised immunoglobulins recognize in the same way apoB-48 in the CMs and plasma. This has been demonstrated by the parallelism of the two competitive curves.

The suitability of the ELISA method has been established during the postprandial follow-up of a group of healthy subjects. We found fasting apoB-48 concentrations of ~0.1 mg/L. In fact, a wide range of fasting apoB-48 values have been reported by different authors, likely because no routine standard method has been established, and the results are expressed as either TRL apoB-48 or plasma apoB-48 (2, 4, 8, 20).

According to the data obtained (Fig. 4), 3 h after a lipid meal, mean plasma apoB-48 was approximately fourfold higher than fasting apoB-48, which is consistent with the three- to fivefold increases reported by others (5, 20, 21). The fact that plasma apoB-48 concentrations were parallel to plasma TGs postprandially illustrates well that the changes in measured apoB-48 correspond to the expected transient accumulation of CM particles in the circulation after a lipid meal (21). The data fit well with the concept that apoB-48 is a reliable marker of CMs and CM remnants, whereas retinyl palmitate could not be (20).

In conclusion, we have developed a competitive ELISA for human apoB-48, using detergent treatment, that makes routine quantification specific, reliable, and easy.

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