Simple, Reproducible Procedure for Selective Measurement of Lipoprotein Lipase and Hepatic Lipase

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Existing methods for determining the release of lipoprotein lipase (EC 3.1.1.34) and hepatic lipase (EC 3.1.1.3) into plasma after heparin injection give highly variable results, primarily traceable to errors in the isolation of labeled oleate from the substrate, triolein. Methods involving anion-exchange resin to bind oleate show high variability and have a low yield. Introducing a strong base in the last step of the assay may spuriously increase the counts from oleate, whereas a detergent such as Triton X-100, used to minimize this problem, has a strong quenching effect.

We report a simple and rapid method in which we eliminate rather than correct for the sources of variation. The substrate, tri[1-14C]oleyl-glycerol, is sonicated under strictly standardized conditions with gum arabic, 50 g/L. Incubation is stopped by addition of a benzene/chloroform/methanol mixture and NaOH, 0.2 mol/L. Labeled oleic acid is extracted with hexane after acidification of the alkaline aqueous (upper) phase, so that no alkali is introduced into the scintillation liquid. For lipoprotein lipase measurement, hepatic lipase is inactivated by a specific antisera, whereas hepatic lipase is measured after lipoprotein lipase is inactivated by NaCl, 1.0 mol/L. The method is efficient and specific, and quenching and chemiluminescence artifacts are avoided.

Additional Keyphrases: triglyceride lipase activity

enzyme activity

Heparin-releasable lipoprotein lipase (LPL; EC 3.1.1.34), a key enzyme in the metabolism of triglyceride-rich lipoproteins, is present in various extrahepatic tissues, chiefly heart, mammary gland, adipose, and muscle tissues (1, 2). After an intravenous injection of heparin, trioleyl glycerol activity appears in plasma (3). LPL is not the only lipase released by heparin into the bloodstream: another lipase originates from the liver (4, 5) and its role is not yet clear. Both enzymes are located in the capillary bed, where lipase activity takes place, and have different characteristics: LPL is stimulated by apolipoprotein C-II (apo C-II) and inhibited by NaCl, 1.0 mol/L, whereas hepatic lipase (HL; EC 3.1.1.3) seems not to require any specific serum factor and is activated by high salt concentrations.

Several methods have been published for the assay of these lipases (6-10); this alone indicates the difficulties inherent in their measurement (11). There are three main groups of problems: (a) stabilization of the substrate, (b) separation assay of LPL and HL, and (c) selective removal of the fatty acids released.

The substrate-stabilizing agents used include lipids (phospholipids or glycerides), detergents (Trition X-100, Tween 60), and polysaccharides (gum arabic) (11). Because the enzymes have different characteristics, they must be assayed under different conditions, and the incubation must include added activators or inhibitors such as preheparin plasma or purified apo C-II, detergents, protamine sulfate, salt, or antiserum.

Methods for removing the liberated fatty acids include anion-exchange resin (10, 12, 13) and (or) liquid partition (7, 14) with an alkaline mixture. In radioassays, addition of any of these components with labeled fatty acids to the counting vials induces high variability and impairs reproducibility. To overcome these problems, we have developed a simple, highly reproducible procedure for the selective measurement of LPL and HL.

Materials and Methods

Apparatus. We used a Sorvall refrigerated general-purpose centrifuge (Model RC-3; Ivan Sorvall Inc., Norwalk, CT 06852) for blood centrifugation. To separate the different lipoprotein subclasses, we used an ultracentrifuge with a type 40.3 fixed-angle rotor (Model L5-65; Beckman Instruments Inc., Fullerton, CA 92634). Incubations were in a Dubnoff metabolic shaking incubator (Fisher Scientific Co., Fair Lawn, NJ 07410). We measured cholesterol, triglycerides, uric acid, and glucose with an ABA-100 bichromatic analyzer (Abbott Laboratories, Mississauga, Ont.). For sonication, we used a 70-W MSE ultrasonic desintegrator (Model MK2; Measuring Scientific Equipment, Crawley, U.K.) at a high 4 setting. To lyophilize milk, we used a Model 10-147 MR BA lyophilizer (VirTis Inc., Gardiner, NY 12525). A Beckman Model LS-350 scintillation counter was used.

Reagents. The substrate was a mixture of tri[1-14C]oleyl glycerol (Amer sham Corp., Arlington Heights, IL 60005) and unlabeled trioleoylglycerol (Sigma Chemical Co., St. Louis, MO 63178). The mixture was purified on an anion-exchange resin (Amberlite IRA-400; Mallinckrodt Chemicals, Pointe Claire, Que.). The elution stabilizer was gum arabic purchased from Sigma Chemical Co. Sodium hydroxide (ACS grade) and tris(hydroxymethyl)methylamine (Tris) HCl (analytical grade) for the buffers were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Bovine serum albumin, essentially free from fatty acid, came from Sigma Chemical Co., sodium chloride (certified) from Fisher Scientific Co., Montreal, Que., and hydrochloric acid (ACS grade) from A and C American Chemicals, St. Laurent, Que.

The organic solvents were benzene, chloroform, and hexane, all ACS grade, from Fisher Scientific Co., Montreal; isopropyl alcohol (reagent grade) from A and C Chemicals Co., and methanol (ACS grade) from Baker Chemical. The scintillation liquid was Aquasol-2 (New England Nuclear, Boston, MA 02118).

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Source of enzymes. Post-heparin plasma: All subjects were healthy normal volunteers and blood was sampled after a 12-h fast into Vacutainer Tubes (Becton Dickinson, Mississauga, Ont.) containing 5 int. units of heparin per milliliter. “Post-heparin” plasma was obtained 10 min after an intravenous injection of 10 int. units of heparin (Harris Co., Montreal, Que.) per kg of body wt. The injection was made in one arm and blood was collected from the opposite one. Plasma was separated by centrifugation at 4 °C and kept frozen at −20 °C until used (within three months).

Other sources: Acetone powder extracts were prepared according to previously published methods from adipose tissue of fed rats (15). Milk LPL was prepared as follows: fresh bovine milk was skimmed and dialyzed, first against water and then against a 0.1 mol/L NaCl solution. After lyophilization, it was kept at −20 °C in Tris HCl (0.2 mol/L, pH 8.4) and thawed just before use.

Preparation of the substrate. The substrate was a tri[1-14C]oleoylglycerol emulsion. Triolein continuously undergoes autohydrolysis, which increases background radioactivity. To decrease the blank counts (by four- to fivefold), we purified labeled triolein by anion-exchange chromatography on a 1.5 × 15 cm column packed with Amberlite IRA 400 resin (H+ form). The triolein was eluted with hexane and the free fatty acids were retained on the column. Aliquots of triolein (spec. acty. 40 mCi/mol) in hexane were dispensed into plastic vials (usually 3 mL for 30 assays), dried under nitrogen, and stored frozen at −20 °C. On each day of assay, after thawing at room temperature (1 h), we added a filtered 50 % solution of gum arabic, in the same volume as the hexane aliquots before evaporation, and sonicated the vials in an ice bath during four 30-s periods at 30-s intervals under strictly standardized conditions. One hundred microliters of this emulsion yielded about 100 000 cpm and a final triolein concentration of 15 mmol/L.

Preparation of the antiserum against hepatic lipase. HL was isolated from human post-heparin plasma by affinity chromatography on heparin–Sepharose CL-6B (Pharmacia, Uppsala, Sweden). The heparin affinity column (25 × 4 cm) was equilibrated with buffer A (5 mmol/L barbital buffer, 0.4 mol/L NaCl, pH 7.4), and post-heparin plasma diluted with an equal volume of buffer A was applied to the column. The column was developed with buffer A, and HL was eluted with buffer B (5 mmol/L barbital buffer, 0.75 mol/L NaCl, pH 7.4). Anti-HL antiserum was obtained from laboratory rabbit after immunization with a mixture of 100 μg of HL and complete Freund's adjuvant. The antiserum totally inhibited lipase activity from HL preparations and we found no cross reaction with preparations containing only LPL activities (milk LPL, rat adipose tissue LPL; see Source of enzymes).

Assay procedure. HL was inactivated with the specific antiserum and the remaining activity due to LPL was selectively measured in the presence of activator. In preliminary experiments the antiserum titer was checked for complete inhibition of post-heparin plasma HL as follows: several dilutions (with control rabbit serum) were preincubated at 0 °C for 1 h with post-heparin plasma and lipase activity measured under the conditions for HL (see below). Inactivation of HL was considered to be complete when less than 3% of initial activity remained.

For duplicate determinations of LPL we preincubate 25 μL of post-heparin plasma on ice for 1 h with 25 μL of anti-HL antiserum, diluted if necessary. During this period, we pipet 300 μL of a mixture of, per liter, 0.2 mol of Tris HCl and 0.15 mol of NaCl, pH 8.4, containing 50 g of bovine serum albumin and 120 mL of pooled human normal plasma as activator into tubes kept on ice. Then we add 20 μL of the preincubation post-heparin plasma–antiserum mixture to these tubes. To start the incubation, we add 100 μL of triolein emulsion and continue at 37 °C for 30 min with gentle shaking. We stop the reaction by adding 3.0 mL of benzene/chloroform/methanol (10/5/12, by vol) containing oleic acid, 0.1 mmol/L, as described by Pittman et al. (16), but with the following modification: after adding 200 μL of 0.2 mol/L NaOH and mixing (30 s), we centrifuge the tubes for 20 min at 2000 rpm, then transfer 1 mL of the 1.95-mL upper phase to a tube containing 1 mL of 0.25 mol/L HCl and 4.5 mL of hexane. After vortex-mixing, we separate the hexane phase by brief centrifugation and transfer it into counting vials. This extraction procedure is repeated twice, pooling the hexane phases, evaporating under a stream of air with heating (50 °C), then redissolving the residues in 10 mL of scintillation liquid for counting. This modification of the procedure of Pittman et al. (16) avoids introducing a strong alkali and interfering substances into the counting vials (see Discussion).

In the assay for HL, we mix 10 μL of post-heparin plasma with 310 μL of Tris HCl buffer (0.4 mol/L, pH 8.9) containing NaCl (1.0 mol/L) and bovine albumin (50 g/L) but without plasma activator, then incubate as for the LPL assay.

For neither assay did maximum hydrolysis exceed 10% of the added substrate. Results are expressed in units per milliliter of post-heparin plasma, where 1 unit represents 1 μmol of fatty acid hydrolyzed per hour.

Results

Selective extraction of oleic acid. Whenever a radioactive assay is used to measure lipase activity, labeled oleic acid must be separated from the excess triolein as well as from the products of lipolysis (radiolabeled di- and mono-olein). For this purpose anion-exchange resins are used in some methods to bind oleate (10, 12, 13) and after numerous washes the radioactivity of the labeled complex obtained is counted in the presence of scintillation liquid. We found that addition of resin to the counting vials increased the counts from [14C]oleic acid by as much as 20%. Moreover, the change was not linear with increasing amounts of resin. On the other hand, we observed that the addition of detergent (Triton X-100), used in some methods, had a sometimes dramatic quenching effect.

In yet another type of method, oleate is extracted with an alkaline mixture and its radioactivity counted directly in the presence of scintillation liquid. We found marked changes (up to 400% for 100 mmol) in the counts obtained with constant amounts of [14C]oleic acid and different amounts of diluted alkali. Surprisingly, in some methods all three interfering elements are used: resin, detergent, and a strong alkali.

Moreover, in our hands the use of resin was not entirely

| Table 1. Analytical Recovery of Labeled Oleic Acid and Triolein by Different Extraction Procedures |
|---|---|---|
| Extraction procedure | [3H]Oleic acid recovery | [14C]Triolein in blank tubes |
| Resin + elution | 63.3 | 2 |
| MeOH/CHCl3/heptane (141/125/100 by vol) | 72.2 | 1 |
| Benzene/CHCl3/MeOH (10/5/12 by vol) | 86.6 | 0.1 |

Labeled compounds were added to diluted plasma samples and extracted in the same way as a reaction mixture.

a Hexane extracts were transferred to resin-containing tubes; resin-bound lipids were eluted with 1 mol/L HCOOH in chloroform/methanol solution (1/1 by vol), transferred to counting vials, evaporated, and their radioactivities counted.

b As indicated by Beifrage and Vaughan (14).

c After Pittman et al. (16) with hexane extraction (see Materials and Methods).
Keep the background count at its lowest.

Validation of the technique. Figure 1 illustrates conditions for maximum enzyme activity. A substrate concentration >15 mmol/L was not rate limiting. Enzyme activity was linearly related to time for at least 60 min and to increasing amounts of post-heparin plasma. Total lipase activity was maximally stimulated with about 12% of added normal plasma (Figure 2).

We observed the same activation with powdered acetone extracts of the adipose tissue of fed rats. The effects of NaCl observed with post-heparin plasma or with rat adipose tissue were characteristic for LPL, i.e., inhibition with increasing molarity of NaCl (Figure 2).

The one-day within-assay precision established with frozen milk LPL thawed just before assay was 3.2% (n = 6).

Day-to-day precision was determined from the means of duplicate milk LPL standard (reconstituted from lyophilized aliquots) included in each assay series; the CV was 12.3% for 24 duplicate determinations over a period of one month.

Effect of anti-HL antiserum. For the selective measurement of LPL, post-heparin plasma was first incubated for different periods of time with antiserum directed against purified human HL. The preincubation was at 0 °C to prevent heat denaturation of enzymes (19). When measured under LPL conditions, i.e., with low NaCl concentration and activator, at least 70% of the initial activity was removed if the

satisfactorily; methanol/chloroform/heptane extraction recovered only 72.2% of the label (Table 1) and even benzene/chloroform/methanol containing oleic acid (0.1 mmol/L) followed by hexane extraction recovered only 86.6%.

Stability of triolein emulsion. To check the stability of our substrate emulsion, we pipetted eight 50-μL aliquots of a freshly prepared substrate emulsion at 0, 1, 3, and 5 h and counted them in liquid scintillation fluid after sonication. The emulsion was found to be highly stable over this period of time. It was highly homogeneous, the within-assay variation was less than 0.65%. The between-assay variation, 0.86%, is representative of a very reproducible substrate. We also observed that this emulsion retained its properties at 4 °C for at least one day if directly resonicated before use. We do, however, recommend use of a freshly prepared emulsion to

Fig. 1. Relation of total lipase activity to (A) substrate concentration, (B) incubation time, and (C) enzyme concentration of post-heparin plasma.

Total lipase activity was measured with 0.1 mol/L NaCl and plasma activator but without inactivation of hepatic lipase.

Fig. 2. Total lipase activities from human post-heparin plasma (PHP) and rat adipose tissue (AT) acetone-powder extract expressed as percentage of the relevant maximum activity, in the presence of NaCl (left) and normal serum (right).

Total lipase activity was measured without inactivation of hepatic lipase.

Fig. 3. Effect of duration of preincubation with an anti-hepatic lipase antiserum on the post-heparin plasma lipase activity measured under conditions optimal for lipoprotein lipase (▲) or hepatic lipase (○).

Activities are expressed as the percentage of lipase activity obtained with normal rabbit serum in place of antiserum.
preincubation time exceeded 30 min. This inhibition varied with the individual sample. When NaCl concentration was increased to 1.0 mol/L after prior incubation, as above in the presence of HL-antiserum, LPL activity was completely inhibited and less than 2% of the initial activity remained (Figure 3). This is actually less than the 5% or less reported when HL and LPL were fully inhibited with selective antiserum (8). Thus in our assay the residual lipase activity after HL-inactivation (upper curve, Figure 3, about 30%) could confidently be ascribed to LPL alone.

Post-heparin lipase activities in normal subjects. Results were obtained in a group of 16 normal volunteers for LPL and HL activities in plasma obtained 10 min after an intravenous injection of 10 int. units of heparin per kg of body wt. The mean ±SD values were 8.02 ± 1.69 unit/mL for LPL and 5.09 ± 1.25 unit/mL for HL. The mean plasma triglycerides for this group of subjects was 790 ± 400 mg/L.

Human very-low-density lipoproteins, prepared by ultracentrifugation of a hypertriglycerideremic plasma at d = 1.006 g/L (116 000 × g, 16 h), were added to normal post-heparin plasma samples to simulate plasma from hypertriglycerideremic subjects. At triglyceride concentrations ranging from 900 to 10 000 mg/mL, we observed no appreciable effect on post-heparin plasma lipase activities.

Discussion

LPL and HL are triglyceride-hydrolysing enzymes found in plasma after intravenous injection of heparin. They have contrasting properties: HL is salt-resistant and to some extent protamine sulfate-resistant (6), whereas LPL is inhibited by NaCl 1.0 mol/L or protamine sulfate and is activated by apo C-II. We report a simple and rapid method of assaying these enzymes with a radiolabeled triolein emulsion as substrate. Although the general principle of the method is similar to that of other published techniques (8), we have introduced several modifications that simplify the procedure, improve its reproducibility, and make it suitable for routine analysis in a general clinical laboratory. After setting up various currently available methods we realized, as have others (7), that there were great discrepancies among the results obtained with different procedures. We critically reviewed every step, using the reports already in the literature, and attempted to resolve several sources of variability or uncertainties as they became evident.

We selected a radioactive substrate because of its high sensitivity, which allows a short period of incubation (30 min or less) and small sample size (10 µL). With a water-insoluble substrate, however, choice of a stabilizing agent is a problem. We chose a gum arabic solution because LPL reportedly gives higher initial rates of reaction with triolein emulsified with this agent than with phospholipids or detergent (17).

The second area of difficulty is isolation of lipase-labeled fatty acids from mono-olein, diolein, and excess triolein. Methods involving union-exchange resins to bind free fatty acids (10–12) introduce an important source of variation, presumably chemiluminescence due to the presence of resin in the counting vials. Although the method of Zieve (20) avoids this pitfall, the use of small columns of resin is time-consuming when a large number of samples are being assayed. Moreover, the yield of oleate from the resins with the reported eluents was only about 60% (Table 1), blanks were high, and both oleate and blanks varied markedly with assay conditions.

Methods involving liquid-partition systems for extraction of fatty acids have the advantage of selectively extracting oleate without introducing resins into the counting solution. Unfortunately, however, they introduce a strong alkali into the counting vial, which we found increased the oleate counts (often dramatically: 400% for 0.1 mol/L KOH), another effect potentially ascribable to chemiluminescence.

Use of a detergent such as Triton X-100 (10) seems to minimize these effects. However, we found that Triton X-100 quenches scintillation strongly (75% with a 10-fold dilution) and contributes to the variability and lack of reproducibility of the results. We were surprised to find that in a recently published method (10) all these interfering components (Triton X-100, 2 mol/L KOH, and ion-exchange resin) were combined in the counting vial and heated up in the final step of the method. Because of the lack of linearity and reproducibility of some of the interfering effects, which may act in opposite directions, the use of quenching curves does not provide a consistent and accurate correction. We therefore eliminated the sources of variation instead of attempting to correct for them. To avoid interference from the presence of strong alkali in the counting solution, we extracted the labeled oleic acid with hexane at low pH. In our hands the liquid-partition system of Pittman et al. (16) seemed to give the best yield and the lowest blanks (Table 1).

Because of uncertainties concerning LPL inhibition by protamine sulfate (11), we used 1.0 mol/L NaCl for complete inhibition of this enzyme. At first we obtained LPL activity by subtracting HL activity measured at high salt concentration from post-heparin plasma total lipase activity obtained at 0.1 mol/L NaCl (21). We now use HL antiserum to inactivate HL specifically, which allows direct measurement of each enzyme and circumvents the problem of measuring total activity under sub-optimal conditions for HL.

With the method described, our normal values for LPL relative to HL are predictably higher than those reported in the literature (6, 8), because both enzymes are now measured under optimal conditions. On the other hand, the lower absolute values obtained here are probably related to the dose of heparin injected. We have selected a nonanticoagulating dose of heparin (10 int. units/kg) (6) to minimize the risk of bleeding. Larger doses (100 int. units/kg) should yield higher values because of the known dose-effect relationship (8). Furthermore, HL and LPL are affected differently at different doses of heparin, and endogenous factors such as adipose and muscle tissue masses and exogenous factors such as diet and physical exercise are known to influence the relative activities of those enzymes, especially LPL (18).

References

Measurement of Nifedipine in Plasma by Gas–Liquid Chromatography and Electron-Capture Detection

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In this method for measuring nifedipine, a calcium-channel inhibitor now widely used in the treatment of cardiovascular disease, the drug is extracted from plasma under basic conditions into toluene, and an aliquot of the extract is injected directly into a gas chromatograph equipped with an OV-101 column and an electron-capture detector. The standard curve is linear between 1 and 100 μg/L; the assay measures not only nifedipine, but also a major metabolic product (found only after oral administration of the parent drug) and photodegradation products. The procedure is rapid and adequately sensitive for routine use in clinical monitoring of nifedipine in plasma.

Additional Keyphrases: calcium-channel antagonist • drug assay • photodegradation • monitoring therapy • heart disease • drug metabolite

Nifedipine, a 1,4-dihydropyridine derivative, recently was introduced into clinical use in the United States as an antianginal agent. By reversibly blocking the cell membrane sites through which calcium moves, the drug inhibits transmembrane calcium ion flux and is known as a "calcium-channel antagonist" (1). Its major actions in vivo derive from its affinity for vascular tissue, where it blocks excitation–contraction coupling. It has therefore been remarkably effective in controlling the variant form of angina pectoris that is thought to be due to abnormal vasoconstriction in the coronary arterial circulation (2). It has also been widely used to treat chronic stable angina (3).

Very little information is available concerning its pharmacokinetics or pharmacodynamics, either in normal subjects or in patients. The compound is light-sensitive, which presents difficulties in developing valid assay techniques for its measurement in plasma. Ramsch developed a fluorescent assay method (4) but this was subsequently shown to be nonspecific. Other techniques used in assay of the drug include gas chromatography (5, 6), "high-performance" liquid chromatography (7), and mass spectrometry (8). None of these methods has been adapted for convenient use in clinical monitoring of the drug in plasma.

Here we describe a simple gas-chromatographic method for measuring plasma nifedipine concentrations, and for detecting its photodegradation products and a major metabolite. This approach has been used in preliminary studies of nifedipine pharmacokinetics in normal subjects, and it can be used to monitor drug concentrations in patients after therapeutic doses.

Materials and Methods

Principle. Nifedipine and an added internal standard (diazepam) partition rapidly into toluene from plasma at an alkaline pH. An aliquot of the toluene phase may be injected directly into a gas chromatograph, equipped with a 3% OV-101 column and an electron-capture detector. The drug’s concentration in plasma is evaluated by comparing the peak-area ratio (nifedipine:internal standard) with that found for standards of known concentrations.

Procedure. Pure nifedipine powder (Pfizer Inc., New York,