Hepatic and Lipoprotein Lipases Selectively Assayed in Postheparin Plasma

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Sensitive, reliable procedures are reported for the selective assay of lipoprotein lipase (LPL) and hepatic lipase (HL) in postheparin plasma samples. LPL is inhibited in the HL assay by inclusion of 0.76 mol/L sodium chloride in the substrate. In the LPL assay, specificity is attained by pretreating the sample with sodium docetyl sulfate, which selectively denatures HL. This LPL method was validated by direct comparison with a procedure in which HL is inactivated by an antiserum to human HL. We used the described assays to quantify LPL and HL activity in 32 normal adults, demonstrating a clear sex difference for both enzymes. On average, the men displayed higher HL and lower LPL activities than did the women.

Indexing Terms: enzyme inhibition assay · sex-related differences · immunosassay compared

The hydrophobic nature of lipid molecules necessitates their intravascular transport as lipoprotein complexes. Two enzymes, lipoprotein lipase (LPL; EC 3.1.1.34) and hepatic lipase (HL; EC 3.1.1.3), catalyze lipoprotein triglyceride and phospholipid hydrolysis, playing a key role in intravascular lipoprotein metabolism. Both enzymes are located on capillary endothelium, LPL being found on most if not all peripheral capillary beds, in contrast to HL, which is almost exclusive to the hepatic sinusoids (1). The enzymes bind to the endothelium via a heparin sulfate-like ligand, projecting into the bloodstream, where they interact with their circulating substrate. However, because their affinity for heparin is higher than that for the heparin sulfate-like ligand, injection of an intravenous heparin bolus displaces both enzymes into postheparin plasma (PHP), where their activity may be quantified.

The enzymes, although apparently similar with respect to their ability to catalyze lipoprotein triglyceride hydrolysis, differ in their substrate requirements, pH optima, activation, and inhibition. LPL, which displays maximal catalytic activity at pH 8.2 (2), is inhibited by 1 mol/L sodium chloride (3–6) and protamine sulfate (2, 3, 5, 7), agents to which HL is resistant (4–6). In addition, LPL displays a requirement for apoprotein (apo) C-II (5–10), which is supplied by adding heat-inactivated human serum to the substrate emulsion. In contrast, HL has a pH optimum of 8.8 (2), shows reduced activity in the presence of inactivated serum (11), and is inhibited by the concentrations of sodium docetyl sulfate (SDS) thought to stabilize LPL (7).

These differences have been exploited to achieve the selective assay of HL by including 1 mol/L sodium chloride in the substrate to inactivate LPL (12). The LPL activity in the sample may then be indirectly estimated as the difference between the total lipase and HL activities. The assay of total lipase activity is, however, unsatisfactory because the optimal substrate, pH, and activator requirements of both enzymes cannot be achieved in a single assay system. Direct assay of LPL can be achieved by use of an antiserum to inactivate HL (4). An alternative may be offered by SDS, an agent known to selectively inactivate HL (7, 10), and a direct LPL assay based on this observation has been reported (7). Despite the ready availability of SDS, this procedure has not been widely adopted, perhaps because of the laborious nature of the anion-exchange procedure used to isolate the fatty acid, and because of doubts regarding the efficacy of the HL inactivation.

In the present study we have developed sensitive, reliable procedures for the selective assay of LPL and HL activities, in which the fatty acid arising from the enzyme-catalyzed triglyceride hydrolysis is isolated by a simple extraction procedure (13). We used SDS to inhibit HL and validated this procedure by comparing our results with those obtained with an anti-HL antiserum. In addition, we evaluated the use of gum arabic and lecithin-stabilized substrate emulsions and investigated the influence of albumin and apo C-II on assay specificity.

Materials and Methods

Materials

Glass scintillation vials, methanol, chloroform, heptane, toluene, and glacial acetic acid were obtained from Fisons Scientific Equipment (Loughborough, UK). "Ecocint" scintillation fluid was purchased from National Diagnostics (Manville, NJ). Sodium chloride, sodium dodecyl sulfate, sodium carbonate, sodium bicarbonate, and ferric chloride were purchased from Merck Ltd. (Dagenham, Essex, UK). Carbonate–bicarbonate buffer (0.1 mol/L, pH 10.5), was prepared by mixing 800 mL of 0.1 mol/L sodium carbonate with 200 mL of 0.1 mol/L sodium bicarbonate. Intralipid 10% was supplied by KabiVitrum Ltd. (Uxbridge, UK). Triolein, bovine serum albumin (BSA) (essentially fatty acid-free), and Trizma ("Trizma Preset 8.2 and 8.8") were obtained from Sigma Chemical Co., Ltd. (Dorset, UK). [14C]Triolein was purchased from Amersham International plc (Aylesbury, Bucks, UK). Plain glass blood-collection tubes (10 mL) were supplied by Sherwood Medical Industries (Crawley,
Sussex, UK). The anti-HL antiserum was a gift from T. Olivecrona, University of Umeå (Umeå, Sweden).

Assay Principle

The procedures described quantify LPL or HL activity in PHP by measurement of the oleic acid produced from the enzyme-catalyzed hydrolysis of a triolein emulsion carrying a $^{14}$C]triolein radiolabel. The liberated oleic acid is isolated by a selective extraction procedure and its mass determined by reference to the $^{14}$C-labeled tracer, which is quantified by liquid scintillation counting. Lipase activity is calculated in terms of micromoles of oleic acid released per hour per milliliter of PHP.

LPL Assay Procedure

Substrate preparation. Two types of substrate emulsion were produced by sonicating triglyceride with two different emulsifiers (gum arabic and lecithin): The gum arabic emulsion consisted of a mixture of 100 mg of unlabeled triolein, 5 μCi of $[^{14}C]$triolein, and 5.68 mL of 90 g/L gum arabic in 0.2 mol/L Tris buffer, pH 8.2; the lecithin emulsion consisted of a mixture of 1 mL of Intralipid, 5 μCi of $[^{14}C]$triolein, and 4.68 mL of 0.2 mol/L Tris buffer, pH 8.2. The mixtures were sonicated in 20-mL glass scintillation vials. The sonication was performed with an MSE Soniprep (Fisons Scientific Instruments, Crawley, UK) at amplitude 10, with 24 cycles of 20 s on, 10 s off, and mixing the vials contents by inversion after every 4th cycle. After sonication we added 2.75 mL of 200 g/L BSA and 0.8 mL of 1.42 mol/L sodium chloride (both in 0.2 mol/L Tris buffer, pH 8.2) and mixed the emulsion by inversion.

Substrate preincubation. Aliquots of substrate emulsion (0.42 mL) were preincubated for 90 min at 37 °C, in 20-mL plain glass blood-collection tubes, with 80 μL of heat-inactivated pooled human serum as the source of apoC-II. The serum pool was prepared from blood donations from ≥50 subjects, heat-inactivated at 50 °C for 45 min, and stored as 2-mL aliquots at −20 °C for ≤1 year before use.

Sample preincubation. Thirty minutes before completion of the substrate preincubation, we mixed in an Eppendorf tube equal volumes (0.25 mL) of sample and 70 mmol/L SDS in 0.2 mol/L Tris buffer, pH 8.2, and kept this at room temperature (20 °C). An aliquot of isotonic saline was treated in the same fashion for use as a blank.

Enzyme-catalyzed triglyceride hydrolysis. On completion of the substrate and sample preincubations, we added 10 μL of sample to each of three tubes of activated substrate and allowed the LPL-catalyzed triglyceride hydrolysis to proceed for 1 h at 28 °C. Triglyceride hydrolysis was stopped by adding 5.33 mL of methanol:chloroform:heptane (56:50:40 by vol).

Extraction of liberated fatty acid. After 20 s of vigorously shaking the hydrolysis mixture, we added 1.5 mL of 0.1 mol/L carbonate–bicarbonate buffer, pH 10.5, shook the contents for 10 s, and centrifuged the contents for 45 min at 1500 × g with a swing-out rotor. To a glass scintillation vial containing 50 μL of glacial acetic acid containing 0.5 g of ferric chloride per liter we added 2 mL of the upper phase, swirled the contents, and mixed this with 16 mL of Ecoscint:toluene (7:1 by vol). The $[^{14}C]$oleic acid was quantified by liquid scintillation counting with a Beckman (Brea, CA) LS 7500 scintillation counter (channel 397–655, counted over 5 min with use of automatic quench correction based on Compton curve). The LPL activity was calculated from the difference in counts per minute between the “blank” and the sample vials.

HL Assay Procedure

The HL assay was identical to the LPL assay but with the following modifications: The 2.75 mL of 200 g/L BSA was added to the triolein/emulsifier mixtures before sonication, the 0.8 mL of 1.42 mol/L NaCl was replaced by 2.58 mL of 3.24 mol/L NaCl, and the solutions were prepared in 0.2 mol/L Tris buffer, pH 8.8; the substrates were used without addition of, or preincubation with, inactivated serum; and no sample pretreatment was used, 10-μL aliquots of PHP being added directly to the substrate.

Optimization of Assay Conditions

Substrate concentration. The substrate triglyceride concentration required to attain zero-order kinetics was evaluated for the gum arabic and lecithin emulsions described. We diluted aliquots of each triglyceride sonicate to 258 μL with saline and supplemented these with BSA, NaCl, and human serum (in the same proportions as the standard sonicate), such that final substrate triglyceride concentrations were in the range 0–8 g/L. We used these substrates to investigate the kinetics of LPL and HL activity in aliquots of a single sample of PHP.

Effect of sonication on lecithin (Intralipid)- and gum arabic-based emulsions. The effect of sonication on the particle size distribution of the gum arabic and lecithin emulsions was assessed by nephelometry (14). Scintillation vials containing either (a) 100 mg of triolein and 5.68 mL of 90 g/L gum arabic in 0.2 mol/L Tris buffer, pH 8.2, or (b) 1 mL of Intralipid diluted to 5.68 mL with 0.2 mol/L Tris buffer, pH 8.2, were subjected to 0, 6, 12, 18, 24, or 30 sonication cycles (as described in LPL Assay Procedure). The light-scattering index (LSI), an index of particle diameter, of each emulsion was determined with aliquots of emulsion diluted 100-fold with 9 g/L sodium chloride solution by use of a Mark IV micronephelometer (Scientific Furnishings Ltd., Poyn- ton, Cheshire, UK).

Effect of albumin incorporation on HL substrate. To assess the effect on HL activity of adding the BSA before the triglyceride sonication, we analyzed 12 samples for HL activity by the standard procedure and by sonicating the substrate before adding the BSA. To minimize the influence of batch differences, we pooled four separate batches of emulsion for each substrate type.

We also evaluated the effect of the presence of albumin at substrate sonication on nonspecific lipase activ-
ity—non-LPL (i.e., salt-resistant), non-HL (i.e., SDS-resistant) lipase activity—by repeating the above experiment with six PHP samples, assaying both before and after pretreatment with SDS (for inactivation of HL).

Comparison of lipase activities observed with gum arabic and lecithin sonicates. We analyzed 10 samples for LPL and HL activity, using the optimized gum arabic- and lecithin-based emulsions described above.

Incorporation of apo C-II activator into LPL substrate. The optimal serum volume and preincubation time for substrate activation with apo C-II was determined by assay of a single sample with aliquots of the gum arabic/triolein substrate at a triglyceride concentration of 9.1 g/L, preincubating the substrate with 0–80 μL of heat-inactivated pooled human serum for ≤90 min at 37 °C. Activation of the lecithin-based substrate was similarly investigated by using 80 μL of serum and preincubating for ≤70 min.

Comparison of SDS and anti-HL antiserum for inactivation of HL. To compare the use of SDS and anti-HL antiserum for the selective assay of LPL, we assayed 15 samples (a) by the standard LPL procedure, (b) by an adaptation in which the SDS pretreatment was replaced by a 2-h preincubation at 4 °C of the PHP with goat antiserum to human HL, and (c) by using the antiserum preincubation but including SDS (0.7 mmol/L final concentration) in the substrate to assess any surfactant effect introduced by the SDS.

Assay Precision
To assess the extent of the within-assay imprecision, we prepared five 100-μL aliquots from a single sample of PHP and treated these as individual samples (each assayed in triplicate) in a single run of both the LPL and HL assay procedures. Because each sample was assayed in triplicate, we were able to assess the within-assay precision for single measurements, the means of two replicates, and the means of three replicates.

Between-assay CV was determined by assaying 1-mL aliquots of a single "control" PHP (stored at −18 °C) in 20 runs of the LPL and HL assay procedures.

Reference Ranges for LPL and HL Activities
Reference ranges for the LPL and HL procedures were evaluated by obtaining blood samples from 32 volunteers with no known metabolic abnormalities. The samples were collected 15 min after intravenous injection of heparin (100 IU/kg body weight). These were allowed to clot for 30 min at room temperature in 20-mL plain glass blood-collection tubes, then centrifuged for 15 min at 1500 × g. Aliquots (1 mL) of each PHP were stored for ≥1 month at −18 °C, before analysis for LPL and HL activity.

Results
Optimization of Assay Conditions
Substrate concentration. The variation of lipase activity with substrate triglyceride concentration observed with the gum arabic and lecithin emulsions is illus-

trated in Figure 1. Both emulsions saturate the available enzyme at triglyceride concentrations >5 g/L. LPL displays a higher maximal activity with the gum arabic emulsion. HL, although achieving the same maximal activity with both emulsions, attains saturation at a lower triglyceride concentration with the lecithin emulsion. On the basis of these results, we adopted a triglyceride concentration of 9.1 g/L for routine assays.

Effect of sonication on gum arabic- and lecithin-based emulsions. The changes in LSI observed on sonicating the gum arabic- and lecithin-based mixtures are illustrated in Figure 2. The gum arabic mixture gave an increase in LSI with successive sonication reaching a maximum after 15 cycles; in contrast, the lecithin emulsion displayed a small decrease in LSI.

Effect of albumin incorporation on HL substrate. The HL activities detected in the 12 samples assayed by using substrate sonicated (x) in the absence of BSA and (y) in its presence were found to be highly correlated (r² = 0.97) around a line y = 1.06x − 1.05. Thus, use of postalbuminated substrate led to a small increase in mean HL activity (mean y = 12.00 ± 7.43, mean x = 12.28 ± 6.90). This increase was not, however, statistically significant (Student's paired t-test, P = 0.258).

The salt-resistant lipase (i.e., non-LPL) activity detected in six samples pretreated with SDS to inactivate HL, when expressed as a percentage of the reported HL activity, decreased from a mean 5.42% (range 4.0–7.3%, median 5.26%) to 3.93% (range 1.2–7.3%, median 3.55%) when the prealbuminized substrate was used. This decrease was statistically significant in a one-sided Student's paired t-test (t = −2.232, P < 0.05).

Comparison of triglyceride hydrolase activities observed with gum arabic- and lecithin-based emulsions. Figure 3 summarizes the LPL and HL activities observed in 10 samples assayed with the gum arabic and lecithin substrates, at a triglyceride concentration of 9.1
g/L. On average, both LPL and HL give a higher lipolytic activity with the gum arabic emulsion. The LPL activities observed with the two substrates were highly correlated (r² = 0.93), as were those observed with HL (r² = 0.85). Use of the lecithin-based substrates yielded a mean of 58% less LPL activity and 34% less HL activity.

**Incorporation of apo C-II activator into LPL substrate.**

Addition of heat-inactivated pooled human serum greatly increased the LPL activity detected with the gum arabic/triolein and lecithin substrates. As illustrated in Figure 4, a further increase in LPL activity was obtained when the gum arabic/triolein emulsion was preincubated with the inactivated serum; LPL activity increased 3.6-fold when the substrate was pre-incubated for 90 min with 80 µL of serum. The lecithin substrate showed no preincubation requirement, the maximal LPL activity being obtained on adding 80 µL of heat-inactivated serum.

**Comparison of SDS and anti-HL antiserum for inactivation of HL.**

SDS and a goat antiserum raised against human HL were evaluated for use in the selective assay of LPL in PHP samples. The LPL activities detected with (x) the antibody procedure and (y) the SDS procedure correlated (r² = 0.84) around a linear-regression line of y = 0.96x + 1.70 (Figure 5, lower axis), indicating that the same enzymatic activity is quantified by each procedure. The increase in mean LPL activity from 6.54 µmol·h⁻¹·mL⁻¹ measured by the antibody procedure...
to 7.98 µmol·h⁻¹·mL⁻¹ with the SDS procedure was statistically significant (Student's t-test, t = -7.80, P = 0.000). The LPL activity measured by the antibody procedure was increased when 0.7 mmol/L SDS was included in the substrate (upper axis, Figure 5): The linear-regression line of y = 1.13x + 0.48 obtained closely resembles that observed with the SDS procedure. The increase in mean LPL activity from (6.54 to 7.85 µmol·h⁻¹·mL⁻¹) was again statistically significant (Student's paired t-test, t = -11.02, P = 0.000). By paired Student's t-test, the data obtained with this modified antibody procedure did not differ from that obtained with the SDS methodology (respective mean LPL activity of 7.85 and 7.98 µmol·h⁻¹·mL⁻¹, t = -0.74, not significant).

Assay Precision

For the gum arabic emulsified substrate, the within-assay CV, calculated from the SD around the mean of five (triplicate) determinations of a single sample within a single assay run, was 6% for the LPL assay and 3% for the HL assay. When only two replicates were used, analytical performance was unaffected. Use of single determinations led to increased imprecision, intra-assay CV's of 8% and 5% being observed with the LPL and HL assays, respectively.

The between-assay CV, calculated on the basis of the SD around the mean LPL and HL activities observed in a single sample assayed in 20 runs of each procedure, was 12% for LPL and 9% for HL (mean activities of 10.8 and 12.4 µmol·h⁻¹·mL⁻¹, respectively).

Reference Ranges for LPL and HL

Limited reference ranges for each procedure were obtained by assay of PHP samples from 32 normal volunteers. As Table 1 shows, the data for the women were skewed toward lower values, mean activities (µmol·h⁻¹·mL⁻¹) of 13.22 and 9.03 and medians of 11.87 and 7.53 being obtained for LPL and HL, respectively. In contrast, the LPL data for men were skewed toward the higher values (mean 13.05, median 13.39). HL data in men's samples showed a slight positive skew (mean 20.47, median 19.64).

Given the distribution of the observed data and the small sample size, we used nonparametric analysis to assess the statistical significance of the observed sex differences in LPL and HL activity. By the Mann-Whitney U test, the data are consistent with a trend in the women toward a higher LPL and lower HL activity (mean women's LPL rank 20.59 vs men's LPL 11.87 µmol·h⁻¹·mL⁻¹, P < 0.01; mean women's HL rank 12.18 vs men's HL 21.40 µmol·h⁻¹·mL⁻¹, P < 0.01).

Discussion

The kinetic studies described show that both LPL and HL require a substrate triglyceride concentration in excess of 5 g/L for maximal catalytic activity with the gum arabic and lecithin substrates. LPL displays a higher maximum catalytic activity with the gum arabic substrate; in contrast, HL displays the same maximal activity with both substrates, although at a lower triglyceride concentration with the lecithin emulsion. For the 10 samples examined, however, higher catalytic activities were obtained for both enzymes when gum arabic-based emulsions were used.

Gum arabic emulsions have previously been shown to give a higher LPL-catalyzed triglyceride hydrolysis than do lecithin emulsions (15), perhaps reflecting a preference for a larger-diameter substrate (16). In the present studies, the gum arabic emulsion yielded a higher LSI value (Figure 2) than did the lecithin emulsion, consistent with the formation of larger vesicles. Furthermore, in the case of HL, higher triglyceride concentrations were required with the gum arabic substrate. This is consistent with the production of an equal number of larger-diameter substrate particles. However, both observations could equally be attributable to nonspecific protein binding by the gum arabic emulsion, leading to decreased triglyceride substrate availability at the oil–water interface (17). Substrate specificity (18, 19) may also contribute to the apparent preference of both enzymes for the gum arabic emulsion. Intralipid is a phospholipid-stabilized soybean oil emulsion, comprising a complex mixture of triglycerides (20), in contrast to the homogeneous triolein content of the gum arabic-stabilized emulsion. Any preferential hydrolysis of non-triolein substrates would result in an apparent decrease in lipase activity (only triolein hydrolysis being detected by reference to the 14C label).

The contribution to the reported HL activity of a salt-resistant (non-LPL) and SDS-resistant (non-HL) activity decreased on use of prealbumininated substrate. On the basis that any increase in specificity is desirable and that the sensitivity of the procedure appears unaffected, we advocate the use of prealbumininated substrates in the HL assay. Nonetheless, use of postalbumininated substrates appears to lead to little if any alteration in HL activity.

In the present studies, optimal conditions for substrate activation were determined for a single substrate triglyceride concentration (9.1 g/L). LPL activity was greatly increased when inactivated serum was added and, when the gum arabic emulsion was used, was further increased by preincubation at 37 °C. In view of the observed variation of particle size with sonication

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<th>Table 1. Lipoprotein Lipase and Hepatic Lipase Activities (µmol·h⁻¹·mL⁻¹) In 32 Normal Subjects</th>
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Mann–Whitney U test:
LPL mean rank 20.59 vs 11.87 P = 0.0087.
HL mean rank 12.18 vs 21.40 P = 0.0055

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(Figure 2), and the known influence of particle diameter on apoprotein uptake (21), we emphasize that investigators should determine the optimal period of incubation and substrate:serum ratio for different sonication procedures and for each serum pool used to supply heat-inactivated apo C-II.

The observed interassay CVs of 12% and 9% for LPL and HL, respectively, are consistent with those reported by previous workers who used gum arabic emulsions (7, 22, 23). In the present studies, using triplicate analysis, we obtained intra-assay CVs of 6% and 3% for the LPL and HL procedures, respectively. Use of duplicate analysis led to no decrease in analytical performance. On this basis, we recommend the use of duplicate analysis in routine assay.

The comparison between SDS and anti-HL antiserum for selective assay of LPL confirms the efficacy of selective inhibition of HL by SDS (7, 10) and the use of SDS for the selective assay of LPL (7). The increase in LPL activity observed with the antiserum procedure on addition of 0.70 mmol/L SDS to the substrate is consistent with a surfactant effect of SDS on the substrate. Although the data obtained by the SDS and antiserum procedures are not directly interchangeable, they are highly correlated. Thus, SDS pretreatment offers a convenient, consistent, off-the-shelf alternative to the use of an anti-HL antiserum in the assay of LPL.

We have applied the described assays in the diagnosis of LPL deficiency and in investigation of the mechanisms underlying hyperlipidemias associated with anti-hypertensive therapy (24), gestational hypertriglyceridemia (25), acute hyperinsulinemia (26), obesity (27), and insulin insensitivity (28).

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References

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