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methods

A novel method for measuring human hepatic lipase activity in postheparin plasma

S. Imamura,* J. Kobayashi,[1,†] S. Sakasegawa,* A. Nohara,† K. Nakajima,§ M. Kawashiri, A. Inazu,†† M. Yamagishi,** J. Koizumi,§§ and H. Mabuchi†**

Diagnostics Research & Development Department,* Diagnostics Division, Asahi Kasei Pharma Corporation, Izunokuni City, Japan; Department of Lipidology,† Kanazawa University Graduate School of Medical Science;§ Nakajima & Associates, Co., Ltd.; Division of Cardiology,** Kanazawa University Graduate School of Medical Science; Kanazawa University,†† Faculty of Medicine, School of Health Science, Laboratory Sciences; and Department of General Medicine,§§ Kanazawa University Hospital, Kanazawa City, Japan

Abstract The objective of this study was to establish a hepatic lipase (HL) assay method that can be applied to automatic clinical analyzers. Seventy-four hyperlipidemic subjects (men/women 45/29) were recruited. Lipase activity was assayed measuring the increase in absorbance at 546 nm due to quinonediimine dye production. Reaction mixture R-1 contained 50 mM Tris-HCl (pH 9.5), 0.5 mM glycerol-1,2-dioleate, 0.4% (unless otherwise noted) polyoxyethylene-nonylphenylether, 3 mM ATP, 3 mM $MgCl_2$, 1.5 mM $CaCl_2$, monoacylglycerol-specific lipase, glycerol kinase, glycerol-3-phosphate oxidase, 0.075% *N,N*-bis-(4-sulfobutyl)-3-methylaniline-2 Na, peroxidase, ascorbic acid oxidase. Reaction mixture R-2 contained 50 mM Tris-HCl (pH9.5), 0.15% 4-aminoantypirine. Automated assay for activity was performed with a Model 7080 Hitachi analyzer. In the lipase assay, 160 μl of R-1 was incubated at 37°C with 3 μl of samples for 5 min, and 80 μl of R-2 was added. Within-run coefficient of variations was 0.9–1.0%. Calibration curve of lipase activity was linear ($r = 0.999$) between 0 and 320 U/l. Analytical recoveries of purified HL added to plasma were 96.6–99.8%. HL activity in postheparin plasma measured in this method had a closer correlation with HL mass by a sandwich ELISA ($r = 0.888$, $P < 0.0001$) than those in the conventional method using [^{14}C-]triolein ($r = 0.730$, $P < 0.0001$). This assay method for HL activity can be applied to an automatic clinical analyzer.—Imamura, S., J. Kobayashi, S. Sakasegawa, A. Nohara, K. Nakajima, M. Kawashiri, A. Inazu, M. Yamagishi, J. Koizumi, and H. Mabuchi. **A novel method for measuring human hepatic lipase activity in postheparin plasma. *J. Lipid Res.* 2007.** 48: **453–457.**

Supplementary key words dioleoylglycerol • quinonediimine dye • automatic clinical analyzer

Hepatic lipase (HL), a lipolytic enzyme that is a secreted glycoprotein, is synthesized by hepatocytes and bound to heparin sulfate proteoglycans at the surface of liver sinusoidal capillaries. HL plays a major role in lipoprotein metabolism as a lipolytic enzyme that hydrolyzes triglycerides (TGs) and phospholipids in chylomicron remnants, intermediate-density lipoproteins (IDLs), and high-density lipoproteins (HDLs). Patients with HL deficiency present with hypercholesterolemia or hypertriglyceridemia and accumulate β-very-low-density lipoproteins (VLDLs), chylomicron remnants, IDLs, TG-rich low-density lipoproteins (LDLs), and HDLs (1–7).

To date, the only available methods for measuring HL activity in postheparin plasma (PHP) have used ^{3}H- or ^{14}C-labeled trioleoyl glycerol as substrates in the presence of 1 M NaCl, because lipoprotein lipase (LPL) activity is known to be completely inhibited and remaining activities are considered to correspond to HL activity under these conditions. This assay procedure is complicated and does not appear to be suitable for routine work (8, 9). Several years ago, a method for measuring HL protein mass by ELISA in PHP was developed (10).

Still, to diagnose HL deficiency, demonstrating the lack of HL activity is considered to be essential. In the present study, therefore, we have developed and established, using dioleoylglycerol as a substrate, a novel and simple assay system for measuring HL activity in PHP that can be applied to an automatic clinical analyzer.

MATERIALS AND METHODS

Subjects

Seventy-four hyperlipidemic subjects (men/women 45/29) were recruited for this study (**Table 1**). The statement of institutional approval of the study was in accordance with the Declara-

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Abbreviations: BMI, body mass index; EL, endothelial lipase; IDL, intermediate-density lipoprotein; LPL, lipoprotein lipase; PHP, postheparin plasma; TG, triglyceride.

[1] To whom correspondence should be addressed.
e-mail: junji@med.kanazawa-u.ac.jp

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TABLE 1. Clinical profile of the study subjects

	Total (n = 74)	Men (n = 45)	Women (n = 29)
Age, years	53 ± 16	52 ± 16	54 ± 16
BMI, kg/m^2	23 ± 3	23 ± 2	22 ± 4
Total cholesterol, mg/dl	259 ± 92	246 ± 90	278 ± 95
TGs, mg/dl	137 ± 87	146 ± 66	105 ± 40
HDL-C, mg/dl	51 ± 17	47 ± 19	60 ± 12
Fasting plasma glucose, mg/dl	96 ± 8.3 (n = 64)	96 ± 7.4 (n = 40)	102 ± 34 (n = 24)
HbA1c, %	5.1 ± 0.5 (n = 53)	5.1 ± 0.5 (n = 32)	5.4 ± 1.1 (n = 21)

BMI, body mass index; HDL, high-density lipoprotein, TG, triglyceride.

tion of Helsinki, and informed consent was obtained from all participants. Exclusion criteria included: age >75 years, body mass index (BMI) >30 kg/m^2, diabetes mellitus, abnormal liver or muscle enzymes, creatinemia, use of antioxidants and lipid regulators, habitual alcohol intake >3 standard drinks/day, or endocrinological disorder. Blood samples were obtained following a fast of ≥12 h. PHP was obtained 10 min after 30 U/kg heparin injection. The monoclonal anti-LPL antibody 5D2 (MAb 5D2) was kindly provided by Dr. John Brunzell of the University of Washington, Seattle.

Method for measuring lipase activity in partially purified HL and LPL fractions

HL and LPL were prepared from PHP using heparin-Sepharose column chromatography (11). HL and LPL were eluted with 0.8 M NaCl and 1.6 M NaCl, respectively. Lipase activity was assayed measuring the increase in absorbance at 546 nm (subwave length; 660 nm) due to the production of quinonediimine dye based on the previously described assay procedure (12, 13). Reaction mixture R-1 contained 50 mM Tris-HCl (pH 9.5), 0.5 mM dioleoylglycerol, 0.4% polyoxyethylene-nonylphenylether, 3 mM ATP, 3 mM $MgCl_2$, 1.5 mM $CaCl_2$, monoacylglycerol-specific lipase (0.75 U/ml), glycerol kinase (0.75 U/ml), glycerol-3-phosphate oxidase (37.5 U/ml), 0.075% *N,N*-bis-(4-sulfobutyl)-3-methylaniline-2 Na, peroxidase (10 U/ml), and ascorbic acid oxidase (10 U/ml). Reaction mixture R-2 contained 50 mM Tris-HCl (pH 9.5), and 0.15% 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one. Automated assay of lipase activity was performed with a Model 7080 Hitachi automatic clinical analyzer. In the lipase assay, 160 μl of R-1 was incubated at 37°C with 3 μl of samples for 5 min, and then 80 μl of R-2 was added. This reaction produced a violet quinonediimine dye with a peak absorbance at 546 nm. HL activity generating 1 μmol of monoglyceride from diglyceride per minute was defined as 1 unit.

The method for measuring HL activity in PHP

We measured HL activity in PHP using the above-mentioned method either in the presence (HL activity) or absence (total lipase activity) of 1 M NaCl.

Separation of HL and LPL by heparin-Sepharose CL-6B

PHP (5 ml) was dialyzed against 1 l of 1.6 M NaCl containing 10 mM PIPES-NaOH (pH 7.5) at 4°C for 18 h. The dialysate was dialyzed against 500 ml of 0.3 M NaCl containing 10 mM PIPES-NaOH (pH 7.5) at 4°C for 18 h.

The dialysate was applied to a heparin-Sepharose CL-6B column (1.6 × 3 cm). The column was washed with 30 ml of 0.3 M NaCl containing 0.1 mM EDTA, 10% sucrose, 0.025% BSA, and 10 mM PIPES-NaOH (pH 7.5).

HL and LPL were eluted with 20 ml of 0.8 M NaCl containing 0.1 mM EDTA, 10% sucrose, 0.025% BSA, 10 mM PIPES-NaOH (pH 7.5), 20 ml of 1.6 M NaCl containing 0.1 mM EDTA, 10% sucrose, 0.025% BSA, and 10 mM PIPES-NaOH (pH 7.5) respectively. These HL and LPL fractions were concentrated with an Amicon Ultra membrane (molecular cut; 30 kDa), reconstituted with 2 ml of 10 mM PIPES-NaOH (pH 7.5) containing 10% sucrose and 0.1 mM EDTA. The concentrated HL and LPL was stored at −20°C.

Effects of MAb 5D2 on lipase activity in PHP- or NaCl-eluted fraction from heparin-Sepharose

To evaluate the specificities of the lipase activity determined by the present method, we investigated the effects of the addition of MAb 5D2 in the reaction mixture against the enzyme activity in either a PHP- or an 0.8 M NaCl-eluted fraction from heparin-Sepharose.

The conventional method for measuring HL activity in PHP

In the conventional method for measuring HL activity in PHP, total lipase activity was measured using Triton X-100-emulsified [^{14}C]triolein, based on the previously reported method (8, 9). The remaining activity in the presence of 1 M NaCl was defined as HL activity.

The method for measuring HL protein mass in PHP

HL protein mass in PHP was detected using a sandwich ELISA following the previously described method (10).

Statistical analysis

Statistical evaluation was performed using StatView-J 5.0 software (SAS Institute, Cary NC, on a Macintosh Computer). Pearson's correlation coefficients analysis was carried out. Results were expressed as mean ± SD, and the significance levels were set at $P < 0.05$.

RESULTS

Effect on lipase activity of addition of polyoxyethylene-nonylphenylether in the reaction mixture

Lipase activity detected in our method increased in a dose-dependent manner with increasing concentrations of polyoxyethylene-nonylphenylether, a nonionic detergent, in the reaction mixture (**Fig. 1**). Lipase activity detected the fractions eluted in 0.3 M, 0.8 M, and 1.6 M NaCl using reaction mixtures containing 0.5 mM dioleoylglycerol (**Table 2**). Lipase activity was detected only in the fractions of heparin-Sepharose chromatography eluted in 0.8 M NaCl, suggesting that this activity corresponds to HL. In

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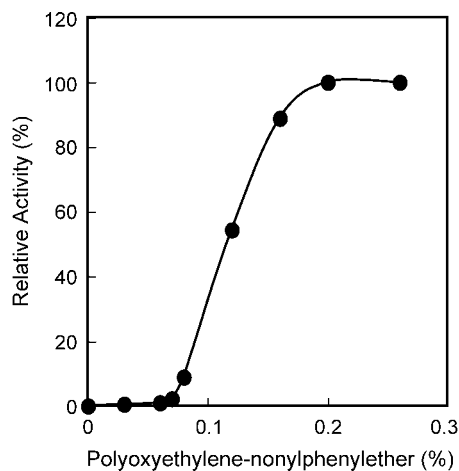

Fig. 1. Effect of increasing concentrations of polyoxyethylene-nonylphenylether on lipase activity in the reaction mixture for HL activity assay. Methods were described in detail in the Materials and Methods section, except that the effect of various concentrations of polyoxyethylene-nonylphenylether on HL activity in the reaction mixture was investigated in this particular experiment. The horizontal line shows the final concentration of polyoxyethylene-nonylphenylether in the reaction mixture.

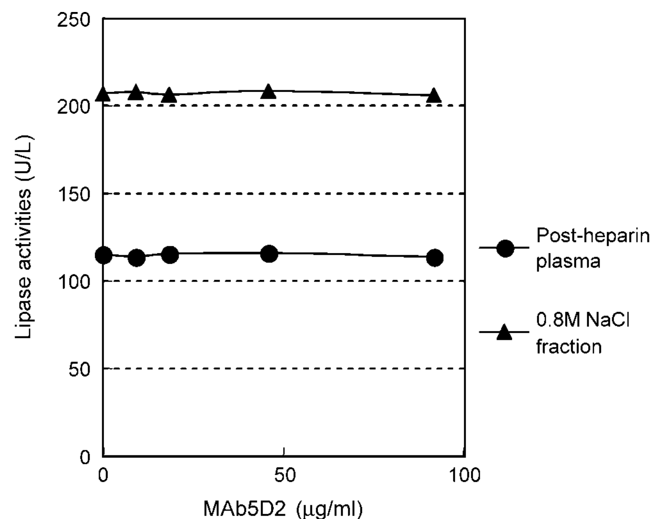

Fig. 2. Effect of anti-lipoprotein lipase monoclonal antibody 5D2 on lipase activity in postheparin plasma (PHP)- or 0.8 NaCl-eluted fraction from heparin-Sepharose. The reaction condition for the measurement of lipase activity was described in Materials and Methods, with the exception that increasing amounts of 5D2 were added in the reaction mixture. PHP was obtained from a normal volunteer 10 min after 30 U/kg heparin injection.

contrast, the LPL fraction (1.6 M NaCl) of heparin-Sepharose chromatography showed no lipase activity, even when apolipoprotein C-II (apoC-II) was added as LPL-specific activator.

Effect of MAb 5D2 on lipase activity in the PHP- or NaCl-eluted fraction

To determine whether the activity measured in the present method is affected by anti-LPL MAb, we studied the additive effect of the MAb 5D2 on lipase activity in the PHP- or 0.8 M NaCl-eluted fraction from heparin-Sepharose (**Fig. 2**). MAb 5D2 did not affect lipase activity at all in either in the PHP- or the 0.8 M NaCl-eluted fraction, whereas lipase activity in PHP was inhibited by 40% in the presence of the MAb 5D2 in an assay system for LPL activity (data not shown).

Correlation of HL activity with HL mass in PHP

HL activity in PHP measured in the present method was highly correlated with HL mass by a sandwich ELISA in 74 hyperlipidemic Japanese subjects (**Fig. 3**). By comparison, HL activity measured in the conventional method using Triton X-100-emulsified [^{14}C]triolein as a substrate [conventional HL (cHL) activity] had a weaker correlation with HL mass.

TABLE 2. Lipase activity in the fractions eluted in 0.3 M, 0.8 M, and 1.6 M NaCl using dioleoylglycerol as substrate

Fractions	Activity (U/l)	Total Activity (mU)
Plasma	252	1,260
0.3 M NaCl	0	0
0.8 M NaCl eluate	38	760
0.8 M NaCl concentrate	320	640
1.6 M NaCl concentrate	0	0

We also analyzed the correlation of HL activity with HL mass in PHP in men (n = 45). HL activity in PHP measured in the present method was highly correlated with HL mass by a sandwich ELISA ($r = 0.886$, $P < 0.001$). By comparison, cHL activity had a weaker correlation with HL mass ($r = 0.676$, $P < 0.001$).

Furthermore, we analyzed correlation of HL activity with HL mass in PHP in women (n = 29). HL activity in PHP measured in the present method was highly correlated with HL mass by a sandwich ELISA ($r = 0.901$, $P < 0.001$). By comparison, cHL activity had a slightly weaker correlation with HL mass ($r = 0.852$, $P < 0.001$).

HL activity in PHP measured in the presence or absence of NaCl

The remaining activity in the presence of 1 M NaCl using Triton X-100-emulsified [^{14}C]triolein as a substrate is usually interpreted as HL activity. Unexpectedly, the correlation coefficient of HL activity in the absence of 1 M NaCl with HL mass was almost equal to that in the presence of 1 M NaCl with HL mass (0.892 vs. 0.901 in women; 0.895 vs. 0.886 in men) (**Table 3**).

Correlation of HL activity with age and BMI

HL activity did not show a significant correlation with age or BMI in the study subjects (Table 3). Even measured separately for each gender, no correlation existed (data not shown).

Correlation of HL activity with lipids and lipoproteins

HL activity in the present method did not have significant associations with total cholesterol or TG, and had

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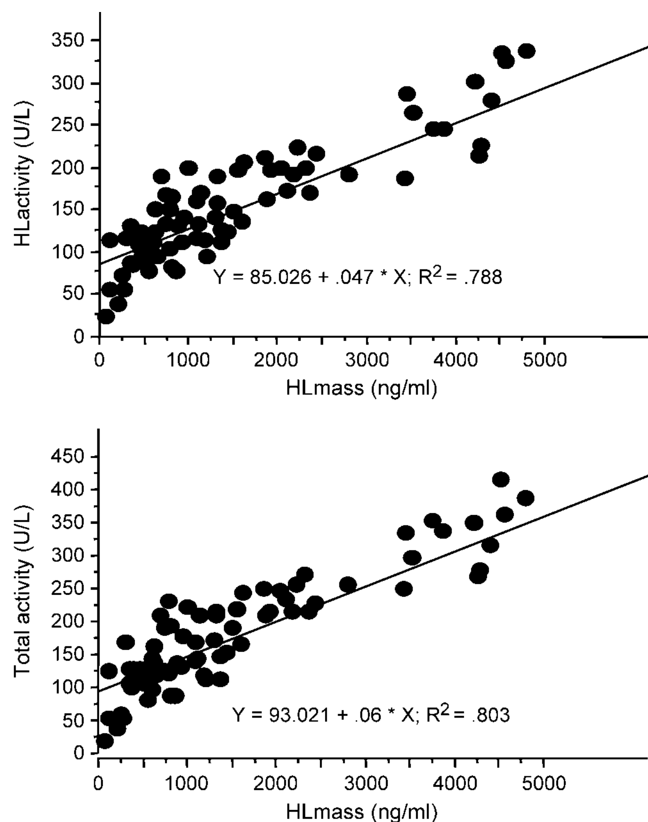

Fig. 3. Relationship of HL mass to HL activity and total lipase activity from PHP in 74 hyperlipidemic Japanese subjects (men/women 45/29). Lipase activity in PHP was measured using the method described in Materials and Methods in either the presence (HL activity) or absence (total lipase activity) of 1 M NaCl. HL activity generating 1 μmol of monoglyceride from diglyceride per minute was defined as 1 unit. HL mass is shown in ng/ml.

an inverse association with HDL-C and HDL_2-C (Table 3). Measured separately for each gender, the observed associations between HL activity and HDL-C did not reach statistical significance, partly because of the small sample size (data not shown).

Other findings

The within-run (n = 20) coefficient of variation was 0.9–1.0%. The calibration curve of lipase activity was linear ($r = 0.999$) between 0 and 500 U/l. Analytical recoveries of purified HL added to plasma were 96.6–99.8%. This method was free of interference by bilirubin C, bilirubin F, ascorbic acid, and intra-lipid. Weak interference by hemoglobin was observed. High activity of human pancreatic lipase (1,000 U/l) showed no lipase activity.

DISCUSSION

In the present study, we have developed a novel method for measuring HL activity in PHP. Instead of radioisotope-labeled substrate, we have used 0.2 mM dioleoylglycerol as a substrate for this new lipase assay.

TABLE 3. Correlation of HL activity and mass with several metabolic parameters in men and women combined (N = 74)

	T-Lipase Activity	HL Activity	HL Mass	cHL Activity
BMI	−0.035	−0.059	0.024	−0.057
Total cholesterol	−0.075	−0.042	−0.047	0.240[a]
TGs	0.144	0.167	0.290[a]	0.119
HDL-C	−0.330[b]	−0.310[b]	−0.356[b]	−0.183
HDL_2-C	−0.317[b]	−0.300[a]	−0.348[b]	−0.231
HDL_3-C	−0.070	−0.051	−0.031	0.159
T-lipase activity	na	0.978[c]	0.896[c]	0.743[c]
HL activity	0.978[c]	na	0.888[c]	0.747[c]
HL mass	0.896[c]	0.888[c]	na	0.730[c]
cHL activity	0743[c]	0.747[c]	0.730[c]	na

T-lipase, total lipase; HL, hepatic lipase; na, not available; cHL activity, HL activity measured in conventional method using Triton X-100-emulsified [^{14}C]triolein.

Lipase activities measured in the absence and the presence of 1 M NaCl were referred to as T-lipase activity and HL activity, respectively.

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

The finding shown in Fig. 1 suggests that the existence of polyoxyethylene-nonylphenylether, a nonionic detergent, in the reaction mixture appeared to be a critical factor in the expression of HL enzyme activity, although at this time, the precise mechanisms have not been elucidated. Glycerol-1,2-dioleate became clear by adding nonionic detergent, suggesting that glycerol-1,2-dioleate came to exist in the reaction mixture as water-soluble mixed micelles with this nonionic detergent.

The result shown in Table 2 that lipase activity was detected only in the 0.8 M NaCl-eluted fraction of the heparin-Sepharose indicated that lipase activity detected in the present method corresponds to that of HL. Also, the finding that the MAb 5D2 did not affect lipase activity in the 0.8 M NaCl-eluted fraction and PHP confirmed that this method specifically detected HL activity (Fig. 2). HL activity obtained using this method showed stronger associations with HL mass by sandwich ELISA than did cHL activity measured using Triton X-100-emulsified [^{14}C]triolein (8, 9). This suggests that the present method could be more reliable for measuring HL activity in PHP than the conventional method using Triton X-100-emulsified [^{14}C]triolein as a substrate.

A previous report has shown extremely high correlation between HL mass and activity in PHP (10). However, their method for measuring HL activity requires ^{3}H-labeled triolein and is labor intensive and time consuming. HL is a lipolytic enzyme catalyzing the hydrolysis of TG and PL in IDL and HDL_2. Whether HL is atherogenic or anti-atherogenic is still the subject of debate (14–17). Because HL lowers plasma concentrations of the pro-atherogenic apoB-containing lipoproteins as well as the anti-atherogenic HDL, the net effect of these HL-induced alterations in plasma lipoproteins on coronary artery disease is not easily predictable. Patients with HL deficiency present with hypercholesterolemia or hypertriglyceridemia and accumulate β-VLDLs, chylomicron remnants, IDLs, TG-rich LDLs, and HDLs (1–7). The clinical profile of this lipid disorder could be quite similar to that of type III hyperlipidemia,

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which makes it difficult to identify this lipid disorder. To identify patients with HL deficiency, it is essential to demonstrate the lack of HL activity in PHP. In contrast, LPL deficiency is relatively easily identified, because patients with this lipid disorder usually have drastic hyperlipidemia due to marked accumulation of chylomicrons in the serum, causing acute pancreatitis (7). To date, as mentioned above, the available method for measuring HL activity in PHP has used ^{3}H- or ^{14}C-labeled trioleoyl glycerol as substrates in the presence of 1 M NaCl (8, 9). However, these assay procedures are complicated and require the use of radioisotopes. In the present study, therefore, we have developed and established a novel assay system for measuring HL activity in PHP using dioleoylglycerol as a substrate, without requiring radioisotope labeling. The results presented in Table 3, showing that total lipase activity (measured in the absence of 1 M NaCl) was similar to HL activity (measured in the presence of 1 M NaCl) in terms of correlation coefficients with HL mass, suggested that this assay could be suitable for measuring HL activity in the presence or absence 1 M NaCl.

With regard to the correlation of HL activity with lipid and lipoproteins, there were inverse correlations with HDL-C (HDL_2-C) in the whole subjects, but there was no correlation with BMI, total cholesterol, TGs, or HDL_3-C. These findings might be compatible with the fact that HL is involved in catalyzing hydrolysis of TG in HDL_2-C (7, 18).

In addition to LPL and HL, in the past decade, considerable attention has been paid to the physiological role of endothelial lipase (EL) (19), which is known to have higher phospholipase activity and lower activity in TG hydrolysis, compared with the other two lipases (20). To our knowledge, however, there has been no report on what proportion of lipase activity in PHP is accounted for by EL. Despite this, we presume that there is little or no possibility that our present method for measuring HL activity overlapped the activity of EL, in view of their high positive correlation to HL mass.

In summary, we have developed a novel and simple method for the assay of HL activity in PHP, which is suitable for application to an automatic clinical analyzer.

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