[Chem. Pharm. Bull. 31(8)2732—2736(1983)]

Spectrophotometric Assay for Lipase in Serum Using a Chromogenic Substrate, Orange I Laurate

SHINICHI KAMACHI,[a] KIYOSHIGE WAKABAYASHI,[a] MASATOSHI YAMAGUCHI[b] and YOSUKE OHKURA*,[b]

New Drug Research Laboratories, Chugai Pharmaceutical Co., Ltd.,[a] Takada, Toshima-ku, Tokyo 171, Japan and Faculty of Pharmaceutical Sciences, Kyushu University 62,[b] Maidashi, Higashi-ku, Fukuoka 812, Japan

(Received December 24, 1982)

A sensitive spectrophotometric method for the assay of lipase in serum is described, based on the use of a chromogenic substrate, Orange I laurate. Orange I liberated from the substrate (used as an emulsion) under the optimal conditions for the enzyme reaction in the presence of sodium cholate (emulsifier of the substrate, activator of lipase, inhibitor of esterases) and sodium dodecyl sulfate (activator of lipase, inhibitor of esterases) is determined spectrophotometrically after adding concentrated sodium cholate to stop the enzyme reaction and to dissolve unreacted substrate, and diluting the mixture with water to reduce the absorbance due to the substrate. Serum lipase activities obtained by this method correlated well with those obtained by a method using olive oil as a substrate. The method is readily performed with good precision, and is suitable for the assay of many samples.

Keywords——lipase; human serum; Orange I laurate; chromogenic substrate; spectrophotometric assay; sodium cholate, emulsion; activator; inhibitor

Lipase (glycerol-ester hydrolase, EC 3.1.1.3) catalyzes the hydrolysis of triglyceride. An increase in the activity of lipase in serum has been observed in patients with pancreatic diseases. This increase is more specific to pancreatic diseases than an increase in the activity of amylase in serum, and so the assay of lipase activity in serum is clinically important.[1-4]

Many methods have been reported for the assay of lipase in serum. Titrimetric and spectrophotometric methods use olive oil as a natural substrate,[2-8] or laurates of phenols (β-naphthyl laurate,[9] phenyl laurate,[10] *p*-nitrophenyl laurate[11]) and tributyroate of dimercaptopropan-1-ol[12] as synthetic substrates. The methods with olive oil (used in titrimetric methods measuring fatty acid formed[2-6] and in spectrophotometric methods based on the formation of copper complex of fatty acid[7,8]) usually require very long incubation times and are insensitive. The methods with the synthetic substrates (used in the spectrophotometric methods[9-12]) are rather simple to carry out and in general are fairly sensitive, but those with β-naphthyl laurate, phenyl laurate and dimercaptopropan-1-ol tributyroate require procedures for color development of the enzyme reaction products (β-naphthol, phenol and dimercaptopropan-1-ol, respectively). The fluorimetric method, which uses fluorescein dilaurate as a fluorogenic substrate, is more sensitive.[13] These substrates are used as emulsions.

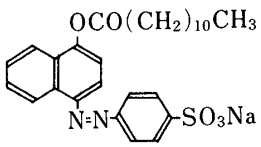

Chart 1

In the previous paper, we showed that laurate of sodium *p*-(4-hydroxy-1-naphthylazo)-benzenesulfonate (Orange I) (Chart 1) is a sensitive and fairly selective substrate of lipase in serum.[14] Recently, we have found that sodium cholate, at high concentrations, inhibits lipase and dissolves Orange I laurate, though it activates the enzyme at low concentrations.[15,16] A sensitive and simple method for the assay of lipase in the serum has thus been developed, based on spectrophotometric determination of Orange I formed from Orange I laurate under the optimum reaction conditions of the enzyme.

Experimental

Materials——All chemicals were of reagent grade, unless otherwise noted. Orange I was purchased from Wako Pure Chemical Industries (Japan). Orange I laurate was prepared as described previously.[14] Lipase (from porcine pancreas, 20000—50000 units/mg protein,[17] lyophilized powder containing approximately 75% protein) was obtained from Sigma Chemical Co. (U.S.A.). Human sera were supplied by Kyushu University Hospital.

Apparatus——Visible absorption spectra and absorbances were taken with a Hitachi 124 spectrophotometer in 10-mm quartz cells. pH was measured with a Hitachi-Horiba M-7 pH meter at 25 °C. Sonication was carried out using a Kaijo Denki T-A-4280 sonifier cell disruptor.

Standard Procedure——Substrate emulsion was prepared by adding Orange I laurate to 0.1 M phosphate buffer (pH 8.0) containing 4 mM sodium dodecyl sulfate (SDS) and 0.5% sodium cholate so as to give 2 mM and sonicating the mixture for approximately 5 min. The emulsion could be used for about 2 d when stored at 4 °C.

A mixture of 1.0 ml of the emulsion and 25 μl of serum was incubated at 37 °C for 30 min, then 1.0 ml of 20% sodium cholate solution was added. After the addition of 2.5 ml of water, the absorbance of the resulting mixture was measured at 475 nm against a blank. For the blank, the order of addition of serum and 20% cholate solution was reversed and the same procedure was carried out, incubation being omitted.

To obtain a standard curve, the same procedure as for the blank was carried out except that 2.5 ml of water was replaced with 2.5 ml of aqueous Orange I solution (30—120 nmol). Lipase activity was expressed as μmol of Orange I formed per liter of serum at 37 °C.

Results and Discussion

The enzyme reaction product, Orange I, had an absorption maximum at 475 nm (molar absorptivity, 20380) in the final mixture obtained in the procedure, though the maximum for

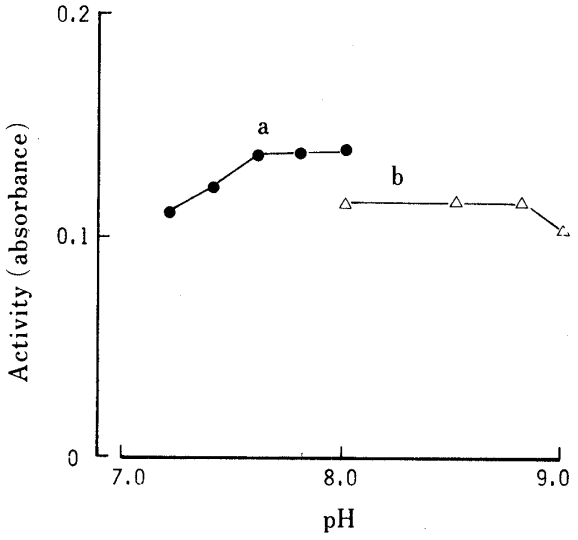

Fig. 1. Effect of pH on Lipase Activity

Portions (25 μl) of serum with lipase activity of 40.7 μmol·min^{-1}·l^{-1} were treated according to the standard procedure with various pH values of the substrate emulsion. Mean values of triplicate determinations were plotted.

a, 0.1 M phosphate buffer; b, 0.05 M borate buffer.

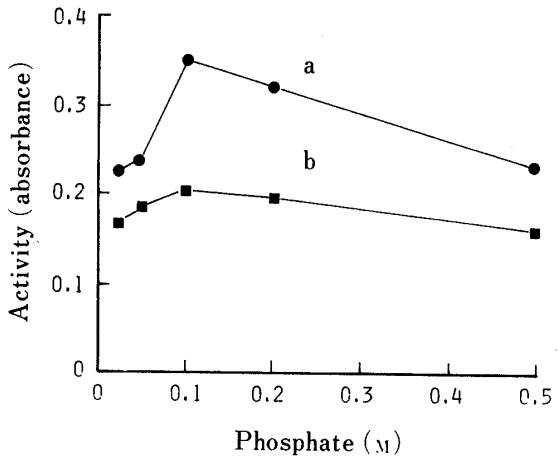

Fig. 2. Effect of Phosphate Concentration on Lipase Activity

Portions (25 μl) of serum or porcine pancreas lipase solution were treated according to the standard procedure with various concentrations of phosphate buffer. Mean values of triplicate determinations were plotted.

a, serum (101.8 μmol·min^{-1}·l^{-1}); b, porcine pancreas lipase (50 μg/ml).

Orange I laurate is at 383 nm (molar absorptivity, 15660) when measured against water (molar absorptivity at 475 nm, 720). The standard curve was linear up to 120 nmol of Orange I and passed through the origin. The absorbance for 120 nmol of Orange I (corresponding to a lipase activity of 160 μmol·min^{-1}·l^{-1} serum) was 0.55.

Lipase in serum was most active at pH 7.6—8.8 in 0.1 M phosphate buffer or 0.05 M borate buffer (Fig. 1). The serum enzyme activities attained in barbital buffer and Tris-hydrochloric acid buffer (both 0.05 M, pH 8.0) were identical with that in the borate buffer (pH 8.0). When these four buffers were examined with porcine pancreas lipase solution (50 μg/ml), the same pH dependency of lipase activity as described above was obtained. Phosphate buffer (pH 8.0) was thus employed for the procedure. Maximum activity was attained at a phosphate concentration of 0.1 M (Fig. 2).

Orange I laurate in the substrate emulsion gave a maximum and constant activity at concentrations of 1 to at least 5 mM with serum lipase and 2 to at least 5 mM with porcine pancreas lipase (Fig. 3), with an observed K_m of 0.3 mM in each case; 2 mM was used in the standard procedure.

It was previously reported that Orange I laurate is hydrolyzed by esterase preparations (carboxyl esterase from porcine liver and aryl esterase from human serum). SDS completely inhibited these esterase activities at a concentration of 4 mM, while it most intensely activated lipase from porcine pancreas at concentrations of 2—4 mM, but in fact the substrate was not affected by carboxyl esterase in serum from a patient with pancreatitis and was only very slightly hydrolyzed by aryl esterase in the serum.[14] It was observed in this study that lipase in serum was activated by SDS in the same way; 4 mM SDS in the substrate emulsion was used in the procedure.

Sodium cholate, an inhibitor of esterases, activates lipase in serum.[15,16] Its effect on the lipase-mediated reaction was determined in the presence of SDS. Maximum activity of lipase was achieved at sodium cholate concentrations of around 0.5% in the substrate emulsion (Fig.

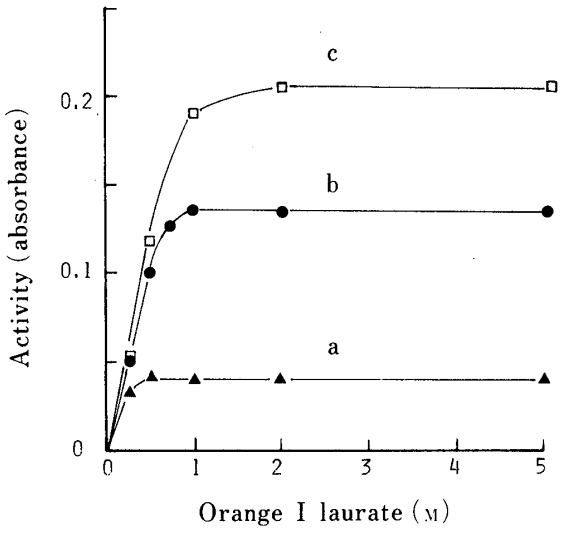

Fig. 3. Effect of Concentration of Orange I Laurate on Lipase Activity

Portions (25 μl) of serum or porcine pancreas lipase solution were treated according to the standard procedure with various concentrations of Orange I laurate in the substrate emulsion. Mean values of triplicate determinations were plotted.

a and b, sera (11.6 and 39.3 μmol·min^{-1}·l^{-1}, respectively); c, porcine pancreas lipase (50 μg/ml).

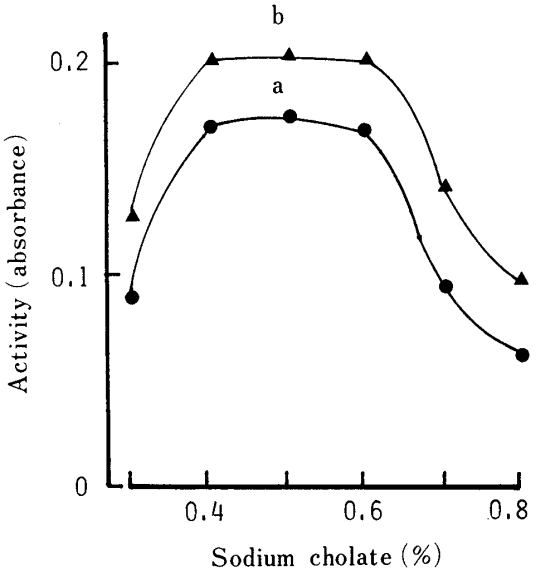

Fig. 4. Dependence of Lipase Activity on the Concentration of Sodium Cholate

Portions (25 μl) of serum or porcine pancreas lipase solution were treated according to the standard procedure with various concentrations of sodium cholate in the substrate emulsion. Mean values of triplicate determinations were plotted.

a, serum (50.9 μmol·min^{-1}·l^{-1}); b, porcine pancreas lipase (50 μg/ml).

4). Sodium cholate also served as an emulsifier of Orange I laurate. At concentrations less than approximately 0.3%, the resulting substrate emulsion was unstable. Thus, 0.5% sodium cholate was used in the substrate emulsion.

Orange I laurate is also hydrolyzed by a lipoprotein lipase preparation (from microorganisms).[14] This enzyme is only present in a very small amount in normal serum and markedly decreases in patients with lipemia.[18] Protamine sulfate, an inhibitor of lipoprotein lipase,[19] inhibits lipase in normal serum by 7% at a concentration of 0.2 mg/ml in the incubation mixture.[14] Therefore, inhibitor was not used in the present assay procedure.

The enzyme activity was linear with time up to at least 60 min and up to an absorbance of at least 0.63 caused by Orange I formed by incubation at 37 °C (Fig. 5); an incubation time of 30 min was selected in the standard procedure to obtain a sufficient absorbance to measure precisely even when a sample with low lipase activity such as normal serum was subjected to the assay. Normal serum usually gave an absorbance of approximately 0.02 on 30-min incubation. The amount of Orange I formed was proportional to serum sample size up to 50 μl or more and to the amount of porcine pancreatic lipase (in 25 μl) up to 5 μg or more.

Sodium cholate stops the enzyme reaction at concentrations of 3% or more and dissolves Orange I laurate to give 14 and 75 mM solutions at concentrations of 4.5 and 20%, respectively, at room temperature. Therefore, 1.0 ml of 20% sodium cholate solution was added to the incubated mixture (1.025 ml) and the resulting mixture was diluted with 2.5 ml of water so as to give a cholate concentration of 4.44% in the final assay mixture. When the dilution with water was omitted, Orange I laurate in the mixture showed a large absorbance at 475 nm (approximately 0.7) which interfered with exact measurement of the absorbance due to Orange I by using a conventional spectrophotometer. When 3.5 ml of 5.7% sodium cholate solution (a mixture of 1 part of 20% sodium cholate solution and 2.5 parts of water) was added directly to the incubated mixture, a long standing-time (*ca.* 5 h) with occasional shaking of the resulting mixture was required to obtain a clear solution. The absorbance of the final

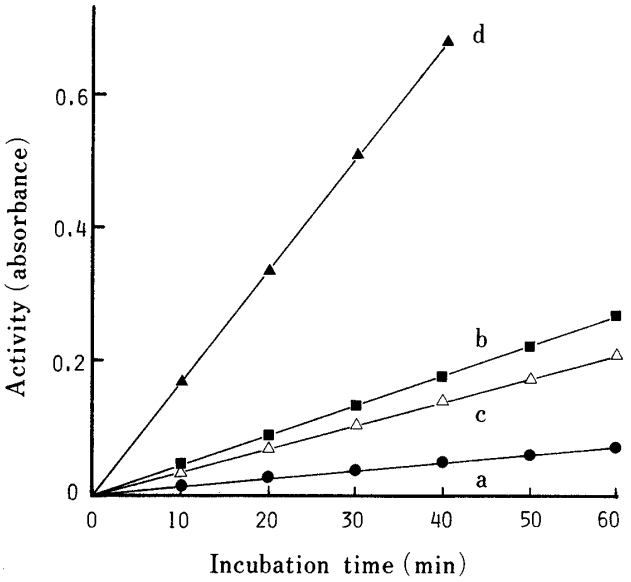

Fig. 5. Effect of Incubation Time on Lipase Activity

Portions (25 μl) of serum or porcine pancreas lipase solution were treated according to the standard procedure with various incubation times. Mean values of triplicate determinations were plotted.

a and b, sera (12.1 and 38.7 μmol·min^{-1}·l^{-1}, respectively); c and d, porcine pancreas lipase (25 and 125 μg/ml, respectively).

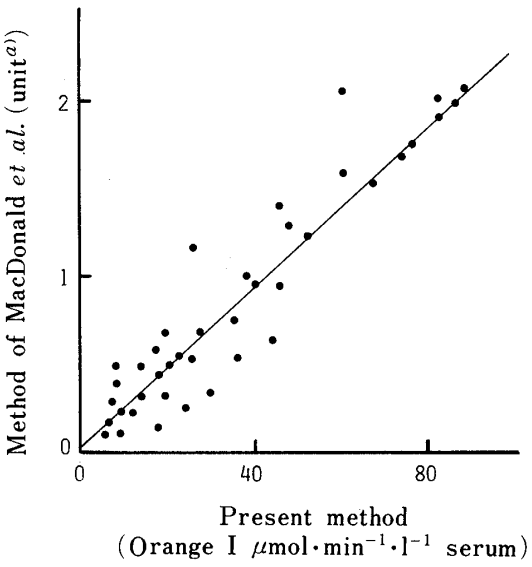

Fig. 6. Correlation between Serum Lipase Activities Obtained by the Present Method and the Method of MacDonald *et al.*

a) expressed as ml of 50 mM sodium hydroxide required to neutralize fatty acids liberated by hydrolysis under the conditions described.[4]

assay mixture did not change at room temperature (25 °C) for about 3 h.

Orange I laurate in the substrate emulsion was not hydrolyzed by incubation at 37 °C for about 1.5 h, and incubation was omitted in the preparation of the blank.

Comparison with the method of MacDonald *et al.*,[4] one of the standard methods (requiring 1.0 ml of sample and a 3-h incubation) which is based on the titration of fatty acids liberated from olive oil, was made for normal and pathological sera (Fig. 6). The correlation coefficient was 0.93 ($n=40$) and the regression equation for the present method (x) against the method of MacDonald *et al.* (y) was $y=0.023x+0.019$.

The within-day precision of the present method was examined. The coefficient of variation was 2.9% ($n=40$) for serum with a mean lipase activity of 62.5 μmol·min^{-1}·l^{-1} serum. The entire procedure takes less than 40 min and many samples can be assayed successively. This method is sensitive, precise, rapid and simple, and should be useful for routine use.

Acknowledgement We are grateful to the Central Clinical Laboratory of Kyushu University Hospital for the supply of sera.

References and Notes

1) P. D. Webster and L. Zieve, *New Eng. J. Med.*, **267**, 604 (1962).
2) I. S. Cherry and L. A. Crandall, *Am. J. Physiol.*, **100**, 266 (1932).
3) N. W. Zietz and E. A. Frierck, *Clin. Chim. Acta*, **13**, 356 (1966).
4) R. P. MacDonald and R. O. LeFaree, *Clin. Chem.*, **8**, 509 (1962).
5) B. Borgstron, *Scand. J. Clin. & Lab. Invest.*, **9**, 226 (1957).
6) C. W. Vogel and L. Zieve, *Clin. Chem.*, **9**, 168 (1963).
7) P. H. Dirstine, C. Sobell and R. J. Henry, *Clin. Chem.*, **14**, 1097 (1968).
8) Y. Suzuki, T. Irie, H. Fujita and E. Maehata, *Rinshobyori*, **23**, 466 (1975).
9) A. M. Seigman and M. M. Nachlas, *J. Clin. Invest.*, **29**, 31 (1950).
10) H. J. Randerechts and H. Moskau, *Clin. Chim. Acta*, **4**, 221 (1959).
11) K. Furuya and S. Furuya, *Seikagaku*, **33**, 615 (1961).
12) S. Kurooka, S. Okamoto and M. Hashimoto, *J. Biochem.* (Tokyo), **81**, 361 (1977).
13) J. G. Meiyer-Bertenrath and H. Kaffarnik, *Hoppe-Seyler's Z. Physiol. Chem.*, **349**, 1071 (1968).
14) S. Kamachi, K. Wakabayashi, M. Yamaguchi and Y. Ohkura, *Anal. Chim. Acta*, **148**, 225 (1983).
15) J. T. Tildon and J. W. Ogilvie, *J. Biol. Chem.*, **247**, 1265 (1972).
16) C.E. Wilde and R. G. O. Kekwick, *Biochem. J.*, **91**, 297 (1964).
17) Unit definition: μ equivalent of fatty acid produced from a triglyceride in 1 h at pH 7.7 at 37 °C.
18) T. Matsuo, Y. Ushihama and K. Niimi, *Rinshobyori*, **23**, 419, (1975).
19) C. Hollet and H. C. Meng, *Am. J. Physiol.*, **151**, 137 (1956).