[Chem. Pharm. Bull. 23(7)1417—1420(1975)]

UDC 577.15.02.08 : 547.29.04

Application of Lipoprotein Lipase for the Assay of Serum Triglyceride[1)]

Mamoru Sugiura, Masakazu Isobe,[2a)] Fumio Hirata, and Toshiwo Inagawa[2b)]

Tokyo College of Pharmacy[2a)] and Research Institute, Ono Pharmaceutical Company[2b)]

(Received September 30, 1974)

A method for the assay of triglyceride in human serum was attempted by hydrolysis of triglyceride with lipoprotein lipase and measurement of released free fatty acid by titrimetry according to the method of Dole. Triglyceride in serum was completely hydrolyzed to glycerol and free fatty acid with the lipoprotein lipase produced from *Pseudomonas* sp. in a short time. It was found that the lipoprotein lipase was useful as a reagent for clinical examination. Data from the Dole method with lipoprotein lipase were parallel to those from the Van Handel and Zilversmit method, which has frequently been employed.

Recently many studies have been reported on abnormal lipid metabolism, and a new method for the assay of serum triglyceride is desired for clinical work. In the Van Handel and Zilversmit method,[3)] which has frequently been employed for the assay of serum triglyceride, it is necessary to extract neutral fats from serum, and after chemical saponification of the fats, released glycerol is assayed by colorimetry. The present paper describes the application of a lipoprotein lipase for the assay of serum triglyceride which is in the form of lipoprotein, and free fatty acid released from triglyceride is assayed by the Dole method.[4)] The lipoprotein lipase used was separated and purified from *Pseudomonas* sp. as reported in our previous paper.[5)]

Methods and Materials

Enzyme——The lipoprotein lipase from *Pseudomonas* sp. is a partially purified preparation which has an activity of 150000 to 300000 U/g.

Human Serum——Human serum, containing a number of sera from hyperlipemia and jaundiced patients, was obtained and used in a fresh state.

Artificial Fat Emulsion——For the preliminary experiments and for the assay of enzymic activity of lipoprotein lipase, artificial fat emulsion "Fatgen" (Dainippon Pharmaceutical Co., Ltd.) was used in the presence of 1% bovine serum albumin.

Assay of Triglyceride by the Dole Method Using Lipoprotein Lipase——The reaction mixture contained 0.5 ml each of serum, phosphate buffer, and the lipoprotein lipase solution (500 U/ml). The enzyme reaction was carried out at 37° for 10 min. To the reaction mixture, 5 ml of stopper solution (isopropanol: heptane: 2N H_2SO_4=40: 10: 1), 3 ml of heptane, and 2 ml of H_2O were added and the mixture was stirred vigorously for 15 sec. After allowing to stand for 20 min, 3 ml of the solution was taken from the upper layer (heptane layer) and the extracted free fatty acid was titrated with 0.01 M KOH–EtOH solution while stirring with a stream of N_2 gas. The amount of triglyceride was recorded as moles of released free fatty acid.

Assay of Serum Triglyceride by the Modified Van Handel and Zilversmit Method——The assay procedures are as follows: A mixture of 0.2 ml of serum. 15 ml of $CHCl_3$, and 1 g of Florisil was shaken for 10 min. After centrifugation, 3 ml of the upper layer was taken out and $CHCl_3$ in it was removed by evaporation. To the

1) Preliminary communication of this article has been presented at the 90th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, July 1970. This forms Part XCIII of "Studies on Enzymes" by M. Sugiura.

2) Location: *a) Ueno-sakuragi 1-chome, Taito-ku, Tokyo, 110, Japan; b) Sakurai, Simamoto-cho, Misima-gun, Osaka, 618, Japan.*

3) E.V. Handel and D.B. Zilversmit, *J. Lab. Clin. Med.*, **50**, 152 (1957).

4) V.P. Dole, *Clin. Invest.*, **35**, 150 (1956).

5) M. Sugiura, M. Ito, and H. Tanaka, *Yakuzaigaku*, **28**, 129 (1968); M. Sugiura, H. Tanaka, and H. Asano, *Annu. Proc. Gifu Coll. Pharm.*, **18**, 47 (1968).

residue, 0.5 ml of 0.4% KOH–EtOH was added and hydrolyzed for 20 min at 60—70°. The mixture was neutralized by the addition of 0.5 ml of 0.2 N H_2SO_4 and EtOH was completely evaporated. After the mixture cooled, 0.1 ml of 0.05 M $NaIO_4$ was added and the solution was kept for a few minutes. To the reaction mixture, 0.1 ml of 0.5 M $NaAsO_2$ and 5 ml of chromotropic acid reagent were added, the solution was heated in a boiling water bath for 30 min, and absorbance of this solution was determined at 570 mμ. The amount of triglyceride was indicated as the amount of the standard triolein that showed equal absorbance.

Results

Action of Lipoprotein Lipase on Triglyceride

In order to elucidate the action of lipoprotein lipase on lipids, hydrolysis products of Fatgen and human serum were assayed by thin-layer chromatography. The enzyme reaction was carried out with 0.5 ml of serum and 1% Fatgen by the addition of 150 U of lipoprotein lipase. After the reaction mixture was extracted and concentrated, it was spotted on a thin-layer of silica gel H. As the developing solvent, petroleum ether: ether: acetic acid (90: 10: 1) was used. The results are shown in Fig. 1.

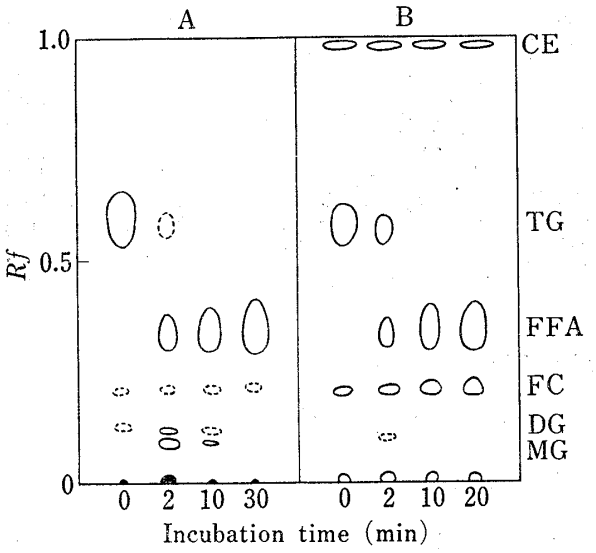

Fig. 1. Thin-Layer Chromatogram of Hydrolyzates of Fatgen and Serum with *Pseudomonas* Lipoprotein Lipase

Fatgen (A) and serum (B) were hydrolyzed with the lipoprotein lipase for various periods and hydrolyzates were spotted on a thin-layer of silica gel H. The plate was developed with a mixture of petroleum ether, ether, and acetic acid (90:10:1). CE: cholesterol ester, TG: triglyceride, FFA: free fatty acid, FC: free cholesterol, DG: diglyceride, MG: monoglyceride

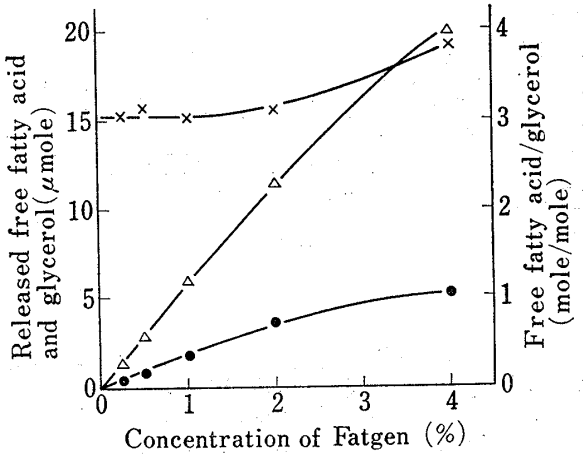

Fig. 2. Relationship of Released Free Fatty Acid and Glycerol in Enzymic Hydrolyzate of Fatgen with *Pseudomonas* Lipoprotein Lipase

Incubation mixture contained 1 ml of diluted Fatgen, 0.5 ml of phosphate buffer (pH 7.0), and 0.5 ml of lipoprotein lipase (300 U/ml). The enzymic reaction was carried out at 37° for 10 min. —△—: free fatty acid, —●—: glycerol, —×—: free fatty acid/glycerol

Triglyceride was rapidly hydrolyzed and decomposed to free fatty acid and glycerol within 10 min, but the cholesterol ester was not hydrolyzed. To ensure the action of lipoprotein lipase, the relationship between the original substrate and products was examined and the results are shown in Fig. 2.

A favorable proportional relationship was seen between the substrate and the released free fatty acid before the amount of Fatgen had reached 2%. The molar ratio of the released free fatty acid and glycerol was approximately 3 under these conditions, and triglyceride had been completely hydrolyzed. It was elucidated from these results, that the assay of triglyceride was possible by the assay of free fatty acid which was released from triglyceride by lipoprotein lipase.

Reaction time was examined, and released free fatty acid and glycerol were assayed. These results are shown in Fig. 3. It has become apparent that the hydrolysis of triglyceride proceeded to approximately 70% after 1 min, 90% after 5 min, and almost completely after 10 min. The ratio of the released free fatty acid and glycerol became constant 3 after 5 min. From these results, the reaction time required for the complete hydrolysis of triglyceride is considered to be 10 min.

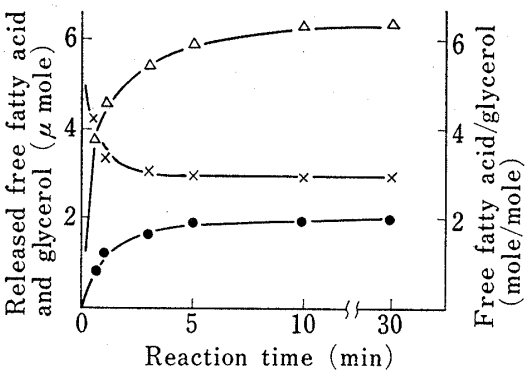

Fig. 3. Time Course of Hydrolysis with *Pseudomonas* Lipoprotein Lipase and Relationship of Released Free Fatty Acid and Glycerol

Incubation mixture contained 1ml of 1% Fatgen, 0.5 ml of phosphate buffer (pH 7.0), and 0.5 ml of lipoprotein lipase (300 U/ml). The enzymic reaction was carried out at 37°. —●—: free fatty acid, —△—: glycerol, —×—: free fatty acid/glycerol

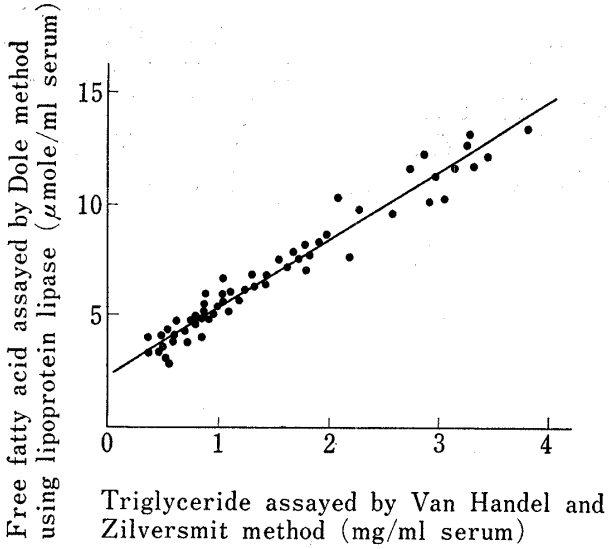

Fig. 4. Relationship between the Dole Method and the Van Handel and Zilversmit Method for the Determination of Triglyceride in Human Serum

The Dole method was applied on 57 cases of human serum. Reaction mixture contained 0.5 ml of serum, 0.5 ml of phosphate buffer (pH 7.0), and 0.5 ml of lipoprotein lipase solution (500 U/ml). The enzymic reaction was carried out at 37° for 10 min, and released free fatty acid was determined.

Reproducibility

On the basis of the results obtained above, following conditions were employed. A mixture of 0.5 ml each of human serum, phosphate buffer (pH 7.0), and lipoprotein lipase solution (500 U/ml) was incubated at 37° for 10 min. Then the released free fatty acid was assayed by the method of Dole. With a same serum, reproducibility of the assay method was examined and the result was FFA (mole/ml)=5.24±0.15 (mean±standard deviation).

Correlationship between the Present Method and the Modified Van Handel and Zilversmit Method

The present method was applied on 57 cases of human serum and compared with the modified Van Handel and Zilversmit method. The results are shown in Fig. 4.

Data from the Dole method (y) and the modified Van Handel and Zilversmit method (x) were treated statistically. There is a regression function of $y=0.03x+2.36$, and correlation coefficient of the two methods was 0.975. The sum of square deviation ($S_{y.x}$) from the regression line was 37.6 and the variance of error ($V_{y\cdot x}$) to $S_{y\cdot x}$ was 0.683.

Stability of Lipoprotein Lipase

The stability of lipoprotein lipase from *Pseudomonas* sp. during a long period of storage was examined. The lipoprotein lipase powder (150000 U/g) was maintained at 22° and 5°, and the remaining activity was determined. It was found that the enzyme, when was kept at 5°, still exhibited the activity as much as the original one after 2 months, while the activity of lipoprotein lipase, which had been kept at 22°, was decreased to 55% of the original after 1 month.

Discussion

The Van Handel and Zilversmit method, which has been frequently employed for the assay of serum triglyceride, is very intricate, and a new method which is simple and accurate has been desired. Recently, Bucolo and David[6] attempted hydrolysis of the serum triglyceride with lipases from various origins and to assay the triglyceride. However, the recovery of the released glycerol was low. Hence they combined a proteolytic enzyme with the lipase and hydrolyzed the serum triglyceride completely.

We examined the application of lipase from *Pseudomonas* sp., which belongs to lipoprotein lipase, for the assay of triglyceride in serum. The triglyceride in serum was completely hydrolyzed in a short time only with lipoprotein lipase. The method reported in the present paper is found to be simple and rapid compared with other methods. The data from this method were reproducible and were comparable with the Van Handel and Zilversmit method.

6) G. Bucolo and H. David, 8th International Congress on Clinical Chemistry, Copenhagen 1972. *Scand. J. Clin. Lab. Invest.*, **29**, Suppl. 126 (1972); G. Bucolo and H. David, *Clin. Chem.*, **19**, 476 (1973).