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Method for Triglyceride Measurement in Small Amounts of Plasma

J. I. Ramírez, M. Llobera and E. Herrera *

Cátedra de Fisiología General
Facultad de Biología
Universidad de Barcelona, and
Departamento de Investigación, Centro «Ramón y Cajal»
(Madrid)

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A method is described for determining triacylglyceride concentration in small amounts of plasma. After ethanolic-KOH digestion of diluted plasma aliquots, samples were neutralized with $MgSO_4$ and glycerol was fluorimetrically assayed in supernatants by the coupling of glycerokinase, pyruvate kinase, and lactate dehydrogenase catalyzed reactions. Values were corrected by free glycerol present in the non-digested samples. Digestions were performed at different times and temperatures in order to establish optimum conditions for recoveries and reproducibility. Parallel determinations before and after phospholipid removal showed that their presence in plasma did not interfere with the obtained values. This method is especially useful for running many samples in parallel and for determinations in small experimental animals in which the amount of plasma is very limited.

Several methods have been described for determination of plasma triacylglycerides (5, 11, 13, 16-18) but widely differing results have often required verification of reproducibility (1, 3, 8, 14). In the present work we adapted the most commonly employed procedure to determine plasma triacylglycerides for measurement in small amounts of plasma. This procedure is based on ethanolic-alkaline hydrolysis and determination of released glycerol by its conversion to glycerol-3-phosphate with ATP and glycerokinase. The consumed ATP is converted to ADP which reacts with phosphoenol pyruvate in the presence of pyruvate kinase to yield pyruvate. This is reduced to lactate by means of NADH and lactate dehydrogenase, the decrease in NADH being followed fluorimetrically:

* Correspondence should be addressed to:
Dr. Emilio Herrera
Departamento de Investigación
Centro «Ramón y Cajal»
Ctra. de Colmenar km 9
Madrid, 34. Spain

Triacylglycerides $\xrightarrow{\text{ethanolic-alkaline hydrolysis}}$ glycerol + FFA glycerol + ATP $\xrightarrow{\text{glycerokinase}}$ glycerol-3-phosphate + ADP phosphoenol pyruvate + ADP $\xrightarrow{\text{pyruvate kinase}}$ pyruvate + ATP pyruvate + NADH $\xrightarrow{\text{lactate dehydrogenase}}$ lactate + NAD^+.

On the basis of recovery experiments and the observed lack of interference with circulating phospholipids, certain modifications are proposed which permit rapid and easy measurement of triacylglycerides in less than 10 μl total plasma.

Materials and Methods

Reagents. Phosphoenol pyruvate, pyruvate kinase (8.4 mg protein/ml, 385 U/mg protein), lactate dehydrogenase (11 mg protein/ml, 900 U/mg protein), and glycerokinase (5 mg protein/ml, 95 U/mg protein, diluted to 0.6 U/ml with 0.05% bovine serum albumin) were purchased from Sigma. All other reagents used were of analytical grade.

Equipment. A Farrand Optical Co. fluorimeter A-44 was used for fluorescence determinations.

Procedure. Plasma samples are diluted 1/8 (v/v) with double-distilled water and 0.05 ml aliquots are pipetted into small (1.5 ml total capacity) narrow bottom, screw cap tubes. 0.125 ml of ethanolic-KOH (0.5 N in 90% ethanol) are also added to the tubes which are then well capped and hydrolyzed in bath water at 80° C for 120 min. When the samples are cool, neutralization is performed with 0.250 ml $MgSO_4$ (0.15 M) and the tubes are recapped. To obtain blank samples (non hydrolyzed plasmas), other 0.05 ml aliquots of diluted plasma are pipetted into new tubes where 0.250 ml $MgSO_4$ (0.15 M) and then 0.125 ml ethanolic-KOH are added. After mixing, the tubes are centrifuged at 1700 x g for 30 min and supernatants are used for glycerol determination. For this purpose 0.1 or 0.05 ml (depending on expected values) aliquots of supernatants or 0.1 ml of glycerol standards (containing 0, 2.5, 5.0, 7.5, or 10.0 nmoles) are added to 1.5 ml of the reaction mixture containing phosphate buffer pH 7.0 (0.05 M), $MgCl_2$ (2.2 mM), NADH (12 μM), ATP (72 μM), phosphoenol pyruvate (36 μM), bovine serum albumin (0.09 mg/ml), lactate dehydrogenase (5 μl), and pyruvate kinase (5 μl). After mixing and remaining at room temperature for at least 20 min, fluorescence is read (F_1), after which 5 μl of glycerolkinase are pipetted into each tube and immediately mixed. Fluorescence is read again after 45 min (F_2) and glycerol values are calculated from the change of fluorescence units found in the two readings of the samples (F_1-F_2) and compared with those of the standards. Triacylglycerides are calculated as glyceride glycerol from the difference between total glycerol values found in the hydrolyzed samples and free glycerol present in the blanks (non-hydrolyzed samples).

Results and Discussion

To determine the influence of saponification temperature and time on the amount of assayed triacylglycerides, 0.05 ml aliquots of undiluted plasma were processed according to the described procedure but using different temperatures and times for the ethanolic-KOH hydrolysis. Total amounts of detected triacylglycerides are summarized in Fig. 1, demonstrating that temperature increase from 60 to 100° C causes progressively diminished triacylglyceride recovery. To determine whether this loss was the result of glycerol instability during saponification, duplicated free glycerol samples (5.88 nmoles/tube) were incubated in the same

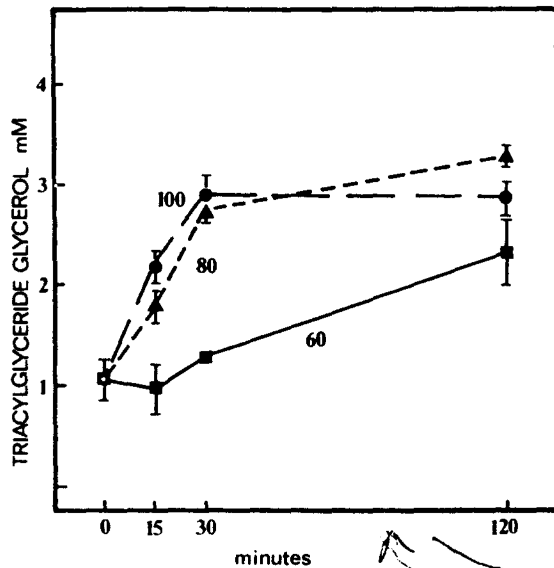

Fig. 1. *Amount of triacylglycerides detected in 0.05 ml aliquots of undiluted rat plasma processed according the described method but using temperatures of 60 (■——■), 70 (▲---▲), 85 (▲— —▲) or 100° C (●——●) and different times for the ethanolic-KOH hydrolysis.*

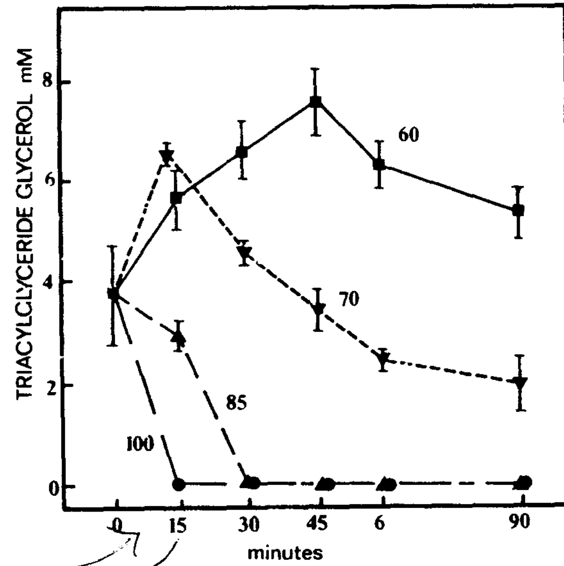

Fig. 2. *Amount of triacylglycerides detected in 0.05 ml aliquots of diluted (1/8/v/v) rat plasma processed according the described method but using temperatures 60 (■——■), 80 (▲---▲) or 100° C (●——●) and different times for the ethanolic-KOH hydrolysis.*

manner as plasma for 0, 15, 30, 45, 60, or 90 min at 60, 70, 80, or 100° C in the presence of ethanolic-KOH. Observed values did not differ from those of nonincubated controls (time 0) at any of the incubation times or temperatures used (data not shown). Since the decrease in triacylglyceride recuperation during incubation could be due to ongoing chemical changes which interfere with the enzymatic-fluorescence determination of glycerol, possible change in the appearance of pyruvate (or pyruvate-like material) during incubation was determined by measuring pyruvate concentration (15) in 5 samples of fed rat plasma before and after the ethanolic-KOH hydrolysis for 120 min at 80° C. Results showed that while pyruvate concentration was 0.7 ± 0.19 mM before digestion, it rose to 2.61 ± 0.14 mM afterward ($P < 0.001$). This enormous increase in pyruvate produced from precursors in plasma (lactate, amino acids, etc.) should consume a high proportion of NADH present in the reaction mixture used for the final evaluation of glycerol, since it contained lactate dehydrogenase, causing a reduced and insufficient amount of NADH and inhibiting adequate recovery. To overcome this problem, the procedure was adapted to assay small amounts of diluted plasma samples (0.05 ml of plasma diluted 1/8 (v/v) with double-distilled water) so that any formation of NADH-oxidizing material is not sufficient to interfere with the assay. With this modification, adequate conditions were determined by making ethanol-KOH digestions at either 60, 80, or 100° C for different periods of time. Results are summarized in Fig. 2 where it is seen that while 60° C digestion did not allow complete hydrolysis of triacylglycerides, this was achieved by incubation at 80° C or at 100° C for 30 min. As no differences were found at either of these