Lipase-Triggered Kinetic Assay of Serum Triglycerides

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We describe a kinetic method for assay of serum or plasma triglycerides, by use of an enzymatic hydrolysis and reaction sequence already described [Clin. Chem. 19, 476 (1973)]. The reaction is triggered by addition of lipase, at a time when free glycerol, or pyruvate (or both) are no longer present. In this method, therefore, there is no need for a blank glycerol assay. In the procedure, reagents are used that are available commercially in the form of stable, dry powders; the method for the preparation of the reagents has been changed to achieve improved stability and performance. Stability and recovery of added triglycerides are satisfactory.

Kinetic assay for several substrates has been described in a number of papers (e.g., 1, 2). In these methods the rate of an enzymatic assay is measured in which the rate-limiting component is the substrate to be determined. Assay procedures based on this principle are particularly attractive when applied to automated instruments, because observation time is considerably shorter than that required by the corresponding endpoint method and thus the analysis is speeded.

The most obvious kinetic adaptation of the enzymatic triglycerides assay we have described (3) would be to trigger the series of reactions described below by adding glycerol kinase to the total assay mixture at a time when all the triglycerides have been completely hydrolyzed by the action of lipase:

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\text{glycerol kinase} \\
\text{Glycerol + ATP} \rightarrow \text{glycerol-1-phosphate + ADP} \\
\text{pyruvate kinase} \\
\text{ADP + P-enolpyruvate} \rightarrow \text{ATP + pyruvate} \\
\text{lactate dehydrogenase} \\
\text{Pyruvate + NADH + H}^+ \rightarrow \text{lactate + NAD}^+ \\
\]

References

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One such adaptation has recently been described (4). This procedure determines total glycerol, thus rendering it necessary to perform a separate blank assay for free glycerol by omitting the hydrolyzing enzymes. Furthermore, the rate of the reaction in this method is not linear during the first minute after addition of glycerol kinase (3, 4). Additionally, the instability of some of the components of the assay results in a relatively unstable reagent; the resulting small changes in the composition of the reagent, which would have a negligible effect on an endpoint assay, are particularly evident in the glycerol kinase-triggered kinetic assay, and are evidenced by a lack of reproducibility of the rate with time. Consequently frequent standardization is required.

In our modification of this enzymatic assay, we incubate sufficiently long for all free glycerol or pyruvate (or both) in the sample to be consumed. Then we add lipase and measure the resulting rate of the slow release of glycerol from triglycerides. By properly modifying the reagent reconstitution volume and by adding stabilizing substances as described below, we have been able to increase the stability of the reagents and to avoid the need for frequent standardization.

Materials and Methods

Apparatus

A Model 2000 spectrophotometer (Gilford Instruments, Inc., Oberlin, Ohio 44704), a Spectronic System 400-4 (Bausch & Lomb, Rochester, N. Y. 14625) and an Reaction Rate Analyzer, Model 8600, with filters for 340 nm, set at 30 °C, and associated recorder, calculator, and mosaic printer (all from LKB Instruments, Inc., Rockville, Md. 20852) were used for the kinetic and endpoint enzymatic assays. An AutoAnalyzer Sampler II system (Technicon Instruments Corp., Tarrytown, N. Y. 10591) with a Model 111 Fluorometer (G. K. Turner Assoc., Palo Alto, Calif. 94303) was used for the automated triglycerides assays.

Chemicals

Three-vial “Stat-Packs” (cat. No. 869263; Calbiochem, La Jolla, Calif. 92037) were used for kinetic and endpoint triglyceride assays. Bovine serum albumin was also a Calbiochem product (cat. No. 126575).

“Tri-EZ” (A. R. Smith Laboratories, Los Angeles, Calif. 90017), Elevated Lipid Control (Lederle Diagnostics, Pearl River, N. Y. 10965) and Elevated Lipid Control (Calbiochem) were used to standardize the kinetic assay, and to prepare calibration standards by appropriate dilutions with a solution of bovine serum albumin, 6 g/dl, in distilled water.

Serum samples were obtained from hospitals in the area. All chemicals used were analytical reagent grade or the otherwise best available grade.

Reagents and Procedures

Reconstitute Vial A of the triglycerides Stat-Pack by dissolving its contents in 10.5 ml of distilled water.

Dissolve Vial B contents in 1 ml bovine serum albumin (1 g/liter in potassium phosphate buffer, 0.1 mol/liter, pH 7).

Dissolve Vial C contents in 0.5 ml of the same albumin-buffer solution.

Do the kinetic assay as follows:

To 10.5 ml of the reagent from Vial A add 1 ml of the reagent from Vial B. This is a total glycerol reagent, including glycerol kinase and a-chymotrypsin. The resulting concentrations of the various components are increased by 35% over those already described (3) because of the smaller volume of reconstitution. To 1 ml of the combined solution add 10 µl of sample. Incubate for at least 5 min. Then add 50 µl of the lipase solution from Vial C and immediately record the rate in the recorder or in the printer of the LKB, with use of a steady-state computer mode. The rate is recorded for 1 min. Use the difference in readings between 20 s and 40 s after addition of lipase for the assay. Prepare a calibration curve by measuring the rate obtained for different concentrations of triglycerides, with the controls described, diluted as required.

Results and Discussion

Figure 1 shows the time course of the lipase-triggered reaction for various concentrations of triglycerides. The reaction is linearly related to all concentrations during the selected time interval. The fast change in absorbance from 0 to 10 s is thought to be a result of a clarification of the reaction mixture (3). The increased concentration (35%) of the assay constituents in Vial A, achieved by the use of a smaller reconstitution volume, is sufficient to obtain the linear rates shown. Any further increase in the concentration of all or some of the assay components did not result in higher changes in absorbance, a distinct advantage, because we could use a reagent already available, without any substantial modifications.

To assess the stability of the combined reagent at room temperature during the assay, we prepared and incubated several sets of standard sera with the combined Vial A and B reagent as described. Each set was assayed at fixed intervals after the standards were added to the reagent, and standard curves were constructed for each set. The standard curves remained unchanged for incubations as long as 45 min, at which time the rates decreased for the standards more concentrated than 300 mg/dl.
When Vials B and C are reconstituted as described above, the glycerol kinase and lipase in these reagents are stable for at least 24 h in the refrigerator. Assays of lipase (6) and glycerol kinase (7) were respectively 2900 and 23 U per vial at 0 h and 2880 and 22 U per vial after 24 h in the refrigerator. It was found that if these vials were reconstituted with 0.1 g/dl bovine serum albumin solution in water, the enzymes were unstable. This instability was ascribed to the acidity of the albumin solution (about pH 5). When the protein solutions were prepared in potassium phosphate buffer (0.1 mol/liter, pH 7), the enzymes were stable for 24 h in the refrigerator. The stability of these two key enzymes, the reproducibility of the same change in absorbance for the same standards, and the linearity of response up to and including the 300 mg/dl standard were the criteria chosen to assess stability of the reagents. Some lots of reagents gave satisfactory results, based on these criteria, after 48 h of storage of the separated reagents in the refrigerator, while others were unsatisfactory after 30 h.

The stability of the reagent is demonstrated further in Table 1. Three sets of standards were assayed with a freshly reconstituted reagent and one that had been stored for 24 h in the refrigerator as separate Vials A, B, and C. Vial A and B reagents were combined before each assay. Table 1 shows that the rates measured are the same for both reagents. The reagents can be stored for as long as 24 h without significant loss of any of the components. Therefore, frequent restandardization is unnecessary if the reagent in Vial A is combined with that in Vial B immediately before assay and if this combined reagent is used within 1 h. After 70 min the linearity does not extend beyond the 250 mg/dl standard.

Figure 2 shows, for 61 sera, how the kinetic assay correlates with the AutoAnalyzer procedure (5). The correlation coefficient (r) is 0.996, with a linear regression of where the points are the actual assays and r = 0.94x. Assay speed and preparation of the reagent more than adequately compensate for a certain lack of sensitivity resulting from the small ΔA during the selected period. It is not possible, in our experience, to increase the size of the sample in order to increase the rate, because this would decrease the upper limit of the assay.

This same assay has been adapted for use in the Bausch & Lomb Spectronic System 404-4 as follows:

Prepare reagents from Vials A and B as already described. Dissolve the reagent in Vial C with 2.2 ml of the bovine serum albumin—phosphate buffer solution.

Dilute 0.1 ml of sample with 1 ml of distilled water by use of the "Dispersa Syringe." With this same syringe add 0.1 ml of the diluted sample to 1 ml of the combined A and B reagents. After a minimum of 5 min incubation at 30 °C, add 0.2 ml of reagent C using the "Reagent Add" syringe. Automatic transfer of the assay mixture into the instrument requires 15 s. The assay is followed for an additional 45 s and the difference in readings between 15 s and 45 s is recorded.

Precision studies were performed with control sera, diluted to contain about 100, 200, 300, and 400 mg/dl of triglycerides. These were stored frozen. The day-to-day precision was respectively 4.2, 4.0, 3.9, and 3.4% (CV). Within-day precision (seven assays of each pool) gave a CV, respectively, of 4, 1, 2, and 2%.

References