A Nonlinear Regression-Kinetic Method for Quantification of Serum Triglycerides

Steven D. Hamilton, John W. Skoug, and Harry L. Pardue

We report a new kinetic approach to the quantification of triglycerides (triacylglycerols) in human sera. The new approach combines a commercially available enzyme-reagent system with a multiple-linear-regression data-processing method. Values of absorbance collected at 3-s intervals between 20 and 305 s are fitted to a pseudo-first-order model to compute the total change in absorbance expected if the reaction were to proceed to equilibrium. The computed absorbance change varies linearly with triglyceride concentration between 3 and 20 μmol/L (after 250-fold dilution of sample). The pooled relative standard deviation was 7.2% for 23 serum samples with triglyceride concentrations between 3 and 20 μmol/L. Comparison of kinetic (y) and equilibrium (x) absorbance changes for the 23 sera gave a least-squares equation of y = 0.994x + 0.00066, confirming good agreement between the methods. The temperature coefficient is less than 0.2% per degree Celsius.

Additional Keyphrases: equilibrium method compared · "kit" methods · triacylglycerols

We have developed a new approach to the quantification of triglycerides (triacylglycerols) with a commercially available reagent system adapted to a nonlinear-regression kinetic data-processing method. Here we describe the approach and its performance characteristics for triglycerides in calibrator solutions and human sera. Procedures commonly used to quantify triglycerides can be grouped into two categories: those that combine a separation step with nonselective reaction (1, 2) and those that eliminate the separation step by use of selective enzymatic reactions (3–5). The enzymatic procedures have been implemented with both equilibrium (3, 4) and kinetic (5) measurement approaches. Kinetic approaches based on initial rates have usually involved two-step reaction sequences in which the triglycerides are hydrolyzed completely before the indicator reactions are started. This approach is used to decrease effects of induction periods that result when hydrolysis and indicator reactions proceed simultaneously.

During preliminary studies with a commercially available reagent system for triglycerides (4) we observed that the reaction approached pseudo-first-order behavior after a short induction period, with triglyceride concentration being the rate-limiting component. Having recently developed data-processing methods that greatly decreased errors ordinarily associated with quantitative procedures based on first-order kinetic processes (6, 7), we decided to evaluate the feasibility of adapting the reagent system and the data-processing method for the quantification of triglycerides.

Results we report here show that the resulting procedure permits measurement of triglycerides in the undiluted sample in 245 to 300 s, with a linear range (expressed in terms of triolein concentration) between 1.0 and 5.0 mmol/L (0.9 to 4.3 g/L), with pooled relative standard deviations of 4.5% for standards and 7.2% for sera, and with a temperature coefficient of 0.15%. This measurement time is fourfold less than that for the recommended (4, 5) equilibrium procedure, and the linear range includes both normal and abnormally high values expected for human sera. Comparison of kinetic (y) and equilibrium (x) results for 250-fold sample dilution gave a least-squares equation of y = 0.994x + 0.0007 μmol/L, with a standard error of estimate of 0.001 μmol/L and correlation coefficient of 0.9996.

Materials and Methods

Instrumentation and Software

We made absorbance measurements with a photodiode array-based rapid scanning spectrophotometer (Model 8450A; Hewlett-Packard Co., Palo Alto, CA 94304) that provides one scan per second of the spectral range between 200 and 800 nm. Although this instrument includes a 16-bit microprocessor that provides all control and measurement functions, 32K words of random access memory (RAM) for data storage, and a wide variety of data-processing options, it was interfaced with a small computer (Model 2100A, Hewlett-Packard) to permit use of the nonlinear regression programs described earlier (6, 7). (We will supply details of the interface to interested persons.) We mixed solutions manually in a temperature-controlled cuvet (rectangular, water-jacketed quartz cell, 1-cm pathlength, Model 160; Helma Cells, Inc., Jamaica, NY 11424).

Reagents

We prepared all solutions and diluted all samples with water that had been distilled, passed through a combined cation/anion exchanger, and filtered through Whatman No. 1 paper.

Reagent. The reagent for triglycerides was prepared by reconstituting each vial of the powder (Single-Vial Colorimetric Triglycerides kit; Boehringer Mannheim Corp., Indianapolis, IN 46250) with 25.0 mL of water. This reagent was stable for seven days when stored in amber-colored glassware at a temperature between 2 and 8 °C.

Calibrators. We prepared stock solutions of triglyceride calibrators by reconstituting each vial of lyophilized human serum standard with 25.0 mL of water. Aliquots of the stock solution, stored at −20 °C, were stable for one month. We prepared diluted standards containing, per liter, 3.9 to 19.5 μmol (3.5 to 17 mg) of triglycerides (expressed as triolein) by appropriate dilution of stock solutions.

Samples. We obtained 23 serum specimens from a local hospital, diluted aliquots of the sera 250-fold, and stored the diluted samples at −20 °C. These diluted samples were thawed and used directly in the measurement step.
Procedure

We initiated the reaction by mixing 0.50 mL of calibrator or diluted serum sample with 0.50 mL of reagent, in the reaction cuvet. Absorbance values at 560 nm were recorded at 3-s intervals between 8 and 1200 s after the reaction was initiated. The 8-s delay allowed time for the reaction process to pass through an induction period and for mixing perturbations to subside. The 1200-s observation period allowed time for the reaction to proceed to completion.

We processed the data for absorbance in three ways so that we could compare results by the nonlinear regression method with results obtained with more conventional rate and equilibrium methods. Rate data used for comparison of temperature dependence were obtained by computing least-squares slopes of response curves between 50 and 110 s. Equilibrium measurements were made at 1200 s. Results for the nonlinear regression method were obtained with a program described earlier (6, 7).

Results and Discussion

Triglyceride concentrations are reported as those equivalent to a 250-fold dilution of sample; initial concentrations in the observation cuvet are twofold smaller than reported values. Uncertainties are reported in terms of one standard deviation unless noted otherwise. Results were obtained at 25.0 ± 0.1 °C with a curve-fitting range between 20 and 305 s unless noted otherwise.

Experimental and Fitted Response Curves

The function of the nonlinear fitting program is to fit data from an intermediate time range to a first-order model, and in the process to compute the initial absorbance (A₀), the equilibrium absorbance (Aₑ), and the rate constant (k) that give the best fit to the data. The absorbance change (ΔAₑ = Aₑ - A₀) is related to triglyceride concentration.

Figure 1 presents two sets of experimental (e) and fitted (f) response curves. The fitted curves are based on data between 20 and 305 s. At lower concentrations (Figure 1A) the response is very close to first-order behavior, as indicated by the close agreement throughout the data range shown. At higher concentrations (Figure 1B) the response curves tend to continue a gradual increase long after curves for lower concentrations have ceased to change. This feature makes it desirable to use data taken during as many as two or three activity half lives to ensure good fits for higher concentrations. The rate constant, averaged for many runs, is \(7.1 \times 10^{-3}\) s⁻¹. This corresponds to a half life of 97.6 s. Thus the 305-s interval chosen for most data reported herein corresponds to about three half lives.

Comparison of Methods

Both equilibrium and regression kinetic data were evaluated for three runs on each of 23 serum samples. For data processed between 20 and 305 s by the kinetic method, the least-squares equation for kinetic (y) vs equilibrium (x) results is \(y = 0.994x + 0.00066\), with standard deviations (s) of slope and intercept of 0.0062 and 0.0005, respectively, standard error (sₑ) of 0.001, and correlation coefficient (r) of 0.9996. For a 20- to 245-s processing range, the least-squares equation is \(y = 0.992x + 0.0018\), with standard deviations of slope and intercept of 0.0079 and 0.00063 respectively, sₑ = 0.0013, and r = 0.9993. Although both data sets suggest very good correlation, all the statistics are degraded slightly in the latter case.

Imprecision

Pooled values of standard deviations and residuals from the regression process were computed with the relationship (8): \(s_y = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2 + \ldots + (n_k - 1)s_k^2)}{(n_1 + n_2 + \ldots + n_k - k)}\). In this expression, s is the pooled standard deviation (or standard error of estimate); \(s_1, s_2, \ldots, s_k\) are the standard deviations (or errors of estimate) for the different sets of replicates; \(n_1, n_2, \ldots, n_k\) are the numbers of data points in each set of replicates; and k is the total number of data sets being pooled.
For the nine calibrators \( n_1 = n_2 = \ldots = n_9 = 3 \) and \( k = 9 \), the pooled within-day standard deviation was 0.0042 absorbance unit; the value for the sera \( n_1 = n_2 = \ldots = n_9 = 3 \) and \( k = 23 \) was 0.0046. These values correspond to relative standard deviations (RSD, based on average concentrations) of 4.5 and 7.2% for calibrators and sera. The larger RSD for sera results from the fact that the average triglyceride concentration in the sera was less than that in the standards.

Data reported further on show that calibration plots have slopes of \( 7.78 \times 10^{-3} \text{ L mol}^{-1} \text{ cm}^{-1} \). Using this value, and the standard deviation of 0.0046, the estimated detection limit for the method is \( 2 \times 0.0046/7.78 \times 10^{-3} \text{ L mol}^{-1} \text{ cm}^{-1} \) or 7.2 \( \mu \text{mol/L} \) for a 500-fold dilution of sample monitored in a 1-cm cell.

Residuals (differences between measured and computed absorbances) were used to compute pooled absorbance uncertainties resulting from the fitting process. For the nine calibrators and the 23 sera, the pooled fitting errors were 0.0015 and 0.0014 absorbance unit, respectively. The within-day scatter is observed to be about threefold the fitting errors.

Linearity
To evaluate the linearity of calibration data we used an F-test (9) and a test based on a fit of data for \( \Delta A_m \) vs concentration to a quadratic model (10).

For a linear data set, the square of the standard error of estimate \( (s_p)^2 \) should be small enough to be explained by the random error associated with the procedure. Specifically, if the variance ratio \( (s_p)^2/(s^2) \) is smaller than the tabulated \( F \)-value at the desired confidence level and appropriate degrees of freedom, then a hypothesis that the data set is linear is supported. For the calibrator data set at 25 °C, the least-squares equation is \( \Delta A_m = (7.80 \pm 0.36) \times 10^{-3} C^o + 0.0016 \pm 0.00045 \) with \( s_p = 0.0054 \) and \( r = 0.993 \), where \( C^o \) is in \( \mu \text{mol/L} \). Given this value of the standard error and the pooled standard deviation reported above \( (s_p = 0.0042) \), the variance ratio \( s_p^2/s^2 = 1.6 \). This ratio is well below the 95% confidence level value of \( F = 3.9 \) for seven and nine degrees of freedom. All other data sets exhibited similar behavior.

The quadratic test proposed by Burnett (10) fits data to a model of the form \( y = a_0 + a_1 x + a_2 x^2 \), and determines if the "\( a_2 \)" coefficient is or is not significantly different from zero. We applied the recommended procedure to our data. For the data set at 25 °C with a fitting range of 20–305 s, the computed \( F \)-value (see ref. 10) was 0.18, which is less than the tabulated value of 1.89 for the 95% confidence level with six degrees of freedom. Other data sets exhibited similar behavior, indicating that the "\( a_2 \)" coefficient is not significantly different from zero at the 95% confidence level.

These two procedures provide independent information, and both indicate that the calibration plots are linear over the concentration range examined.

Temperature Dependence
We collected data at 24, 25, and 26 °C for three runs each on the nine calibrator concentrations (3.9 to 19.5 \( \mu \text{mol/L} \)) described above. For a 20–305 s data-processing range, least-squares equations for the calibration plots were

\[
\Delta A_m = 7.78 \times 10^{-3} C^o + 0.0019 \quad \text{with} \quad s_{yx} = 0.006 \quad \text{and} \quad r = 0.991 \quad \text{at} \quad 24 \degree C
\]

\[
\Delta A_m = 7.80 \times 10^{-3} C^o + 0.0016 \quad \text{with} \quad s_{yx} = 0.005 \quad \text{and} \quad r = 0.995 \quad \text{at} \quad 25 \degree C
\]

\[
\Delta A_m = 7.79 \times 10^{-3} C^o + 0.0022 \quad \text{with} \quad s_{yx} = 0.005 \quad \text{and} \quad r = 0.993 \quad \text{at} \quad 26 \degree C
\]

Standard deviations for the three slopes are 0.41, 0.36, and 0.36, and standard deviations for the three intercepts are all 0.006. Thus, any systematic difference among the three temperatures is well below the random variation of the data. For a data-processing range of 20–275 s, the slopes \( (10^{-3} \text{ L mol}^{-1} \text{ cm}^{-1}) \) were 7.74, 7.77, and 7.78, with standard deviations of 0.4, 0.35, and 0.39. This supports the above conclusion.

We also extracted rate data from three response curves. Calibration slopes \( (10^{-3} \text{ L mol}^{-1} \text{ cm}^{-1} \text{ s}^{-1}) \) were 3.04, 3.14, and 3.2 at the three temperatures. These data correspond to a temperature coefficient of 2.5% per degree Celsius. The nonlinear regression method offers substantial improvement in the temperature coefficient.

Other Considerations
For triglyceride concentrations approaching the upper limits of the concentration range examined in this work, one or more components in the reaction mixture begin to become rate limiting, and deviations from pseudo-first-order behavior are observed. For this reason, a slightly wider data range (in terms of half lives) is desirable for this system than was needed for previously reported reactions (6, 7). The method was applied successfully in this study for data ranges down to 245 s. We cannot recommend shorter time intervals unless one is willing to accept a narrower linear calibration range. The triglyceride concentration range for first-order behavior can be moved to higher values by increasing the reagent concentration; however, deviations from first-order behavior are then observed at lower concentrations.

Because this reaction does include a short induction period, and because the nonlinear regression projects the \( \hat{A}_o \) value to \( t = 0 \) as if no induction period existed, computed values of \( \Delta A_m \) will be somewhat larger than the true \( \Delta A \). However, as shown by the comparison of kinetic and equilibrium data above, the effect is quite small.

One attractive feature of the nonlinear regression method is its ability to compensate for variations in reagent composition (6, 7). Although that is also true to some extent for the present system, it is more limited than with other systems we have examined, because the kinetic behavior tends to change with reagent composition. A reagent system optimized for this measurement approach might resolve this problem. Variations among reagent lots may require adjustment of the data-processing range for best performance; it is for this reason that we prefer a data range approaching three half lives.

The reagent decomposes slowly when exposed to ultraviolet radiation, with an increase in absorption at 560 nm. In this study, the ultraviolet source in the spectrophotometer was turned off to avoid this complication.

This multiple-linear-regression kinetic method offers advantages of decreased dependence on temperature and other experimental variables as compared with rate methods and reduced monitoring time as compared with equilibrium methods.

This research was supported in part by Research Grant GM13336-15 from the National Institutes of Health.

References