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Manual and Continuous-Flow Colorimetry of Triacylglycerols by a Fully Enzymic Method

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We describe a fully enzymic method for manual and continuous-flow colorimetric assay of triacylglycerols (triglycerides) in serum. Triglycerides are enzymically hydrolyzed in 10 min by lipase and microbial esterase. The resulting free glycerol is measured enzymically by glycerol kinase and glycerol-3-phosphate dehydrogenase. The NADH so formed is oxidized by coupling with a tetrazolium salt/diaphorase system. The test follows Beer's law to 8 g/L, and the final color is stable for at least 1 h for serum, 15 min for aqueous triolein standards. The manual assay requires only 25 μL of serum and few manipulations. A specific triolein standard was developed for calibrating the manual method. For the continuous-flow method, calibration is made with four concentrations of glycerol standard. The procedure is sensitive, has good precision and accuracy, and gives results that compare well with chemical and enzymic commercial kit methods.

Additional Keyphrases: *intermethod comparison · normal values*

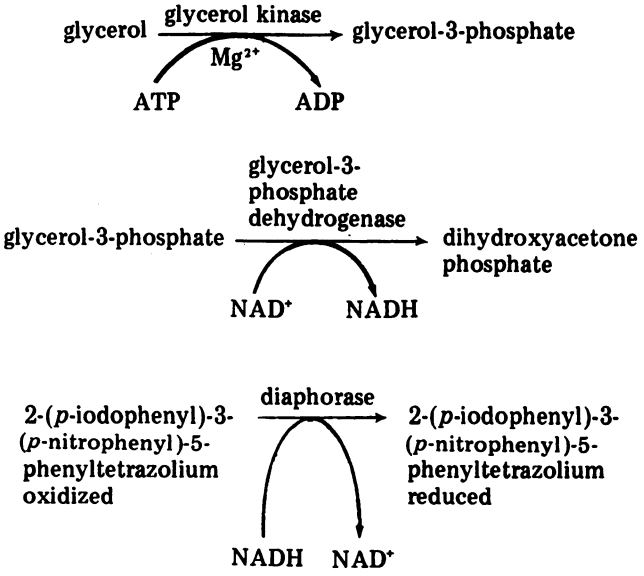
Methods for measuring serum triacylglycerols (triglycerides) include extraction (*1–3*), a combination of chemical and enzymic procedures (*4*), and fully enzymic techniques (*5, 6*). Extraction colorimetric methods usually include some combination of adsorbent, alcohol, base, and organic solvents in the lipolytic system. Color is developed as a result of the Hantzsch reaction or by use of chromotropic acid. In combination tests, lipolysis is usually done chemically; the resulting glycerol is measured spectrophotometrically in the ultraviolet range by use of glycerol-specific enzymes that produce either NAD^+ or NADH. In fully enzymic assays, combinations of lipase, proteases, and esterases are used for lipolysis, and glycerol is measured enzymically in the ultraviolet range.

This report describes a fully enzymic test in which NAD^+ reduction is linked to a tetrazolium dye; the stable formazan formed is measured in the range 500–550 nm. The principal reactions are shown below.

Enzymic lipolysis occurs by the following reaction:

$$\text{triglycerides} \xrightarrow[\text{esterase}]{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

The glycerol is then determined colorimetrically after the following series of reactions:



Materials and Methods

Apparatus

We used a Model 24 double-beam spectrophotometer (Beckman Instruments, Inc., Fullerton, CA 92634) for spectrophotometric assays. All readings at 340 nm were in matched silica cuvets (Coleman Instruments Division of Perkin-Elmer Corp., Oak Brook, IL 62501) or 1-cm square plastic cuvets (Bio-Dynamics/*bmc*, Indianapolis, IN 46250). Absorbance measurements for colorimetric assays were made with a Digitek® and a Unimeter® 300 (Bio-Dynamics/*bmc*) using 15-mm (o.d.) round glass cuvets (Unitubes®, Bio-Dynamics/*bmc*).

For continuous-flow measurements, an AutoAnalyzer I® was used (Technicon Instruments Corp., Tarrytown, NY 10591).

Chemicals

Glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase; EC 1.1.1.8) and glycerol kinase (ATP:glycerol 3-phosphotransferase, EC 2.7.1.30) were purchased from Palomar Chemicals, Carlsbad, CA 92008. Lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3), microbial esterase (carboxylic-ester hydrolase, EC 3.1.1.1), ATP, NAD, and NADH were products of Boehringer Mannheim Biochemicals, Indianapolis, IN 46250. Diaphorase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) was from American Chemical Enterprises, St. Louis, MO 63103. Bovine serum albumin was purchased from Miles Laboratories, Elkhart, IN

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64514; triolein from P-L Biochemicals, Milwaukee, WI 53205; 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride from Leon Laboratories, St. Louis, MO 63114; and triethanolamine from Fisher Scientific, Pittsburgh, PA 15219. Triton X-100 (octylphenoxy polyethoxy ethanol) is a product of Rohm and Haas Co., Philadelphia, PA 19105. All inorganic chemicals were the purest grades obtainable.

Preparation of Reagents

Manual Method

Serum comparisons were made by using the triglycerides procedure of Bucolo and David (*5*), a Hantzsch test (*7*), and the ultraviolet manual test of Boehringer Mannheim (*6*).

Enzyme–coenzyme reagent for the present method was prepared in bulk, dispensed into individual 15 × 60 mm vials, and lyophilized. In this way, consistent single tests[4] could be done without the need to prepare fresh reagents frequently. Reagents were prepared as follows:

Enzyme-coenzyme solution, per liter

Bovine serum albumin	5.0 g
ATP	1.2 g
NAD^+	4.2 g
Lipase	600 000 U (25 °C)
Esterase	200 U (37 °C)
Glycerol kinase	6000 U (30 °C)
Glycerol-3-phosphate dehydrogenase	16 000 U (25 °C)
Diaphorase	6000 U (25 °C)

The reagent is adjusted to pH 7.0, and 0.5-mL quantities are dispensed into screw-cap vials, 15-mm in diameter, and lyophilized.

Color reagent. Triethanolamine-HCl buffer, 0.1 mol/L, pH 7.5, containing 250 mg 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride per liter.

Acid reagent. Hydrochloric acid, 0.1 mol/L.

Triolein standard. Two standards were prepared, one containing 1.00 g of triolein per liter, the other 4.00 g of triolein per liter. Standards were prepared by the method of Chong-Kit and McLaughlin (*8*), with the following exceptions. Triolein is solubilized in a solution containing 9.0 g of sodium chloride and 1.00 g of ethoxylated tridecyl alcohol (Lipal-610, PVO International, Inc., Boonton, NJ 07005) per liter. Magnesium ions, 6 mmol/L, are added as activator, and propionic acid, 13.5 mmol/L, as preservative.[5] These standards are stable for at least a year when refrigerated and well stoppered.

Continuous-Flow Method

Color reagent. This is the same as used in the manual method. Sufficient quantity for 4 h of running time on the AutoAnalyzer I is approximately 200 mL.

Enzyme–coenzyme reagent. For the continuous-flow application the materials were not freeze-dried; rather, they were blended together as a powder. The glycerol-3-phosphate dehydrogenase, lipase, and esterase are prepared as a separate reagent because they are suspensions in ammonium sulfate solution. The powder reagent contains sodium bicarbonate to contribute bulk and maintain a neutral pH in the solution.

A quantity sufficient for 4 h of running time on an AutoAnalyzer I is:

NAD	500 mg
$NaHCO_3$	600 mg
Bovine serum albumin	600 mg
Magnesium acetate-$4H_2O$	120 mg
ATP	215 mg
Glycerol kinase	720 U
Diaphorase	720 U

Dissolve the blended powder mixture in 50 mL of reagent water. To complete the working reagent, add 5 mL of the enzyme suspension described below.

Enzyme suspension. An enzyme suspension in 3.2 mol/L ammonium sulfate contains, per 5 mL:

Glycerol-3-phosphate dehydrogenase	1900 U
Lipase	72 000 U
Esterase	24 U

When 5 mL of the above suspension is added to 50 mL of enzyme–coenzyme reagent, the working solution is complete.

Acid detergent solution. To prepare 3 L, a quantity sufficient for 16 h of running time and subsequent cleaning purposes, add 3 g of Triton X-100 and 1.5 mol of hydrochloric acid to about 2.5 L of reagent water, dilute to 3 L, and mix.

Glycerol standards. Four different concentrations of glycerol standard are used to calibrate the AutoAnalyzer I. A stock glycerol solution equivalent to 4.0 g/L (as triolein) is prepared by adding 416.0 mg glycerol per liter of solution containing 9.0 g of NaCl and 5 g of bovine serum albumin. Dilutions of 1:4, 1:2, and 3:4 are made in saline–albumin solution to give, respectively, 1, 2, and 3 g/L (as triolein) standards. Bovine serum albumin is added to help stabilize the formazan and aid lipolysis by complexing with fatty acids formed.

Sample predilution is made with a solution of 1 mL of a 300 g/L solution of Brij-35 (ICL United States, Inc., Wilmington, DL 19897) per liter of reagent water.

Manual Procedure

Reconstitute freeze-dried vials as required with 2.0 mL of color-reagent solution. Cap, mix gently to dissolve, and preincubate at 37 °C for 5 min. Add 25 μL of serum, mix, and incubate at 37 °C for at least 10 min. For the reagent blank, add 25 μL of reagent water. Add 1 mL of acid solution, mix, and measure the absorbance at 540 nm in the DigiTek or at 525 nm in the Unimeter 300. All samples, whether assayed by the present or comparative methods, were run at least in duplicate, and the results were averaged.

To assay for free glycerol, use enzyme–coenzyme reagent without the lipase and the esterase.

Instrument calibration. The DigiTek photometer was calibrated at 540 nm according to the instrument manual, with use of aqueous triolein standards at 1.00 and 4.00 g/L.[5] The DigiTek has a series of three test-factor switches, which sets up an internal calibration curve, allowing direct digital readout in concentration units. By definition, the DigiTek test factor is equal to one-half the inverse slope. The Unimeter-300 calibration was checked with Pontacyl standard dye solutions of 0.125 and 0.456 absorbance at 525 nm. In the continuous-flow method, the AutoAnalyzer I was calibrated with 1.0, 2.0, 3.0, and 4.0 g/L (as triolein) glycerol standards.

Continuous-Flow Procedure

The system requires a sampler, pump, colorimeter, and stripchart recorder. Working solutions are brought to 20–25 °C. Connecting lines between parts A-12 and C-5 should be glass, and should be connected as closely as possible. Plastic tubing should not be used because it absorbs the red formazan.

[4] Available as the "Unitest System Triglycerides Test" (cat. no. 6600) from Bio-Dynamics/*bmc*, Indianapolis, IN 46250.

[5] Available as "BioTrol T" (cat. no. 6010) from Bio-Dynamics/*bmc*, Indianapolis, IN 46250.

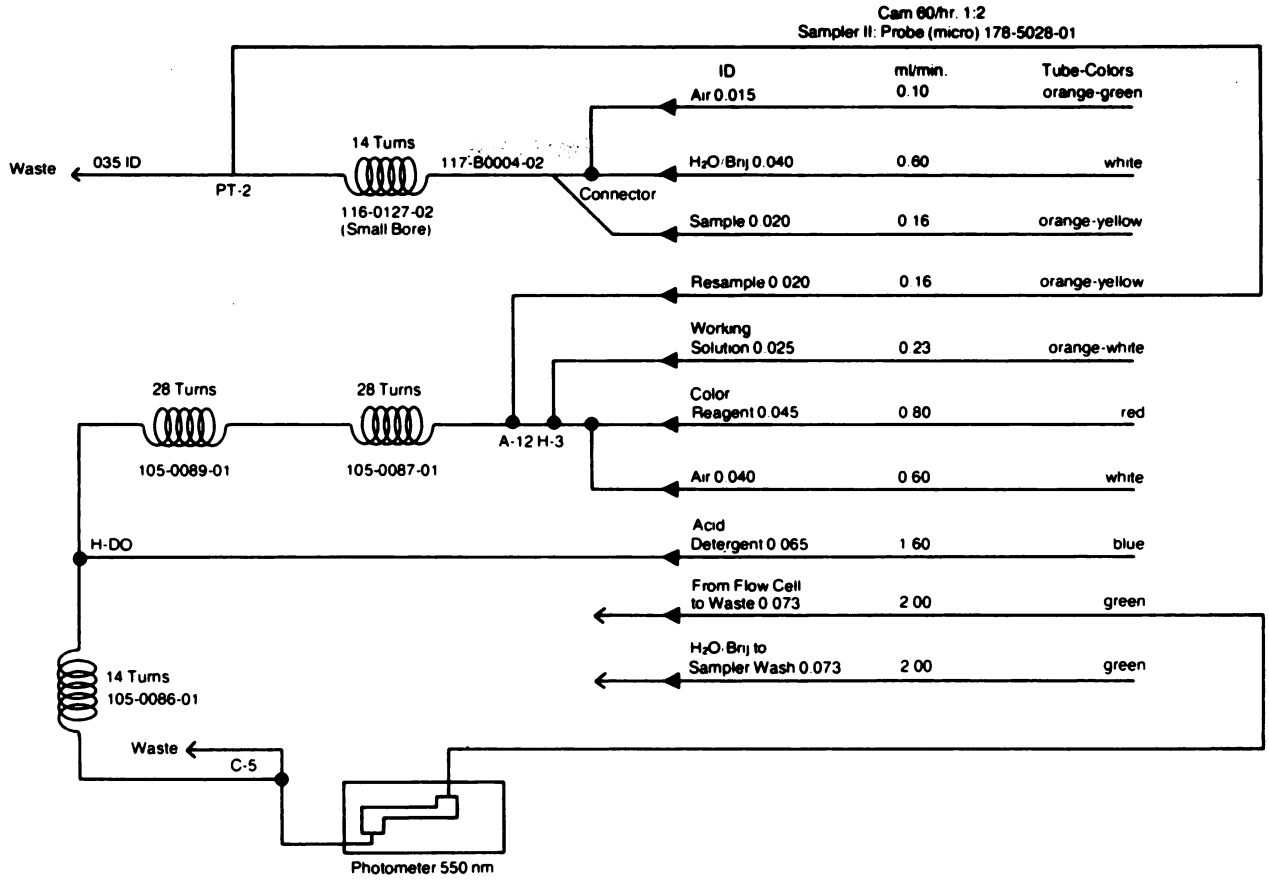

Fig. 1. Schematic diagram of continuous-flow system
Internal diameters of tubing are in inches

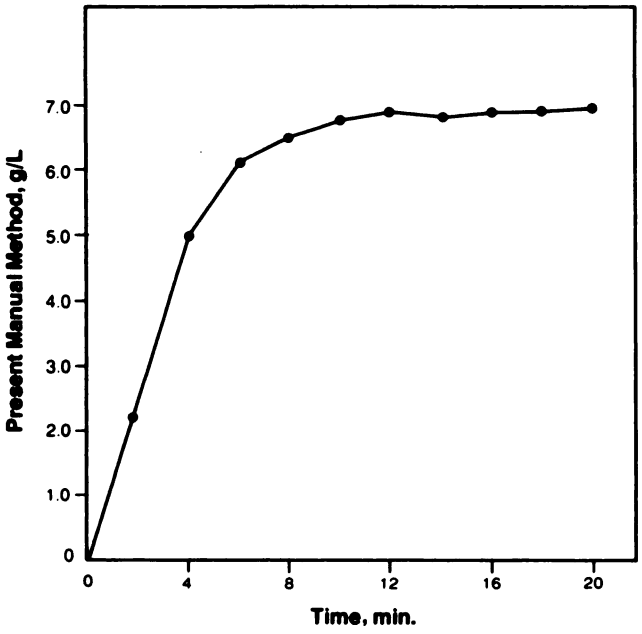

Fig. 2. Time course of color development.
Ten replicate assays were run according to the procedure described in text and stopped at timed intervals. Assay values in g/L were read in the Unimeter

The reagent lines (not the sample or resample lines) should be rinsed daily with a 2-min run of acid detergent followed by a 5-min run with reagent water. This procedure should also be used for new tubing. To help maintain an optimal bubble pattern in the sample predilution line, orient the PT-2 connector to keep the resample line perpendicular, and use a waste line with inside diameter of 0.035 inch. The reagent baseline is set at 95–98% transmittance. Calibration is with 1.0, 2.0, 3.0, and 4.0 g/L glycerol standards. For optimum results, dwell time between A-12 and H-DO must be 9 to 10 min. This is usually no problem when the correct coils are used, as shown in the flow diagram (Figure 1).

Results

Manual Method

Best results were obtained by using lipase purified from *Rhizopus arrhizus*. Following the report of Bucolo and David (*5*), we evaluated the addition of various proteases, including α-chymotrypsin, to shorten lipolysis time and obtain complete hydrolysis. In the present system, these additions made very little difference. We also evaluated esterase as described by Wahlefeld (*6*), but replaced the hog-liver esterase with microbial esterase. Figure 2 illustrates that hydrolysis of a serum sample with a high triglyceride concentration (6.85 g/L) was essentially complete in 10 min. The color developed in the present procedure followed Beer's law to at least 8.00 g/L, as illustrated in Figure 3. Evidently the test has ample sensitivity.

Comparisons of serum samples run by the present method and by three established triglyceride procedures were made. Figure 4 shows a plot of results determined with the present method as compared to the automated procedure of Biggs et al. (*7*), which has a heptane/isopropanol extraction step, followed by the Hantzsch–Nash reaction. For the Unimeter, the correlation coefficient was 0.993 and the regression equation was $y = 1.02x + 0.04$, where x represents comparison procedure results and y results by the present method. DigiTek results gave a correlation coefficient of 0.992 and the regres-

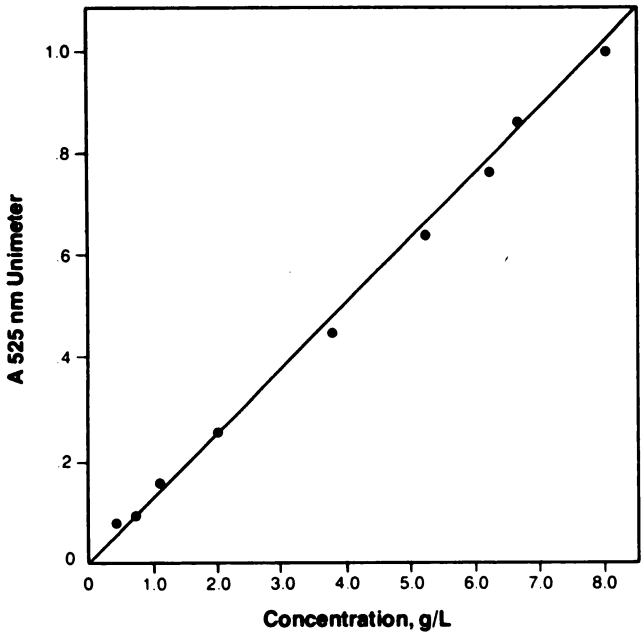

Fig. 3. Linearity of manual method. Serum samples of known concentrations were plotted against absorbance in the Unimeter

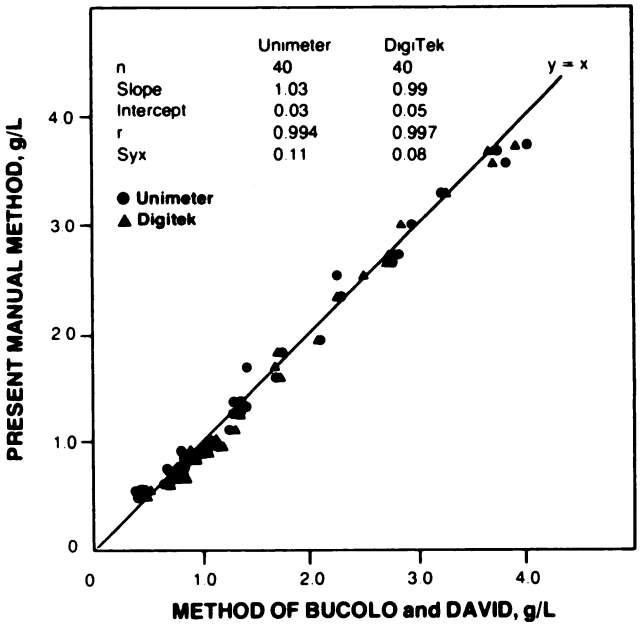

Fig. 5. Correlation of present manual method with the Bucolo and David enzymic method (*5*)

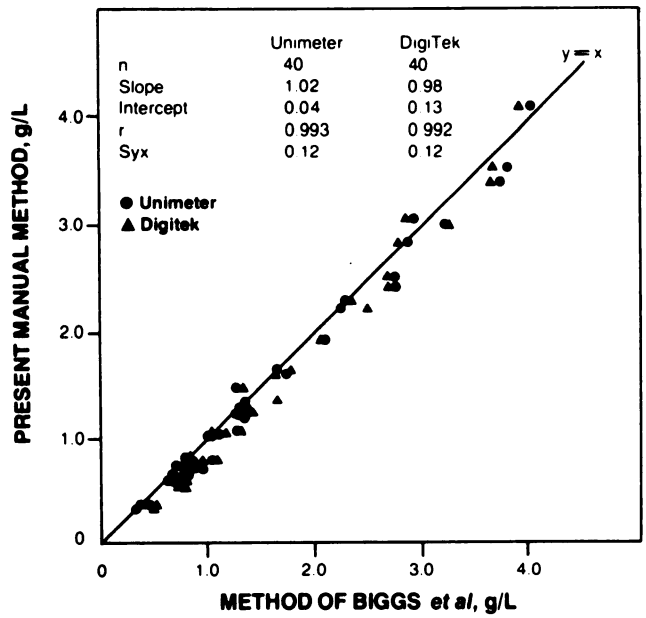

Fig. 4. Correlation of present manual method with that of Biggs et al. (*7*)

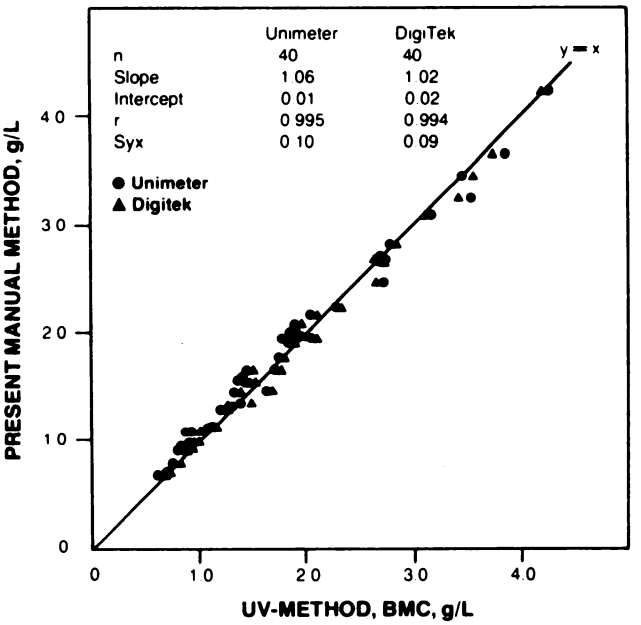

Fig. 6.Correlation of present manual method with Boehringer Mannheim ultraviolet method

sion equation $y = 0.98x + 0.13$, where x represents results as determined by the method of Biggs et al. (*7*).

The second study was made by comparing results of the present method to those of the totally enzymic method of Bucolo and David (*5*). Figure 5 shows plots and linear regression curves for results with the Unimeter and DigiTek vs. the comparison procedure. The Unimeter gave a correlation coefficient of 0.995, and the linear regression equation was $y = 1.03x - 0.03$, where y is Unimeter results and x the results by the comparison procedure. The DigiTek comparison yielded a correlation coefficient of 0.997 and the regression equation, $y = 0.99x + 0.05$, where y represents DigiTek values and x the results by the comparison procedure.

The third comparison was with the Boehringer Mannheim ultraviolet manual kit. Figure 6 shows a plot of Unimeter and DigiTek results vs. those by this procedure. Readings in the Unimeter vs. ultraviolet method gave a correlation coefficient of 0.995 and a linear regression equation $y = 1.06x - 0.01$, where x represents results by ultraviolet procedure and y the Unimeter values. DigiTek results gave a correlation coefficient of 0.994 and a linear regression equation of $y = 1.02x - 0.02$. It can be seen that the present procedure, with results read in the Unimeter and DigiTek, correlated well with those by the three different older procedures.

We assessed analytical recovery by adding glycerol to assayed serum base material and assaying it again. The results (Table 1) indicate that added glycerol was accounted for reasonably well. As shown in Table 2, day-to-day precision for the assay of standard solutions, performed in a physician's office, was quite good as evidenced by low coefficients of variation at both low and high concentrations. Within-run precision, where 30 replicate samples each were run at high and low concentrations, was also quite good for both the Unimeter and DigiTek (Table 3).

Interfering substances. The following common interfering

Table 1. Glycerol Recovery Studies

Actual value	Assay value	Recovery, %
g/L		
	Unimeter	
1.79	1.80	101
3.26	2.06	94
	DigiTek	
0.70	0.77	110
3.55	3.24	91

"Actual" values are the sum of base assay value plus weigh-in value of added glycerol (as triolein). Results are the average of duplicate assays.

Table 2. Day-to-Day Precision of the Manual Method[a]

n	Standard	$\bar{x}$	SD	CV, %
		g/L		
		Unimeter		
10	1.00	1.007	0.030	3.0
10	4.00	4.085	0.086	2.1
		DigiTek		
10	1.00	1.045	0.056	5.4
10	4.00	3.984	0.019	0.5

[a] Triolein standards were measured in duplicate for five days.

Table 3. Within-Run Precision of the Manual Method[a]

n	Standard	$\bar{x}$	SD	CV, %
		g/L		
		Unimeter		
30	1.000	1.000	0.028	2.8
30	5.000	5.030	0.077	1.5
		DigiTek		
30	1.000	1.070	0.039	3.6
30	4.000	3.980	0.090	2.3

[a] Thirty replicate samples with normal or above-normal values were assayed. Samples were aqueous triolein standards.

substances were tested at various concentrations and found to have no significant effect (±6%) at or below the value indicated:

	Concn, mg/L
Hemoglobin	2000
Bilirubin	40
Ascorbic acid	200
Uric acid	200

Interference from turbidity or color is corrected by use of a serum blank.

Reagent stability. The lyophilized reagent is stable for nine months at 2–8 °C. The color reagent and acid stabilizer solutions are stable for longer than a year at room temperature. Reconstituted enzyme reagent is stable for 2 h at room temperature and 8 h at 2–8 °C.

Final color stability. The final developed color of a serum sample containing 2.75 g of triglycerides per liter showed no significant decline in 2 h.

Anticoagulants. Blood was drawn into Vacutainer Tubes (Becton-Dickinson and Co., Rutherford, NJ 07070) containing either no anticoagulant, or (final concentrations) sodium EDTA (1.0 g/L), lithium heparin (14 300 USP units per liter), sodium citrate (380 mg/L), or a mixture of potassium oxalate (2.0 g/L) and sodium fluoride (2.5 g/L). Ten serum samples

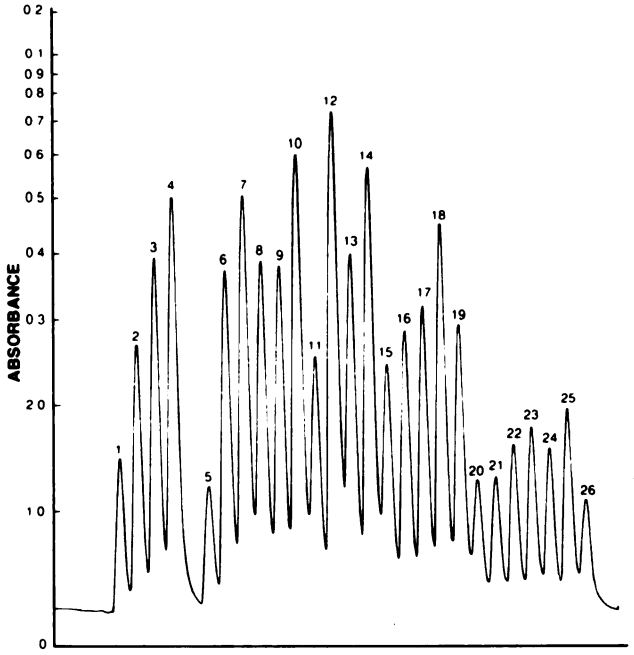

Fig. 7. Stripchart recording of glycerol standards, serum controls and serum samples

Peaks 1, 2, 3, and 4 are glycerol standards 1, 2, 3, and 4 g/L, respectively, as triolein. Peaks 5 and 6 are serum controls Precilip (0.87 g/L) and Precilip P (2.94 g/L), respectively. Peaks 7 through 26 are serum samples

were collected in each type of tube and assayed in duplicate. We found that the presence of oxalate/fluoride and citrate in plasma resulted in values that were 9 to 11% low, while EDTA-treated and heparinized plasma showed no significant difference ($p < 0.05$) from serum values.

Effect of magnesium ions. Magnesium ions are commonly added to enzymic triglycerides tests to activate kinases. We tested several concentrations of magnesium ions, both the chloride and acetate salts. In the present method, concentrations up to 10 mmol/L produced no increase in triglycerides values. Thus we added no exogenous magnesium to the reagent mixture. We find, however, that magnesium ions are required for full recovery of triolein in aqueous standards, and we include 6 mmol/L magnesium acetate in the standard triolein solutions. Magnesium acetate is also included in the enzyme–coenzyme continuous-flow reagent.

Expected values. Data for this study were collected on 54 healthy employees, 32 men and 23 women. Ages ranged from 21 to 48 years for the men and 21 to 41 for the women. All subjects fasted overnight before blood collection. Data for the male population had a gaussian distribution, as determined by the Kolmogorov–Smirnoff test. Distribution was non-gaussian for the female population; therefore, log transformations were used. Expected values were taken as the central 95% of the groups. For men this was (as triolein) 0.05 to 2.19 mmol/L and for women, 0.4 to 1.67 mmol/L. We saw no correlation between age and triglyceride values for males or females, probably because of the small size of the populations used.

Table 4. Precision of the Continuous-Flow Method

	n	$\bar{x}$	SD	CV, %
		g/L		
Day-to-day[a]	10	1.864	0.078	4.20
Within-run[b]	30	2.940	0.022	0.75

[a] Av of duplicates run on five different days.

[b] Results of 30 replicate analyses.

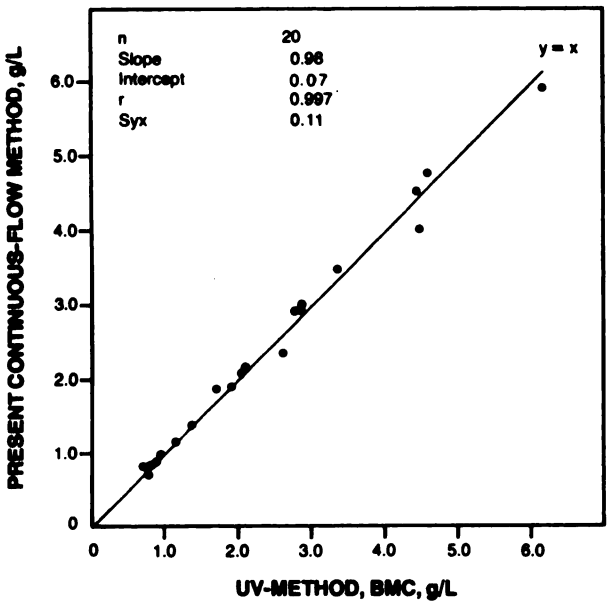

Fig. 8. Correlation of continuous-flow method (using serum results shown in Fig. 7) with Boehringer Mannheim ultraviolet method

Continuous-Flow Method

Figure 7 shows a strip-chart recording of glycerol standards, serum controls, and normal and above-normal serum specimens. Sample interaction amounts to 4% when going from 30 to 0.8 g/L. Baseline drift is about 0.015 *A* in 1 h. Color build-up in the cuvet is easily controlled by periods of flushing reagent lines with acid detergent or hypochlorite solution.

Results of precision studies are shown in Table 4. The precision of the method is acceptable, well within that for other methods.

Results obtained by the Boehringer Mannheim ultraviolet method were compared with results by the present continuous-flow method (Figure 8). The close correlation (0.997), with negligible bias between the methods, is evident.

Discussion

During development of this method we found it important that lipase be screened for ATPase activity. On occasion, poor recovery of triglycerides was related to loss of ATP activity. By process of elimination, we located the ATPase contaminant in the lipase suspension. Lipase obtained from Boehringer Mannheim Biochemicals was found to be free of significant ATPase activity.

When phenazine methosulfate was used as intermediate electron carrier in the color formation sequence, there were always some blanks with high absorbances; the cause was traced to a serum-mediated, nonspecific reduction of INT. This and the light-sensitive nature of phenazine methosulfate led us to use *C. kluyveri* diaphorase as the preferred electron-transfer agent.

The manual procedure described here is simple, easily performed, and dependable, and the reagents are stable. This method eliminates the tedious steps of adsorption and extraction, and is specific because of the use of enzymes. The use of few steps in the procedure facilitates overall precision. Analytical recovery is good.

The continuous-flow method embodies a reasonably simple flow diagram. It requires no dialysis or elevated temperatures. It is essential, however, to clean new tubing throughly before running serum samples. New tubing contains a plasticizer that reacts as glycerol in the enzymic color system. Color build-up in the flow-through cuvet may be minimized or avoided by attention to the cleaning procedure.

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