A Manual Colorimetric Assay of Triglycerides in Serum

Submitters: Homer G. Biggs, John M. Erikson, and Wells R. Moorehead, *Department of Clinical Pathology, Indiana University School of Medicine, Indianapolis, Ind. 46202*

Evaluators: Leila Edwards, *Erie County Laboratory, Buffalo, N. Y. 14215*
Lowell B. Foster, *Patterson Coleman Laboratories, Tampa, Fla. 33622*
Dan Beals and J. C. Wolford, *East Tennessee Baptist Hospital, Knoxville, Tenn. 37901*

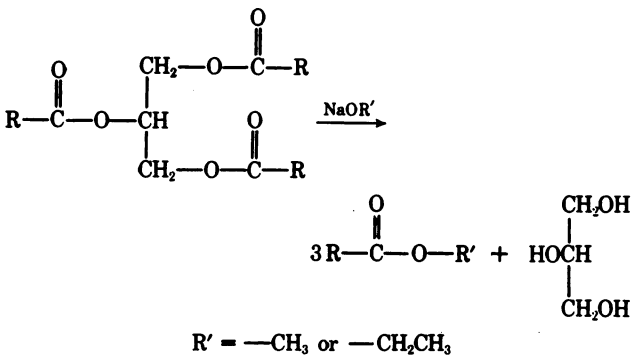
Introduction

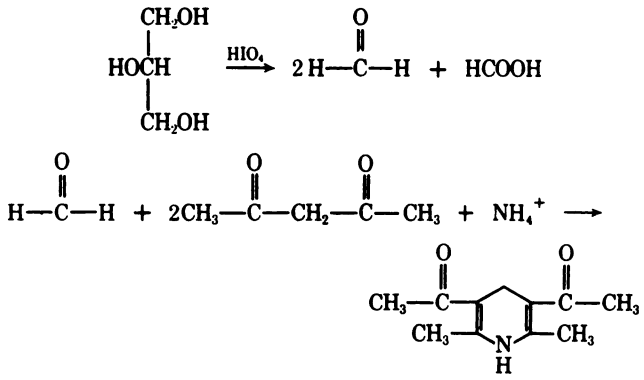
Measurement of serum triglycerides (triacylglycerols) is indispensable for diagnosis of hyperlipemia. Correct diagnosis and treatment depend on triglyceride methodology that is accurate and precise. Many methods involve extraction of the triglycerides, release of glycerin, oxidation of glycerin to yield formaldehyde, and conversion of the formaldehyde to a colored or fluorescent material (*1*). In the procedure of Nash (*2*) 3,5-diacetyl-1,4-dihydrolutidine is formed from formaldehyde and acetylacetone; this procedure has been used extensively in continuous-flow procedures (*3, 4*).

Presented here is a manual colorimetric method based primarily on three reports (*5–7*) in which the Nash sequence of reactions is used. This method is simple, fast, and requires no special equipment. It is precise and accurate and is ideal for use in laboratories that have few requests for triglyceride assays. A continuous-flow procedure has been reported in which the same reactions have been used (*8*).

Principle (*2, 9*)

The sequence of reactions of the extracted triglycerides is:





Materials and Methods

Reagents

Heptane, reagent grade.

Isopropanol, reagent grade.

Sulfuric acid, 40 mmol/liter: Dilute 2.2 ml of concentrated sulfuric acid to volume with water in a liter volumetric flask.

Sodium alkoxide reagent, 28 mmol/liter: To a *dry* 100-ml volumetric flask add 150 mg of sodium methoxide, or 190 mg of sodium ethoxide, dilute to volume with isopropanol, and mix. The solution will be clear if the sodium alkoxide does not contain carbonate. When carbonate is present a turbid solution will result, but this does not seem to alter the usefulness of the reagent (see below). Sodium methoxide dissolves quickly in isopropanol, but sodium ethoxide requires about 40 min to dissolve, even with constant stirring. For best results this reagent should be prepared freshly each day.

Sodium metaperiodate reagent (*7*), 3 mmol/liter: Dissolve 650 mg of sodium metaperiodate and 77 g of anhydrous ammonium acetate in about 800 ml of water in a liter volumetric flask. Add 60 ml of glacial acetic acid and dilute to volume with water. This reagent is stable for six months.

Acetylacetone solution, 73 mmol/liter: Transfer 0.75 ml of acetylacetone (2,4-pentadione) to a 100-ml volumetric flask. Dissolve and dilute to volume with isopropanol. This reagent is stable for six months at room temperature.

Stock triglyceride standard solution, 10.00 g of triolein per liter: Into a 100-ml volumetric flask, weigh 1000.0 mg of triolein. Dilute to volume with

isopropanol and mix well. The C. P. grade triolein we used was obtained from Schwarz/Mann, Division of Becton, Dickinson and Co., Orangeburg, N. Y. 10962.

Working triglyceride standard solutions: In separate 50-ml volumetric flasks, dilute 2.5, 5.0, 10.0, 15.0, and 20.0 ml of the stock triglyceride standard solution to volume with isopropanol and mix. The concentrations of these solutions are 50, 100, 200, 300, and 400 mg/dl, respectively.

Apparatus

The procedure requires screw-topped tubes, 16 × 125 mm with Teflon-lined[1] caps; a vortex-type mixer; a water bath at 60 °C and a photometer to measure absorbances at 400–420 nm. Photometers with flow-through cuvets require a special technique, described under *Photometry*.

Procedure

1. Label a series of screw-cap tubes blank, 50 standard, 100 standard, 200 standard, 300 standard, 400 standard, control, and unknown.
2. To the standard and blank tubes add 0.50 ml of water.
3. To each of the standard tubes add 0.50 ml of the appropriate working triglyceride standard solution (e.g., 50 mg/dl standard into the "50 standard" tube).
4. To the tubes labeled control and unknown add 0.50 ml of the appropriate sample.
5. Add 2.0 ml of heptane to all tubes.
6. To the blank, control, and unknown tubes add 3.5 ml of isopropanol.
7. To each of the standard tubes add 3.0 ml of isopropanol.
8. Add 1.0 ml of 40 mmol/liter H_2SO_4 solution to each tube.
9. Mix the contents of each tube on a vortex-type mixer for about 20 s, then let the tubes stand for about 5 min to allow the phases to separate.
10. Label a series of tubes to correspond to those prepared in step 1 and add 2.0 ml of sodium alkoxide reagent to each.
11. To each of the correspondingly labeled tubes containing sodium alkoxide transfer 0.20 ml of the upper layer from the extraction tubes.
12. Mix well and incubate at 60 °C for 5 min.
13. Add 1.0 ml of the sodium periodate reagent to each tube and mix.
14. Add 1.0 ml of acetylacetone solution to each tube, mix well, and incubate at 60 °C for 20 min.
15. Allow the tubes to cool to room temperature and then measure the absorbance of each solution at 410 ± 10 nm after adjusting the instrument to zero absorbance with the blank solution for single-beam instruments or water for split-beam photometers.
16. Construct a standard curve on linear graph paper by plotting the absorbances of the standard solutions versus their concentrations. The plot should yield a straight line. Concentrations of unknowns are found from this standard curve.

Results

Analytical Variables

Extraction. Nonane has been used for extraction in several procedures (*1, 4–6*). We found that, in this procedure, nonane and heptane give the same results. An advantage of heptane is that its cost is about a tenth that of nonane. Nonane has been used by many workers because it is less volatile. The evaporation rates of both solvents were studied by placing about 3 ml of each in 22 × 45 mm vials and measuring the weight loss in duplicate for several hours at 23 °C. The mean heptane loss was 1.316 mg/min and that of the nonane was 0.135 mg/min. Clearly, heptane evaporates faster than nonane, but this is minimized by using closed tubes. Results were consistent for repeated analyses of heptane extracts (step 9) kept in closed tubes for several days, indicating that loss by evaporation is not a problem.

The effectiveness with which heptane extracts triglycerides was studied by comparing the absorbances of the final colored solutions obtained with triolein standards and corresponding amounts of glycerin. The triolein standards were used as described above and the equivalent amounts of glycerin were dissolved in isopropanol and added to the isopropanol solution of alkoxide as in step 11 of the procedure. Absorbances were the same in both cases, indicating that triglycerides were quantitatively extracted.

The capacity of the heptane extraction was evaluated by analyzing a serum specimen containing 7000 mg of triglycerides per deciliter according to the procedure. Dilutions of the specimen before extraction and equivalent dilutions of the heptane extract from the undiluted specimen provided the same results. This indicates that heptane quantitatively extracts triglycerides with concentrations as great as 70 g/liter.

Transesterification. Either sodium methoxide (*5*) or sodium ethoxide (*1*) can be used, and both provide similar analytical values.

Some lots of sodium methoxide dissolve completely in isopropanol, but others contain insoluble material.[2] Either completely or incompletely soluble sodium methoxide gives the same results, as might be expected because of the large excess used. Sodium ethoxide dissolves slowly in isopropanol, but no insoluble material has been observed. Ethanol is a better solvent than isopropanol for sodium ethoxide, but the difficulty in obtaining and maintaining anhydrous ethanol discourages its use. An additional difficulty is that heptane and ethanol form a two-phase system in step 11.

[1] Trade name for polytetrafluoroethylene (E. I. du Pont de Nemours and Co., Wilmington, Del. 19898).

[2] This insoluble material was collected by centrifugation and when added to dilute hydrochloric acid it effervesced, indicating that it was a carbonate.

Table 1. Added Triolein Analytically Accounted for in Serum[a]

Added	Observed	Calculated	Recovery, %
Triglycerides, mg/dl, as triolein			
0	62		
50	113	112	102
100	167	162	105
200	259	262	98

[a] Values are means of triplicate assays.

Table 2. Precision of the Present Method (Lutidine) and the Modified Method of Van Handel and Zilversmit (*11*) (Chromotropic)

Method	n	$\bar{x}$	SD	Range
		mg/dl		
Lutidine				
Methoxide	21	97.6	3.59	94–106
Ethoxide	26	100	5.11	92–107
Chromotropic acid	22	107	5.27	97–114

Table 3. Results by the Proposed Procedure (Lutidine) and a Modification of the Van Handel and Zilversmit Method (*11*) (Chromotropic) for Two Different Groups of Sera Chosen at Random

Method	n	$\bar{x}$	SD	Range
		mg/dl		
Lutidine-methoxide	30	126	54.1	55–240
Chromotropic acid	30	138	37.7	52–205
Lutidine-ethoxide	30	160	73.9	60–340
Chromotropic acid	30	164	64.7	70–314

The rates of transesterification with standard solutions and serum samples were studied by measuring the final absorbances after allowing the reaction to proceed for various times. Absorbances were maximum within 2 min in all cases at 60 °C, but longer than 15 min was required at 25 °C. The transesterification was shown to be quantitative in the experiments described above in which glycerin gave results identical with those for equivalent triolein standards.

The use of sodium hydroxide in isopropanol as recommended by Giegel et al. (*6*) gave very low absorbance values in all cases, suggesting that only small amounts of glycerin were liberated.

Oxidation with periodate. The oxidation of glycerin by periodate was allowed to proceed for various periods up to 20 min at room temperature and 60 °C. The same results were obtained in all instances, which means that the reaction is very fast or that it occurs during the next step, or both.

When the periodate reagent and acetylacetone solution are combined before use, the resulting solution quickly becomes pale yellow. Spectral analysis shows that this yellow color cannot be attributed to formation of the lutidine derivative obtained from formaldehyde (*9*). If the combined solution is used in the assay procedure, after standing at room temperature for 90 min, all samples have absorbances no greater than the blank, which indicates that the periodate has been consumed in a reaction with acetylacetone. The reaction of periodate with compounds containing active methylene groups is well documented (*10*).

In some methods (*1, 11*) arsenite is used to consume excess periodate. Whether or not we added arsenite in this procedure, the results were the same.

Formation of lutidine derivative. At 60 °C this reaction produced maximum absorbance at 20 min. On varying the pH of the ammonium acetate solution from 5 to 7.8 by adding ammonia or acetic acid, we found that maximum color was produced at pH 6, but sufficient color was obtained throughout the range studied that any pH between 5 and 7.8 can be used. The solution used to develop color in the present procedure, prepared as described under *Reagents,* has a pH of 6.0–6.2.

Photometry. The 3,5-diacetyl-1,4-dihydrolutidine has a symmetrical absorbance spectrum, with a maximum at 410 nm and a molar absorptivity of 7000 cm^2 mol^{-1} (*9*). The absorbance of solutions of the compound is affected by temperature, in that cooling results in greater absorbance, probably because of increased dimer formation (*12*). When solution temperature is increased, absorbance is decreased. If a split-beam photometer without a thermostatted sample compartment is used, the absorbance of the blank, standards, controls, and unknowns are measured vs. water as a reference. This is necessary because the absorbance of the blank solution also changes with temperature.

Photometers with flow-through cuvets, such as the Gilford 300 N (Gilford Instrument Laboratories, Inc., Oberlin, Ohio 44074) would seem to be ideal for use in this procedure. However, if such an instrument has previously been used to measure absorbance of aqueous solutions, it is necessary to purge the flow-through system with about 30 ml of a solution consisting of three volumes of isopropanol and one volume of water before stable readings can be obtained.

Recovery. Recovery was measured by adding 0.50 ml of the 50, 100, and 200 mg/dl working standard solutions to tubes in triplicate and evaporating at 60 °C. To the residue in each tube, 0.50 ml of pooled serum was added and the resulting solutions were analyzed for triglycerides by the above procedure. Table 1 shows the results.

Precision and comparative studies. The same pooled serum sample was analyzed by the proposed procedure with the use of both sodium methoxide and sodium ethoxide and by a modification of the method of Van Handel and Zilversmit (*11*). The modification consisted of the use of 2 g of silicic acid, previously heated at 115 °C for 12 h, in place of zeolite (*13, 14*). Results are summarized in Table 2.

Table 4. Summary of Results of Analyses of Serum Pools[a]

Evaluators:	A	B	C	D
	Pool 1			
n	20	20	20	16
$\bar{x}$	127	129	139	132
SD	6.13	2.87	7.76	6.65
Range	113–137	125–135	129–154	118–141
	Pool 2			
n	20	20	20	16
$\bar{x}$	171	172	181	177
SD	7.38	2.72	10.1	8.73
Range	158–190	168–179	158–202	163–190

[a] Results are in mg/dl, as triolein.

Analyses of variance (*15*) for these data indicated that the results obtained by the two methods are different and that the results also differed significantly according to whether methoxide or ethoxide was used.

The same approach was used to compare results obtained by analyses of serum samples selected without conscious bias (Table 3). Application of the paired *t*-test (*15*) to these two sets of data showed no significant difference between the results obtained by using the modified method of Van Handel and Zilversmit and by the present method with either methoxide or ethoxide.

Evaluation. The Evaluators assayed serum samples and compared results with those obtained with other methods.

The sodium metaperiodate reagent and the acetylacetone solution were initially reported to be stable for only a few days by two of the Evaluators. In both instances the instability was found to be due to contaminated glassware; subsequent use of acid-washed containers resulted in the stability described above.

To determine the accuracy and precision of the method, each Evaluator analyzed samples from two serum pools provided by the Lipid Standardization Laboratory of the Center for Disease Control, Atlanta, Georgia. Their results are summarized in Table 4. Data on control samples obtained by the Evaluators showed similar precisions. The values assigned by the Lipid Standardization Laboratory for pools 1 and 2, as established by an unpublished modification of the procedure of Carlson (*16*), were 138 ± 9.7, SD < 8 and 181 ± 10.6, SD < 9.7 as mg/dl triolein, respectively.

Two Evaluators compared analytical values obtained with the present method with those from two continuous-flow procedures. These data are summarized in Table 5. The method used by Evaluator B (*17*) gave results that are not significantly different, $P < 0.01$. For these same data the correlation coefficient is 0.926 and the regression equation is $y = 0.945\ x + 7.99$, where y represents continuous flow values and x represents values from the present procedure. Results obtained by Evaluator D by essentially the same method (*18*) were different for the two procedures, even though the same standard solutions were used for both. The differences are most probably attributable to the fact that the values produced by the latter continuous-flow system did not include blank corrections.

Evaluator C compared the present method with one in which alumina (*7*) is used to analyze serum samples selected at random and obtained the data shown in Table 6. The correlation coefficient for these data is 0.987 and the regression equation is $y = 0.991\ x - 7.22$, where y represents results obtained with alumina and x represents those by the present method. Analysis of variance showed that the results from the two methods are not significantly different, $P < 0.01$.

Each Evaluator supplied his or her own chemicals, prepared his or her own reagents, and obtained the triolein used for the standard solutions from different sources. The serum pools supplied by the Lipid Standardization Laboratory were received by the Evaluators over a period of eight months. Any or all of these factors may explain the difference in the data shown in Tables 4 and 5.

Discussion

The method described here is well suited for measurement of triglycerides in small batches. Thirty determinations can be performed in less than 3 h.

Evidence is provided demonstrating that heptane can be successfully used in place of the more expensive nonane and that either methoxide or ethoxide

Table 5. Comparison of Results by the Present Method (Manual) and by Continuous-Flow Procedures (Conflo)[a]

	Evaluator B[b]		Evaluator D[b]			
			Pool 1		Pool 2	
	Manual	Conflo	Manual	Conflo	Manual	Conflo
n	77	77	16	16	16	16
$\bar{x}$	135	136	132	144	177	188
SD	50.5	51.5	6.65	8.59	8.73	12.2
Range	54–275	47–262	118–141	130–160	163–191	161–212

[a] Results are in mg/dl, as triolein.
[b] Evaluator B assayed serum samples selected at random and Evaluator D analyzed samples of pools 1 and 2 (see Table 4).

Table 6. Results Obtained by a Method in Which Alumina Is Used (7) and the Method Described Here

	Alumina	Present method
	Triglycerides, mg/dl, as triolein	
n	45	45
$\bar{x}$	175	169
SD	106	107
Range	70–575	60–560

may be used for transesterification. Ethoxide has the disadvantage of being slower to dissolve in isopropanol.

The chromogenic reaction time has been increased to allow full color development.

The results of the precision study (Table 2) indicate that the proposed method with the use of methoxide or ethoxide and the modified Van Handel and Zilversmit procedure, all yield results that are statistically different. However, these differences are probably not meaningful in clinical applications, in that significant differences were not found in assaying serum samples selected at random (Table 3).

Clinical Interpretation

Normal concentrations of serum triglycerides for fasting individuals are reported to be 30–135 mg/dl (*11, 14*).

References

1. Soloni, F. G., Simplified manual micro-method for determination of serum triglycerides. *Clin. Chem.* 17, 529–534 (1971).
2. Nash, T., The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochemistry* 55, 416–421 (1953).
3. Noble, R. P., and Campbell, F. M., Improved accuracy in automated fluorometric determination of plasma triglycerides. *Clin. Chem.* 16, 166–170 (1970).
4. Royer, M. E., and Ko, H., A simplified semiautomated assay of plasma triglycerides. *Anal. Biochem.* 29, 405–416 (1969).
5. Levy, A. L., and Keyloun, C., A colorimetric assay for triglycerides: Automated and manual. *Clin. Chem.* 17, 640 (1971).
6. Giegel, J., Soloni, F., Trinidad, E., et al., Manual and semiautomated procedures for the measurement of triglycerides. *Clin. Chem.* 18, 693 (1972). Abstract.
7. Foster, L. B., and Dunn, R. T., Stable reagents for determination of serum triglycerides by a colorimetric Hantzsch condensation method. *Clin. Chem.* 19, 338–340 (1973).
8. Holub, W. R., Rapid, simplified continuous-flow colorimetry of serum triglycerides. *Clin. Chem.* 19, 1391–1393 (1973).
9. Erikson, J., and Biggs, H. G., 1,4-Dihydro-3,5-diacetyl lutidine. A basis for triglyceride determination in biological samples. *J. Chem. Educ.* 50, 631–632 (1973).
10. Fleury, P. F., Quelques aspects non classiques de l'action oxydante de l'acide periodique sur les composés organiques. *Bull. Soc. Chim. Fr.* 1126–1135 (1955).
11. Van Handel, E., and Zilversmit, D. B., Micromethod for the direct determination of serum triglycerides. *J. Lab. Clin. Med.* 50, 152–157 (1957).
12. Belman, S., The fluorometric determination of formaldehyde. *Anal. Chim. Acta* 29, 120–126 (1963).
13. Galletti, F., An improved colorimetric micro-method for the determination of serum triglycerides. *Clin. Chim. Acta* 15, 184–186 (1967).
14. Carlson, L. A., and Wadstrom, L. B., Determination of glycerides in blood serum. *Clin. Chim. Acta* 4, 197–205 (1959).
15. Sokal, R. R., and Rohlf, J. F., *Biometry*, W. H. Freeman and Co., San Francisco, Calif., 1969, pp 300–340.
16. Carlson, L. A., Determination of serum triglycerides. *J. Atheroscler. Res.* 3, 334–336 (1963).
17. Edwards, L., Falkowski, C., and Chilcote, M. E., Semiautomated fluorometric measurement of triglycerides. *Stand. Methods Clin. Chem.* 7, 69–78 (1972).
18. Kessler, G., and Lederer, H., Fluorometric measurement of triglycerides. In *Automation in Analytical Chemistry, Technicon Symposia 1965;* L. T. Skeggs, Jr., et al. Eds. Mediad Inc., New York, N. Y., 1966, pp 341–344.

No reprints of this paper will be available