Triglyceride Measurements: a Review of Methods and Interferences

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The National Cholesterol Education Program has emphasized the need to identify individuals at risk for coronary artery disease (CAD). Because increased triglycerides may be a risk factor for CAD and because triglycerides are used to estimate concentrations of low-density lipoprotein (LDL) cholesterol, which has definitely been shown to be a risk factor for CAD, it is important that reliable results be obtained. Many methods are available for measuring triglyceride concentrations in serum or plasma, but there is no definitive method that confirms the reliability of any of these procedures. Accuracy and precision guidelines are extremely difficult to determine, owing to broad biological variability both within and among individuals. Here, we review the major triglyceride quantification methods in the literature, some of the potential interference problems, and the limitations regarding standardization that should be addressed when establishing such guidelines.

Assessment of the Needs for Accuracy and Precision in Triglyceride Quantification

The National Cholesterol Education Program Adult Treatment Panel has recommended that triglyceride concentrations <2.82 mmol/L (<250 mg/dL) be classified as desirable, 2.82–5.65 mmol/L (250–500 mg/dL) as borderline, and >5.65 mmol/L (>500 mg/dL) as distinctly abnormal (1). These ranges are relatively broad, but it has been difficult to assign specific concentrations at which medical intervention is recommended because of controversy regarding the role of triglycerides in coronary artery disease (CAD). The association of triglycerides with CAD has been variable in population studies that evaluated triglyceride concentration as an independent risk factor (2, 3).

If triglyceride concentrations in serum were not considered to be an important risk factor for CAD, if only extremely high concentrations were felt to be important in terms of diagnostic value, or if low-density lipoprotein (LDL) cholesterol concentrations could easily be determined independently of triglycerides, then efforts toward standardization of laboratory methodology might not be necessary. If, however, triglyceride concentrations do indicate some degree of risk, even if only in a certain subset of individuals (4, 5), and if triglyceride concentrations continue to be a necessary tool for estimating LDL cholesterol (6–8), then it is important to eliminate as much assay variability as possible. In the laboratory, this means reduction of inaccuracy and imprecision to values comparable with those for cholesterol. In patient management this means setting fasting times and pretesting dietary guidelines to minimize biological variation. It also means careful interpretation of laboratory results (6, 9, 10).

Defining the reliability required for any triglyceride method being considered for laboratory standardization is difficult, because biological variability of triglyceride concentrations differs greatly among individuals (11, 12). Our studies show marked differences among subjects receiving the same amount of fat per kilogram of body weight (12). In addition, some individuals show variability in their fasting triglyceride concentrations when sampled on different days within a short period (unpublished data, J.R.M).

The ubiquitous nature of glycerol interference must be taken into account. Glycerol may derive from the sample itself (13, 14) or from medications given to the patient (15–18), or samples, sample cups, tubing, etc., used in the laboratory may contain traces of lubricants that cause glycerol interference (20). The amount of glycerol present in skin-care products is not negligible (21, 22). A sample probe in a random-access analyzer aspirating 5 µL of sample, equivalent to an average of 0.5 ng of free glycerol in the assay mixture, is conceivably delivering extraneous glycerol or glycol exceeding this amount into the system.

As a first step toward a critical evaluation of triglyceride determinations we have reviewed available methodology, interferences, and standardization limitations.

History and Principal Methods

Early analytical methods for determining triglycerides involved titrimetric procedures of total lipids. After extraction with organic solvents, extracts were saponified and then back-titrated to assess the amount of alkali that was not neutralized by the released fatty acids. Later methods quantified the glycerol that was formed (see Table 1), as first described by Van Handel and Zilversmit (23), who extracted the lipids with chloroform, removed phospholipids by adsorption on silicic acid, and then saponified the glyceride esters to glycerol. They determined glycerol by oxidation with periodate, using a colorimetric measurement of the product from the reaction of formaldehyde and chromotropic acid in sulfuric acid. Although some mono- and diglycerides of fatty acids are present in serum (~3%), results were calculated as "triglycerides." Subsequently, the term triglycerides was commonly used for the total of free and protein-bound glyceride esters.

Kessler and Lederer (24) adapted this method to the AutoAnalyzer® (Technicon, Tarrytown, NY). They used isopropanol extracts of serum, added zeolite/Lloyd's reagent to adsorb phospholipids and other interferences, and then introduced an on-line saponification step. The glycerol reacted with periodate and produced formaldehyde, which

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3 Nonstandard abbreviations: CAD, coronary artery disease; CDC, Centers for Disease Control; BSA, bovine serum albumin; and GPO, glycerol-3-phosphate oxidase.

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was measured fluorometrically after a Hantzsch-type condensation with diacetylacetone and ammonia.

Carlson and Wadström's modification (25) of the above methods became the procedure used by the Lipid Research Clinics population studies. The assay was standardized by the Centers for Disease Control (CDC)—National Heart, Lung, and Blood Institute Lipid Standardization Program. High accuracy and good precision make this procedure an excellent reference, but only CDC calibrators, or a secondary calibration material that has been analyzed by the CDC method, can be used if interlaboratory results are to be compared. The various analytical steps require a high degree of technical competence.

The measurement of glycerol by an enzymatic spectrophotometric procedure was introduced by Wieland (26) after the enzyme glycerol kinase (ATP:glycerol 3-phosphotransferase; EC 2.7.1.30) became commercially available. Eggstein and Kreutz (27) used the reverse enzymatic reaction, after a chemical saponification step, to assay neutral fats. Both methods are based on the oxidation/reduction of NADH/NAD+, and the corresponding change in absorbance. Chemical saponification is usually performed in an alkaline medium, with subsequent neutralization by perchlorate or magnesium sulfate (42), whereby most proteins are inactivated. Phospholipids (42) also hydrolyzed, which necessitates adsorption on zeolite if the Wieland method is used, because the subsequent step quantifies the glycerol 3-phosphate produced. A semiautomated enzymatic analysis, introduced by Klotzsch et al. (28), added a colorimetric indicator reaction to the Wieland principle and introduced the method to the AutoAnalyzer.

The colorimetric reaction took place after samples were subjected to the adsorption step with zeolite, before on-line saponification with alcoholic KOH. This step eliminated interference from endogenous alkaline phosphatase (EC 3.1.3.1) and lactate dehydrogenase (EC 1.1.1.27). However, the technique did not save much time by automation and was later improved by Stavropoulos and Crouch (29-31). They avoided extraction and adsorption by combining alcoholic base saponification with the precipitation technique of Schmidt and von Dahl (42) using magnesium salts. Chernick (43) claimed that the use of tetraethylammonium hydroxide hydrolyzed triglycerides without cleavage of phospholipids.

Bucolo and David (32-34) substantially improved the ultraviolet test of Eggstein and Kreutz by using microbial lipase together with a protease, instead of chemical hydrolysis. This procedure became the most popular ultraviolet technique for determining triglycerides. It could be used with most manual and automated laboratory instruments and received widespread recognition in clinical laboratories. Free glycerol could be blanked by a separate assay with use of reagents without lipase or glycerol kinase. The colorimetric method of Klotzsch et al. (28), in conjunction with lipase instead of chemical hydrolysis, was published by Megraw et al. (35) and became a convenient technique for use with centrifugal analyzers and other medium-sized instruments (44). The enzyme glycerol dehydrogenase (glycerol:NAD+ 2-oxidoreductase, EC 1.1.1.6) was introduced by Hagen and Hagen (45) in 1962, when it was suggested for determining glycerol. Bowie and Gochman (36) extended this principle to triglyceride assays. Rautela et al. (37) and Grossman et al. (46) modified it to be applied to the aoa (Du Pont Instruments, Wilmington, DE) and Centrifichem (Baker Instruments, Evansville, WI).

Because the equilibrium of the enzymatic reaction is unfavorable to the production of dihydroxyacetonate, a kinetic (pseudo-first-order) mode was chosen to overcome the
time constraints of the instruments. Sigiuera et al. (47) modified the reaction for a colorimetric measurement in combination with a tetrazolium salt; Winartasaputra et al. (38) introduced the reaction for fluorometric assays.

During the following years, many additional modifications were made to apply the enzymatic methods to the newly developed automated laboratory instrumentation (38, 48–53). When glycerol oxidase (glycerol:oxidoreductase; EC not assigned) became available, a new approach was presented. Terada et al. (39, 54) and Gaulh et al. (55) combined the oxidase reaction with the reaction sequence of Trinder (40), which coupled peroxidase to a phenol derivative and 4-aminophenylene. Glycerol oxidase, however, is nonspecific and reacts with many glycols, such as ethylene glycol and propylene glycol (16). Greater specificity was achieved by oxidative phosphorylation. Fossati and Prencipe (41) and McGowan et al. (56) used the enzyme glycerol-3-phosphate oxidase (GPO; sn-glycerol-3-phosphate:oxidoreductase, EC 1.1.3.21) in conjunction with a Trinder-type reaction. This method continued to be modified as its application to new instruments nurtured innovative changes (57).

Other methods have been developed, such as bioluminescence assays (58), but have not become popular, either because of improper blanking that results in poor precision or because commercial availability has been limited. An HPLC procedure based on adsorption chromatography and refractive index detection of triglycerides, presented by Ambrose and Smith (59), appears to be highly specific because it does not include di- or monoglycerides in the measurements. The method has its constraints, owing to the instrumentation and the time required, but may be worth considering as a reference procedure.

Factors Affecting Test Results

Problems with Chemical Saponification Methods

The hydrolysis of triglycerides to glycerol and fatty acids can be achieved by chemical saponification, in which the sample is treated with alcoholic KOH or NaOH at high temperatures and subsequently neutralized with magnesium sulfate. Enzymes in the samples are inactivated and fatty acids are coprecipitated as magnesium salts. After centrifugation, the clear supernate is used for the assay (42). Ascorbate and other unstable compounds, such as conjugated bilirubin, are decreased or eliminated simply by controlling the time in which the sample is subjected to heat and alkali.

However, glucose, lactate, hydroxybutyrate, and residual alcohol remain candidates for potential interference. Phospholipids are hydrolyzed to glycerophosphates, which are stable in alkaline solutions. Only those methods that measure the formation of glycerol 3-phosphate require their removal by adsorption, unless the procedures of Stavropoulos and Crouch (29) or Chernick (43) are used. Technical problems are mainly related to the high temperature, the caustic nature of the reagents involved, and the length of time. The temperature inside the sample tubes (not the water bath) must be the same for calibrator and blank; the length of time required varies with the temperature and must be equal for each sample within a series. The stoppers of the sample tubes must be vented to avoid breakage, but evaporation of the alcohol must be prevented. The subsequent precipitation of Mg(OH)\textsubscript{2} causes volume displacement of the liquid from which an aliquot is taken for further testing. The reagent blank must include the saponification step because preparations of KOH are not always free of glycerol. The volume of magnesium sulfate needed to neutralize the alcoholic base in aqueous solutions (reagent blanks and calibrators) is different for proteinaceous samples. To keep all volumes the same, it is advisable to add fatty acid-free bovine serum albumin (BSA), 60 mg/mL, to blanks and calibrators.

Problems with Enzymatic Cleavage of Triglycerides

Choosing the best lipase (triacylglycerol acylhydrolase; EC 3.1.1.3) requires selection from a wide variety of commercially available preparations. We have found that the source of lipase determines the rate and degree of lipolysis: lipase from Chromobacterium sp. acts with higher specificity toward the glycerides of long-chain fatty acids, whereas the lipase from Pseudomonas sp. hydrolyzes the short-chain esters much faster. Microbial lipases on the market have distinct properties, and use of several sources may yield the best results for a specific instrument application (60–62).

Lipases require emulsified substrates, rather than true solutions, which explains the different rates of hydrolysis for tri-, di-, and monoglycerides. Many substances can act as emulsifiers, but lipase preparations that are inhibited by specific surfactants such as Triton\textsuperscript{a} (Rohm and Haas, Philadelphia, PA) are offered commercially. Other lipase preparations are markedly activated by such non-ionic surfactants, by bile acids, and by lauramide or cocoamide esters. Furthermore, the effects of mono- and divalent ions need to be tested because various lipases depend on either potassium, calcium, or magnesium ions for optimal performance. Instead of surfactants, BSA has been suggested as an emulsifier for lipid micelles (32).

The presence of a colipase is not required for these assays. Colipase, a pancreatic protein, negates the inhibition of pancreatic lipase by bile acids (63). In contrast, the microbial lipases used for triglyceride determinations are not inhibited, but activated, by bile acids.

Esterase in the lipase mixture (for instance, carboxylesterase, EC 3.1.1.1; or triacylglycerol-protein acylhydrolase, EC 3.1.1.34) increases the rate of hydrolysis (64, 65). Microbial lipases generally exhibit both lipolytic and hydrolytic activity, which are stimulated by proteases, e.g., chymotrypsin (EC 3.4.21.1), as demonstrated by Bucolo and David (32). The presence of proteases can have a negative effect on the stability of a reagent solution. Digesting proteases can attack other enzymes in the assay system, leading to poor stability of glycerol kinase and pyruvate kinase at pH ≥7.0.

Interference from Extraneous Sources: Reagents

Manufacturers of enzymes and biochemical substrates offer products of refined purity (66); however, they cannot predict the use of a particular reagent component in a specific assay system. Although a listed contamination of 0.01% may not appear to be significant, its effects may accumulate when three or four enzymes are used in a reaction sequence. The following interferences (in alphabetical order) have been detected in our laboratory and can be present in commercially available materials.

Adenylate kinase: ATP-AMP phosphotransferase (EC 2.7.4.3) can be a contaminant in glycerol kinase (EC 2.7.1.30), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), and glycerol-3-phosphate dehydroge-
nase (NAD⁺) (EC 1.1.1.8). Depending on the assay conditions, interference results from the formation of ADP, which reacts with the AMP present in ATP. (AMP is a normal contaminant in ATP.)

Adenosine 5-diphosphate: ADP is present in ATP, especially if reagent solutions are stored or if ATP has been processed with other reagents such as in a lyophilized product. ADP can also be generated by the action of alkaline phosphatase at pH 7.0–9.0. Its effect can be negated with a reagent blank; but consumption of NADH during pre-incubation reduces the dynamic range of the reaction, and samples with high concentrations of alkaline phosphatase may no longer give results that are in the linear range of the assay, in the presence of this contaminant.

Alcohol dehydrogenase: Alcohol:NAD⁺ oxidoreductase (EC 1.1.1.1) is a likely contaminant of enzymes isolated from yeast. Its presence in glycerol kinase from Candida sp. can produce a side reaction with NAD⁺ and alcohol that is carried over from chemical saponification or introduced as a contaminant in NAD⁺. If blanking is performed without glycerol kinase, the total assay will produce falsely high (NAD⁺-coupled reactions) or falsely low (NADH-dependent reactions) values.

Alkaline phosphatase: Orthophosphoric-monoester phosphohydrolase or nonspecific phosphatases are the most significant and severe interferents in triglyceride assays, regardless of methodology. Alkaline phosphatase activity is found primarily in lipase, but some contamination in other enzymes is possible. Alkaline phosphatase reacts with glycerophosphate, dihydroxyacetone phosphate, phosphoenolpyruvate, and ATP at neutral or alkaline pH and can be inhibited by EDTA and phosphates (67).

Catalase: Hydrogen-peroxide-hydrogen-peroxide oxidoreductase (EC 1.11.1.6) is a contaminant present in oxidases and will, therefore, affect assays based on the Trinder sequence, where glycerol oxidase or glycophosphatase oxidase is utilized. Because catalase competes with peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) for H₂O₂, interference is less when test conditions are optimal for peroxidase, with regard to pH and hydrogen donor.

Ethylene glycol: Propylene glycol and other glycols react nonspecifically with glycerol oxidase, which is used in the method developed by Terada et al. (39), and give falsely high values. Ethylene glycol is frequently used to stabilize control sera.

Hexokinase: ATP:p-hexose 6-phosphotransferase (EC 2.7.1.1) can be present in yeast enzymes, especially in glycerol kinase from Candida sp. Its presence results in falsely high values for the ultraviolet method with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase, because hexokinase will generate additional ADP from serum glucose and ATP.

Lactate dehydrogenase: (S)-Lactate:NAD⁺ oxidoreductase, a trace contaminant in most enzymes, will interfere with the INT/diaphorase method if present in glycerol-3-phosphate dehydrogenase, or with the direct glycerol dehydrogenase method with NAD⁺, because it reacts at a slow rate with lactate and hydroxybutyrate in serum (68). The interference caused by this reaction is masked if the rate techniques measure apparent pseudo-first-order kinetics.

NADH-oxidase: "NADH-oxidase" refers to nonspecific activities in the presence of coenzyme. NADH, for instance, can be oxidized at a slow rate without any apparent substrate. NADH-oxidase, which is often listed in manufacturers' certificates of analysis, is always associated with dehydrogenases and need not be a separate protein. Although very low rates may not contribute to a significant bias, accumulation of several substances that oxidize NADH should be avoided.

Oxidases: Oxidases, namely glucose oxidase (EC 1.1.1.4), lactate oxidase (EC 1.1.1.24), and pyruvate oxidase (EC 1.2.3.3), must be absent in preparations of GPO to avoid interference with the Trinder reaction. If the reaction is initiated with glycerol kinase or lipase, the normal pre-incubation time of approximately 5 min with GPO may not be sufficient to remove endogenous glucose and lactic acid that generate additional H₂O₂.

Peroxidase: Peroxidase reacts nonspecifically with NADH, and the rate is increased by ketoadiogenic substances, e.g., ketobutyrate (unpublished data, S.G.K.). In a single-cuvet sequential determination of triglycerides and cholesterol (69, 70), a significant side reaction can be seen in samples from patients with ketoadiogenic, because the NADH-coupled triglyceride reaction mixture contains the peroxidase necessary for the Trinder-type cholesterol assay.

Peroxides: Peroxides are frequently found in surfactants and contribute to the pink color that forms during storage of reagents for assays based on the Trinder-type reaction, but they do not interfere if the reagents are blanked correctly.

Phospholipase: Phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3) and phosphodiesterase (sn-glycerol-3-phosphorylcholine glycerophosphatehydrolase, EC 3.1.4.2) in lipase preparations act on phospholipids and, in conjuction with peroxidase, result in falsely high values for incorrectly blanked triglyceride assay. Mainly diglycerides are generated from phosphatidylcholine; therefore, it is advisable to test the lipase mixture for nonspecific activity before use.

Pyruvate: Pyruvate is a breakdown product of phosphoenolpyruvate and can be present in the reaction mixture. It contributes to NADH consumption before the start of the glycerol reaction; thus the amount of NADH present may not be sufficient to assure the range of linearity.

Quantities of enzyme: Quantities of enzyme used for the assays are of great importance. All contaminants that may be present in commercial materials can be additive. Use of too little enzyme, or too much, increases the possibility for failure. Enzymes stored for a long period may lose activity. If the loss of activity is compensated for by the use of increased amounts of raw material, the contaminants may be correspondingly increased. Contaminants can be more stable than host enzymes.

Interference from Endogenous Substances in Serum

As stated previously, the routine determination of triglycerides without extraction or deproteinization may trigger endogenous substances that cause interference in the reaction. Interference may not always cause a significant bias, but this potential problem should be anticipated when processing patients' samples.

Alcohol/Alcohol dehydrogenase, lactate/lactate dehydrogenase, and glycerol/glycerol dehydrogenase: These enzymes and their substrates are often increased in serum or plasma specimens from patients with liver diseases or with myocardial infarction/ischemia (71). They can cause a slow steady increase in absorbance with all methods that utilize NAD⁺ and alkaline conditions, because the reaction is not completed within the normal pre-incubation time. Interfer-
ence can be negated by analyzing serum blanks at the same time and temperature.

Alkaline phosphatase: Alkaline phosphatase significantly interferes with all methods, whether as a contaminant of the reagent preparations, as previously discussed, or as increased concentrations in the specimens. Specimens with increased concentrations of this enzyme require special attention (e.g., pediatric samples). Triglyceride results may become apparently increased owing to hydrolysis of phosphoenolpyruvate in the ultraviolet assay involving lactate dehydrogenase and pyruvate kinase, or to hydrolysis of glycerol phosphate or dihydroxyacetone phosphate in other procedures. The degree of interference depends on individual assay conditions and whether serum blanks have been included. Although alkaline phosphatase activity is optimally measured at alkaline pH, its activity at pH 7.0 is significant (67). As mentioned previously, phosphate ions and EDTA inhibit the activity of alkaline phosphatase and are, therefore, often included in commercial formulations.

Ascorbic acid: Ascorbic acid interferes with methods involving 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride with diaphorase (EC 1.6.4.3), by decolorizing the formazan, and in the Trinder method, by reacting nonspecifically as a hydrogen donor in the peroxidase-catalyzed reaction. Ascorbate oxidase (l-ascorbate:oxygen oxidoreductase, EC 1.10.3.3) added to the reagents effectively removes this interference, provided the enzyme is pure and does not generate H2O2 (72). (Contrary to most oxidases, ascorbate oxidase generates H2O, not H2O2.)

Ascorbate oxidase need not be added in routine assays, because the small amount of ascorbate ordinarily found in the assay mixture can be disregarded. The threshold limit of ascorbate excretion is about 0.1 mmol/L, the interference from which is equivalent to only 5% of the upper normal triglyceride concentration. Serum-blanks methods are not affected.

Bilirubin: Bilirubin interference with colorimetric methods is well documented and presents a potentially serious problem (73). The interference is probably both spectral and chemical and will be different for conjugated and unconjugated bilirubin (74). Bilirubin is oxidized in a kinetic reaction with H2O2, and its decrease in absorbance is superimposed on the main triglyceride reaction. A serum blank does not compensate sufficiently because the substrate, H2O2, is not formed from free glycerol in equivalent amounts.

The various chromogens used for the peroxidase-coupled assays are affected differently by the bilirubin side-reaction; some are less susceptible to the interference than others, presumably because of a higher affinity of peroxidase for the chromogen (75). The addition of ferrocyanide ions has shown various degrees of success, depending on the chromogens, pH, and spectral wavelength used for measurements (76). Adding bilirubin oxidase appears to give inherently better results and should be considered as a pretreatment for icteric samples (77).

Bilirubin does not interfere with ultraviolet methods when measured with precision spectrophotometers vs blanks, because NADH has a much lower oxidation potential than does H2O2. Bichromatic readings in some instruments, however, do not provide sufficient spectral accuracy. The absorbance of NADH at 380 nm lies within a spectral shoulder extending from 340 to 400 nm. This spectral insensitivity can result in apparent interference. The absorbance of unbound bilirubin peaks at around 420 nm and is still measurable at the range of 380-400 nm. The overlapping spectra of icteric samples are often not considered in the calculation of a bichromatic factor.

Alkaline and acid phosphatases: Phosphatases that originate from erythrocytes may liberate glycerol from glycerol esters and cause chemical interference during hemolysis. Also, spectral interference occurs in some of the colorimetric methods if no serum blank is performed.

Phospholipids: Phospholipids from erythrocyte membranes can also cause falsely increased triglyceride values in unblanked assays. This will occur particularly when there is a delay in separation of the serum from the clot through the action of phosphoesterases.

Uric acid: Uric acid has been reported as an interferent in the Trinder reaction (78). Peroxidase reacts nonspecifically with many reducing compounds as a hydrogen donor; thus increased uric acid concentrations, as well as ascorbic acid, will affect the results of unblanked techniques.

Problems in Laboratory Technique

Plasma: Plasma treated with heparin or EDTA can be used for the triglyceride assay, provided that most platelets have been removed. Platelet membranes will rupture in the presence of surfactant and will release alkaline phosphatase and lactate dehydrogenase into the assay mixture (79).

Highly lipemic specimens: Highly lipemic specimens contain enough chylomicrons to agglomerate as a layer on top of the samples. If sample cups for automated instruments are left in the waiting position for more than 30 min, the samples are no longer homogeneous. Lipemia also interferes if no "clearing factor" is used, namely, if the sample blank solution does not contain agents to solubilize the lipid micelles. This effect is not always visible, and considerable light scattering can be measured even if the cuvets appear to be clear. Patents exist that claim removal of turbidity from lipids and (or) proteins by surfactants (64, 80-82); however, the degree to which surfactants can clear lipids completely, with no optical interference, has been challenged (83).

Triglyceride concentrations measured by the GPO-coupled reaction reportedly are decreased in grossly lipemic samples because of rapid oxygen utilization that, through producing a temporarily anaerobic environment, leads ultimately to decreased color generation (84). Similar results were obtained in the laboratory of one of us (unpublished data, S.G.K.) with the use of the Cobas-Bio centrifugal analyzer (Roche Diagnostic Systems, Nutley, NJ) but not when measurements were taken in a manual spectrophotometer. Adding a liquid with high oxygen retention, such as a fluorocarbon, can eliminate the problem.

Hydrazine-containing buffers: Hydrazine-containing buffers are sometimes advocated for methods based on the reduction of NAD+ in alkaline pH ranges (28). The rationale is to trap the aldehyde group of the product, dihydroxyacetone phosphate (or dihydroxyacetone), in order to achieve a more favorable equilibrium. However, NAD+ and hydrazine form an ultraviolet-absorbing complex and generate a steady increase in absorbance ("creep-reaction"). Tris is a better agent for trapping aldehyde groups.

Creep-reactions: "Creep-reactions" are those in which the absorbance of a reaction mixture continues to change after the main reaction has reached an endpoint. These are definite signs of side reactions and are not obvious in
automated instruments. Test results can be either positively or negatively affected. The validation of new test reagents should include an experiment that traces absorbance time on a recording spectrophotometer, with use of a known serum sample or standard.

**Blanks:** Rate measurements based on pseudo-zero-order kinetics have the obvious advantage of not needing to be blanked, but will mask all creep reactions caused by extraneous or endogenous sources. Enzyme inhibitors may be added to increase the apparent $K_m$ of glycerol kinase (85, 86) and to provide surplus enzyme activity, but contaminants may not be inhibited. Endpoint assays determine the absorbance of sample–reagent mixtures before the reaction has taken place (incubation with reagent not containing glycerol kinase or lipase) and again after the reaction has been triggered and has come to completion. In this case, contaminants in the trigger reagent are not eliminated.

The most nearly accurate, but certainly the most expensive, blanking technique constitutes a separate serum blank for each sample (87). The assay is performed with the complete reagent and then repeated with the reagent lacking lipase. This glycerol blank, however, introduces a negative error caused by the clearing effect of lipase. A separate free glycerol blank needs to be performed as a serum blank with glycerol reagents, lacking glycerol kinase, or with the lipase-containing reagent, lacking one of the chromogenic compounds. The latter approach was patented by Tsuda et al. (88) because of the obvious advantage of introducing sequential analysis to automated instruments. The requirement of a serum blank in addition to a free glycerol blank, described by Artias et al. (83), stressed that surfactants alone clear turbidity only partially, while the glycerol blank is assayed. The sample blank is especially important when special medications have been administered to the patient, because absorbing and (or) fluorescent compounds may be present. Davies et al. (89) found that metronidazole produced a characteristic spectrum in the ultraviolet range. Later et al. (90) reported a fluorescence of unknown origin. Hydroxyureas has also been found to inhibit glycerol oxidase (91).

The bichromatic measurement of triglyceride assays may be a good alternative, but spectral conditions greatly affect the results. The spectrum of hemoglobin, for instance, changes with sample handling. While hemoglobin has its major peak at 500 nm, the formation of oxyhemoglobin decreases the maximum to 415 nm. Bilirubin, if unconjugated, peaks at 460 nm; but in the presence of surfactants that split the protein bonds, unbound bilirubin absorbs at 420 nm. Many automated instruments use a bichromatic pair of 340/380 nm for ultraviolet tests. If the bandwidth of the respective filter is wide, considerable absorbance is measured at 380 nm and thus affects the result, owing to the bilirubin shift. The bichromatic factor is an arithmetic mean of empirical data from many assays; it will correct for the majority of specimens, but not necessarily for abnormal samples.

**Sample-to-reagent ratios:** Most laboratory instruments are preset by the manufacturer with regard to sample and reagent volume, wash volume (if applicable), and instrument settings. By keeping the sample fraction low, i.e., 0.01 (5 μL), endogenous interfering substances are diluted, so that the actual effect has minor significance. The instrument readout, however, must include a high multiplication factor. Errors caused by exogenous sources such as reagent impurities are then multiplied, and the overall low sensitivity is reflected in the imprecision of the method. The delicate balance between sample volume, sensitivity of the method, and instrument-related optical fluctuation must be taken into account when applying specific methodology to laboratory equipment. Glick and Ryder (82) show the effect of common serum indices such as hemolysis and icterus or results with various laboratory instrumentation when used as prescribed by the manufacturers.

**De-ionized water:** De-ionized water, when freshly taken from a system, does not contain sufficient oxygen for the peroxidase-coupled reactions. The enzyme will display suboptimal behavior until all solutions are oxygenated. As a result, calibration factors and blank values may change during the first day. Reagents will equilibrate during the first 24 h, provided enough "headspace" is given for absorbing oxygen from the air.

**Drugs:** The direct effects of drugs on the triglyceride methods have not been studied extensively. Stored samples from patients receiving intravenous administration of heparin have shown decreased stability of triglycerides, with a corresponding increase in the concentration of free glycerol (19, 23). Other investigators have claimed high apparent concentrations of triglycerides when unblanked techniques were used, because of increased free glycerol concentrations after treatment with nitroglucose (16). As stated previously, others have found interference problems under certain conditions, such as during treatment with metronidazole or hydroxymes. Little research data on the effects of special patients' specimens are available, e.g., those from uremic patients, pre- and postdialysis. When selecting a triglyceride method and its technical application (instrument setting, blanks, etc.), special attention should be given to the expected peculiarities of the patients to be tested.

**Calibrators:** A major problem for triglyceride standardization is achieving a consensus on calibration materials. Because the end product of most triglyceride reactions is free glycerol, glycerol itself has been used as a convenient calibrator. Glycerol, however, can be considered only a primary standard for the indicator reaction; it is not the analyte itself and does not participate in all steps of the reaction. Ideally, a primary triglyceride standard should contain, in a pure, well-defined medium, triglycerides representative of the average composition and complexity of those found in adult humans. Owing to the hydrophobic nature of triglycerides and the complex packaging of lipoprotein particles, a truly parallel primary standard is probably not possible.

Triglycerin, although not entirely representative of human triglyceride fatty acid composition, has a similar average chain length and is hydrolyzed in the reaction process. Because triglycerides in serum contain many fatty acids with various lengths and numbers of unsaturated bonds, the rate and degree of hydrolysis in any system will depend on the esters involved and on the ability of the specific lipases in the reagent to cleave them. The hydrolysis of oleate, although it represents an average human fatty acid, will not be completely representative of the hydrolysis of all fatty acids. However, it is probably a reasonable alternative.

Another alternative, that of using well-characterized secondary standards, has also been shown to be accurate and precise (94). Chromatographic methods, e.g., HPLC could be used to validate the hydrolysis of serum-based secondary standards having complex fatty acid composit


66. Biochemical catalogs and specifications of Boehringer Mannheim Corp., Indianapolis, IN; Genzyme Corp., Boston, MA; Sigma Chemical Corp., St. Louis, MO; US Biochemical Corp., Cleveland OH.


