Preanalytical Factors

Lipid/lipoprotein analyses may be affected by a number of preanalytical factors. Patients should be advised to maintain their customary diet and lifestyle for three days prior to testing.
Evaluation of lipid/lipoprotein status should not be performed during stress or acute illness, e.g., recent myocardial infarction, stroke, pregnancy, trauma, weight loss, use of certain drugs. It is also recommended by many lipid specialists that these laboratory assays should not be performed on hospitalized patients until 2 - 3 months after the illness.

It is necessary for patients to fast for at least 12 hours, and abstain from alcohol for 24 hours, before samples are drawn for analysis of triglycerides, apolipoprotein B, or calculated LDLcholesterol. Fasting is not required, but is usually preferred, for measurement of total cholesterol, HDL-cholesterol, LDL-cholesterol by a direct method, apolipoprotein Al and All, and lipoprotein (a).

To avoid hemoconcentration of blood samples resulting in falsely elevated lipid/lipoprotein results, patients should be seated for at least 15 minutes prior to sample collection, and phlebotomists should minimize tourniquet use.

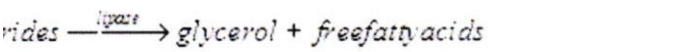
Testing is usually on serum or EDTA plasma; concentrations in EDTA plasma have been reported to be 4.7% lower than serum due to osmotic shifts. For long-term sample stability, EDTA can reduce oxidation and enzymatic cleavage of lipoproteins by chelating metal ions. Heparinized plasma may be acceptable; however, heparin activates lipoprotein lipase which may interfere with laboratory tests such as triglycerides.

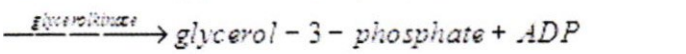
Due to intraindividual and analytical variation, it is recommended that lipid/lipoprotein testing be performed on more than one occasion when evaluating a patient.

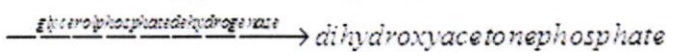
Measurement of Lipids

Triglycerides

All assays for triglycerides currently involve cleavage of serum/plasma triglycerides to free fatty acids and glycerol. The liberated glycerol is measured by one of a variety of methods, one of which is shown below.

Triglyceride: 

glycerol + ATP 

glycerol — 3 — phosphate + -VAD+ - VADH 

This reaction is measured by spectrophotometry at a wavelength setting of 340 nm. At this wavelength, the production of NADH is monitored. As the level of serum/plasma triglycerides increases, the amount of NADH produced increases and the absorbance detected by spectrophotometry increases. Thus, we can say there is a direct relationship between absorbance at 340 nm and triglycerides concentration. Recall, this is similar to the hexokinase method

discussed earlier for glucose measurement.

Some assays will include a trapping agent for dihydroxyacetonephosphate. The purpose of this trapping agent is to prevent the build up of dihydroxyacetonephosphate thus preventing the possible reversal of the third step of this reaction. The most common trapping agent used in this assay is hydrazine.

Since all of the enzymatic methods currently in use for the measurement of triglycerides include the reaction converting glycerol to glucose-3-phosphate, any pre-existing glycerol in the sample will cause a falsely elevated triglycerides result. Glycerol is typically very low in normal serum/plasma. However, glycerol levels may be increased in certain situations including:

- stress resulting in the release of epinephrine
- treatment with mannitol (Osmitrol) - a diuretic • treatment with nitroglycerin - an anti-anginal medication • diabetes mellitus • certain liver diseases • infusion of glycerol-containing intravenous fluids • glycerol-coated stopper used in Vacutainer phlebotomy tubes • hemodialysis for kidney disease

Correction for pre-existing glycerol by a glycerol-blanking method is recommended when one of the above situations exist. This glycerol-blanking method involves measurement using the above reaction sequence without the hydrolysis of triglycerides by omitting the first step. By doing this, any pre-existing glycerol will be measured but no triglycerides will be measured. This would be followed by running the complete reaction sequence above on the same sample to measure triglycerides. The pre-existing glycerol level would be subtracted from the triglycerides result to give the corrected triglycerides level.

Due to the low frequency of problems with endogenous glycerol, many laboratories do not blank for glycerol.

Desired triglycerides levels are currently set at 150 mg/dL or less.

Total Cholesterol

Total cholesterol has become one of the most precise and accurate measurements in the clinical laboratory thanks to lipid standardization efforts. Because of matrix effects inherent in most lipid measurements, calibration of methods must be verified using fresh specimens analyzed by reference methods. Use of such calibrators provided by lipid reference laboratories has improved performance markedly in most laboratories in the past 10 years.

The Abell-Kendall reference method is used at all lipid reference laboratories as well as the CDC. In this method, cholesterol esters are hydrolyzed and then cholesterol (in the free form) is extracted. Cholesterol is then oxidized by Liebermann-Burchard reagent (containing acetic acid, acetic anhydride, and sulfuric acid) to fom colored compounds that can be measured spectrophotometrically. A modification of this procedure complexes the colored product with ferric iron. This produces a very stable product that is approximately seven times more intense, and thus seven times more sensitive.

The Liebermann-Burchard procedure (without extraction) was used extensively in the clinical

laboratory for the measurement of cholesterol in the past. The biggest problem with this assay is interference from bilirubin and other chromagens. (The purpose for the extraction step in the Abell-Kendall method is to remove these chromagens.) Because of these interference problems with the Liebermann-Burchard, enzymatic methods have become frequently used in everyday clinical laboratory assays.

The most commonly used enzymatic assays for total cholesterol begin with two coupled reactions that produce peroxide. Peroxide is typically coupled with a chromagen to produce a colored product that can be read spectrophotometrically. This is illustrated below.

cholesterol ester --→ cholesterol + fatty acids cholesterol

Cholersterol + Oxygen ____> Cholest-4-ene+3-one+peroxide

Peroxide+ 4 aminoantipyrine + Phenol ____> quinoneeimine

As enzymatic assays replaced colorimetric assays, normal reference ranges for total cholesterol dropped by approximately 30 mg/dL. The reason for this drop is that enzymatic assays are not affected by chromagens (e.g. bilirubin). Enzymatic assays correlate well with reference methods.

A variation on the enzymatic reaction shown above involves use of the first two steps of the reaction and measuring the consumption of oxygen in the reaction system using an oxygen electrode.

Desired total cholesterol levels are currently set at 200 mg/dL or less.

Measurement of Lipoproteins

Ultracentrifugation

Ultracentrifugation methods take advantage of two properties of the lipoproteins. First, by virtue of their lipid content, they have lower densities than other plasma macromolecules. Second, each class of lipoproteins has a different density. Thus, by this technique, lipoproteins can be separated from each other as well as from other plasma macromolecules.

This method is not used in most clinical laboratories because it requires special skills and equipment that are not easily adapted for clinical laboratory purposes. Ultracentrifugation is, however, the reference method with which other lipoprotein methods are compared.

Electrophoretic Methods

Electrophoresis has been widely used in the clinical laboratory to separate and measure lipoproteins, but the limitations of this methodology, and the realization that it is not really needed for diagnosis of most dyslipoproteinemias, have considerably limited the use of lipoprotein electrophoresis for routine clinical practice.

Lipoprotein electrophoretograms are usually visualized with a lipid-staining dye such as Oil Red O, Fat Red 7B, or Sudan Black B. Attempts have been made to quantitate the lipoproteins by densitometry. In general, this approach has not met with much success for reasons that include

incomplete resolution of beta and pre-beta lipoproteins, the presence of minor or unusual lipoproteins, and differences in the intensity of staining. These problems are what led Fredrickson to recommend that his classification of hyperlipoproteinemias no longer be used (as noted in the section on lipid and lipoprotein abnormalities).

Again, as noted in the lipid and lipoprotein abnonnalities section, even though the Fredrickson classification is no longer recommended, this classification is still frequently discussed. Thus, there is still need to understand the Fredrickson classification.

Another application of electrophoresis is in the determination of the amount of cholesterol in each lipoprotein. This approach is very similar to that for lipoprotein electrophoresis with the exception of the visualization step. This approach involves pouring enzymatic cholesterol reagent over the electrophoretic membrane following separation. Once the enzymatic reaction is complete, the cholesterol in each lipoprotein fraction can be measured. Using densitometry, the percentage of cholesterol in each fraction can be determined. This percentage would then be multiplied by the total cholesterol value obtained, for example, by a spectrophotometric technique, resulting in the amount of cholesterol in each lipoprotein fraction. This application suffers from the same limitations discussed above for lipoprotein electrophoresis.

Also note that it would be possible to measure the triglycerides levels in each lipoprotein by a method very similar to that described for cholesterol. The difference would be the use of enzymatic triglycerides reagent in place of cholesterol reagent.

Polyanion Precipitation Methods

Lipoproteins are precipitated with polyanions such as heparin sulfate, dextran sulfate, phosphotungstate, and others, in the presence of divalent cations such as Ca+2, Mg^{*2}, and Mn+2 . Precipitation is influenced by factors such as reagent concentration, pH, ionic strength, and so on. Conditions have been established in which the major classes of lipoproteins can be precipitated in stepwise fashion beginning with the lower-density, lipid-rich, lipoproteins. The more dissimilar the lipoproteins, the more satisfactorily they can be separated from each other. Of particular interest is the fact that apoB-containing lipoproteins can be precipitated from most samples under conditions in which virtually all the HDL remains soluble.

Methods for Determining HDL-Cholesterol Values

The methods most commonly used to determine HDL-cholesterol levels involve the use of polyanion-divalent cation precipitation. In this technique, apoB-containing lipoproteins [chylomicrons, VLDL, IDL, LDL, Lp(a)] are precipitated leaving HDL free in the supernatant fluid. An aliquot of this supernatant fluid is then analyzed using the enzymatic total cholesterol procedure which measures the cholesterol in HDL, hence HDL-cholesterol.

Several combinations of polyanion-divalent cations have been used, and not all of them give precisely the same result. HDL-cholesterol values determined with heparin-Mn+2 procedures agree closely with those obtained by ultracentrifugation. This heparin-Mn+2 method is the standard by which other precipitation methods are assessed.

Dextran sulfate-Mg^{+2} and sodium phosphotungstate-Mg^{+2} have been shown to give results approximately 5% lower than ultracentrifugation, and heparin-Ca^{+2} appears to give results that are about 10% higher than ultracentrifugation.

Methods for LDL-Cholesterol Measurement

The two traditional methods have been ultracentrifugation or use of the Friedewald formula.

Since most clinical laboratories do not have ultracentrifugation capability, the Friedewald formula has been extensively utilized. The Friedewald formula is shown below.

$$\text{LDLcholesterol} = \text{total chol esterol} - \text{HDL cholesterol} - \frac{\text{tri de s}}{5}$$

This equation assumes that there are no lipoproteins in the circulation other than HDL, LDL, and VLDL (i.e., there is no consideration of chylomicrons). Therefore, LDL-cholesterol estimation by the Friedewald equation must be performed on fasting specimens.

In this equation, VLDL-cholesterol is estimated by triglycerides divided by 5. This is based on the fact that VLDL particles have a mass ratio of triglycerides to cholesterol of approximately 5.

This equation is not reliable if triglycerides are over 400 mg/dL. It does agree well with LDLcholesterol measured by ultracentrifugation for triglycerides below this level.

In the last few years, direct LDL-cholesterol measurements have been developed. One example of a procedure for the direct measurement of LDL-cholesterol is a method that allows direct measurement without any sample manipulation. The advantage of this method is that it is easily adapted for use on automated chemistry analyzers. In this procedure, the first reagent addition specifically disrupts lipoproteins other than LDL, causing their cholesterol release. This cholesterol proceeds through de-esterification and is reacted with cholesterol oxidase to generate hydrogen peroxide, which is further reacted to form a colorless compound. The second reagent contains a detergent that now releases cholesterol from LDL. After de-esterification, the LDLcholesterol proceeds through a similar set of reactions, except the final step generates a colored compound. The intensity of color produced is directly proportional to the concentration of LDLcholesterol.

Even though methods are now available for the direct measure of LDL-cholesterol, this direct measurement is not always needed. In most cases, total cholesterol, HDL-cholesterol and triglycerides are needed before a definitive diagnosis is made and treatment initiated. Since these tests are relatively inexpensive, highly reproducible, and needed anyway, calculation of the LDLcholesterol when triglycerides are below 400 mg/dL would be more prudent since the Friedewald calculation adds no time or expense to the laboratory evaluation. The direct measurement of LDL-cholesterol is useful when triglycerides exceed 400 mg/dL since these direct methods are not subject to interference by triglycerides.