UNIT: Lipid Studies

Task

Determination of serum cholesterol and triglyceride concentration.

Objectives

Upon completion of this exercise, the student will be able to:

1. Define hyperlipoproteinemias.
2. Outline the three-step coupled enzyme reaction to determine cholesterol concentration.
3. Perform cholesterol and triglyceride procedures.
4. Describe why anticoagulants containing oxalate or fluoride affect cholesterol and triglyceride results.
5. State normal cholesterol values for males and females.
6. State normal triglyceride values.
7. Outline the four-step coupled enzyme reaction to determine triglyceride concentration.

Reagents, Supplies and Equipment

1. Seradyn Cholesterol (Enzymatic) Reagent Set/Kit
2. Sigma Triglyceride (UV) Kit (procedure No. 334-UV)
3. Spectrophotometer(s) capable of measuring transmitted light at 340 nm and 500 nm
4. 37°C constant heat source, timer & pipeting devices for the accurate delivery of volumes required for assays (3 ml, 1 ml, 0.025 ml, 0.02 ml)

References

Kaplan et al. Clinical Chemistry (3rd ed.)
Seradyn enzymatic cholesterol product insert (#862G)
Sigma product insert, procedure No. 334-UV, Triglyceride (UV)
Teitz, N. W. Fundamentals of Clinical Chemistry.

Testing Purpose

Lipids (cholesterol and triglyceride) circulate in the blood associated with proteins in the form of water-soluble complexes termed lipoproteins. Sufficient elevation in the concentration of any lipoprotein can result in hyperlipoproteinemia, a metabolic disorder which may be inborn or due to a number of acquired conditions including endocrine disorders, specific organ failures, or external causes.

In 1967, Fredrickson et. al. introduced a system for classification of hyperlipoproteinemias. The five (5) types of hyperlipoproteinemias are not disease entities, but are groups of disorders that affect plasma lipid and lipoprotein concentrations. Disturbances which are known to cause hyperlipoproteinemia include diabetes mellitus, nephrosis, and biliary obstruction.

Elevated levels of plasma triglycerides and cholesterol are also associated with risk factors related to atherosclerosis. Recently, a number of researchers have advocated using a ratio of the total cholesterol value divided by the HDL cholesterol value as an indicator of potentially serious heart disease risk. Ratios of 4.5 or more are at greater risk.
Procedure I
Manual Cholesterol Determination

Principle

In this coupled enzyme system, cholesterol esters are enzymatically hydrolyzed to cholesterol and fatty acids. The cholesterol produced and the free cholesterol in the sample are enzymatically oxidized to form cholest-4-en-3-one and hydrogen peroxide. A quinoneimine chromogen with an absorption maximum at 500 nm is produced when phenol is oxidatively coupled with 4-aminoantipyrine in the presence of hydrogen peroxide and the enzyme peroxidase. The absorbance is proportional to the amount of total cholesterol in the test sample.

Reaction

Step 1:
\[
\text{cholesterol esters} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids}
\]

Step 2:
\[
\text{cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + \text{H}_2\text{O}_2
\]

Step 3:
\[
2\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine chromogen}
\]

Reagents

Warning—causes irritation. Harmful if swallowed. Do not get in eyes, on skin or clothing. In case of eye or skin contact or irritation, immediately flush the affected area with running water for at least 15 minutes and get medical attention. If swallowed, get medical attention immediately.

2. Seradyn Cholesterol (Enzymatic) Reconstituting Reagent — protect from light and refrigerate.

Specimen

Serum separated from the clot as soon as possible is the specimen of choice. EDTA or heparinized plasma is also suitable, but plasma containing oxalate or fluoride will produce results approximately 15% lower.

Cholesterol is serum or plasma is stable for approximately 5 days at room temperature, longer when refrigerated, and reportedly stable for 5 years at -20°C.

Steps 1 and 2 for Multiple Determination Vials

1. Add 15.5 mL of Reconstituting Reagent to each five-test cholesterol (Enzymatic) Reagent vial. Invert gently to dissolve contents. (The reconstituted reagent, when protected from light, is stable for eight hours at room temperature or five days refrigerated.)
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2. Transfer 3.0 mL of the reconstituted reagent into appropriately marked empty Seradyn vials (or clean 13 x 100 test tubes).

Steps 1 and 2 for Single Determination Vials

1. Appropriately mark Cholesterol Enzymatic Reagen vials: Blank, Standard(s), Control(s), and Patient(s).

2. Add 3.0 mL Cholesterol (Enzymatic) Reconstituting Reagent to each single-test reagent vial. Invert gently to dissolve contents. (The Reconstituted reagent, when protected from light, is stable for eight hours at room temperature or five days refrigerated.)

Steps 3 - 6 for Either Determination

3. Preincubate all vials in a 37°C heat source for at least seven but no longer than 45 minutes.

4. Add 0.025 mL water, standard, control, or serum to the Blank, Standard, Control, and Patient vials/tubes respectively.

5. Mix each vial well by inverting, and return to the 37°C heat source.

6. After 10 minutes incubation at 37°C, read absorbance at 500 nm using distilled water to zero the colorimeter/spectrophotometer.

Calculation

\[
\text{cholesterol mg/dL} = \frac{\text{Abs patient test} - \text{Abs reagent blank}}{\text{Abs standard} - \text{Abs reagent blank}} \times 200 \text{ mg/dL}
\]

Abs = absorbance
200 mg/dL = standard cholesterol concentration

Normal Ranges
Procedure II
Manual Triglyceride (sample start procedure)

Principle

Triglycerides are hydrolyzed to glycerol and fatty acids by the enzyme lipase. The produce glycerol is subsequently phosphorylated by adenosine triphosphate (ATP) to produce glycerol-1-phosphate and adenosine diphosphate (ADP) in a reaction catalyzed by glycerol kinase. ATP is then regenerated in the pyruvate kinase catalyzed reaction between ADP and phosphoenolpyruvate. The pyruvate formed is subsequently reduced to lactate with the simultaneous oxidation of equimolar amounts of NADH in the presence of lactate dehydrogenase. The decrease in absorbance at wavelength 340 nm (from the oxidation of NADH to NAD\(^+\)) is directly proportional to the triglyceride concentration in the sample.

Step 1: \[ \text{Triglycerides} \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids} \]

Step 2: \[ \text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-1-phosphate} + \text{ADP} \]

Step 3: \[ \text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{PK}} \text{pyruvate} + \text{ATP} \]

Step 4: \[ \text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LD}} \text{lactate} + \text{NAD} \]

Reagents

Triglyceride (UV) reagents are for in vitro diagnostic use only. Normal precautions exercised in handling laboratory reagents should be followed.

1. Triglyceride (UV) Reagent A
2. Triglyceride (UV) Reagent B

Specimen

Clear serum or plasma (EDTA or heparinized) collected from fasting patients. Triglycerides in these specimens are reportedly stable for as long as a week at 2-6°C. Blood collection tubes, including stoppers and pipetting devices must be free of glycerol contamination.

Prepare Sample Start Reagent

1. Reconstitute Triglyceride (UV) Reagent A and Triglyceride (UV) Reagent B with volume of deionized water indicated on vial labels.
2. Once dissolved, add 0.25 mL of Reagent B to 10 mL of Reagent A.

3. Into test tube labelled Blank and Samples, pipet 2.0 mL Sample Start Reagent and warm to assay temperature (ambient, 30°C or 37°C).

Procedure

1. To Blank, add 0.04 mL deionized water; to Samples, add 0.04 mL of their respective sera. Mix by gentle inversion.

2. Incubate all tubes at assay temperature for 10 minutes.

3. Read and record absorbance of Blank and Samples at 340 nm. Zero instrument with deionized water.

4. Determine change in absorbance ($\Delta A$) for each sample by subtracting the $A_{Sample}$ from $A_{Blank}$.

Calculations

Calculation of serum triglyceride concentration in mg/dL is based on the molar absorptivity of NADH which is $6.22 \times 10^3$ at 340 nm, and according to the following formula:

\[
\text{triglyceride mg/dL} = \frac{\Delta A \times 885 \times 1.02 \times 100}{6.22 \times 10^3 \times 0.02 \times 1}
\]

Where $\Delta A = \text{change in absorbance for each sample}$

- 885 = Mol. wt. of triglycerides expressed as triolein
- 1.02 = Total volume (mL)
- 0.02 = Sample volume (mL)
- $6.22 \times 10^3$ = Molar absorptivity of NADH at 340 nm
- 1 = 1-cm light path
- 100 = Conversion of mg/mL to mg/dL

>>> After mathematical rearrangement, the calculation is simplified to:

\[
\text{triglyceride mg/dL} = \Delta A \times 726
\]

(To convert to SI units, multiply triglyceride mg/dL by 0.0113.)

Limitations

If triglyceride concentration exceeds upper limit of linearity (500 mg/dL), dilute sample with one part isotonic saline, reassy and multiply result by two.

Normal values

Normal value for serum triglycerides is generally considered to be < 150 mg/dL.
A small, under-developed female patient was seen by a pediatrician. The parent’s concern was the child appeared to have convulsions in the morning before eating breakfast. After eating, the child was more calm. The child appeared to be well fed. Physical exam revealed an enlarged liver. The physician ordered a fasting serum glucose measurement, which was significantly decreased below the reference value.

1. What is the probable diagnosis?

2. What other laboratory results might be abnormal?

3. What is the abnormality associated with this child’s condition?

4. What test could be done to confirm diagnosis?
Lipids Report Form

Name________________________
Date________________________

Spectrophotometer Used________________________

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Basic Calculation

Examples

Over for evaluation of controls.
### Study Questions

**Instructions:** Legibly write your answers in the space provided. Use the back of the page if more room is needed. Unless otherwise indicated, each question is worth one point.

1. What is the specimen of choice for lipid studies?

2. Oxalate of fluoride plasma specimens will produce false decreased results in cholesterol and triglyceride tests (also many other tests). What is the main reason behind this?

3. What is the basis of the complex calculation used to determine the triglyceride concentration?

4. In the triglyceride procedure, the ΔA is obtained from subtracting the sample’s absorbance from the blanks. Why is this different from many of the other procedures where the blank is subtracted from the specimen?

5. Briefly define hyperlipoproteinemias.
6. List normal values for cholesterol in men and women. (2 pts)

7. According to the information presented in the lab, list each step of the coupled enzyme reaction process to determine cholesterol concentration. Then explain in writing how the product(s) of each reaction step are used to determine the cholesterol concentration. (6 pts)

8. According to the information presented in the lab, list each step of the coupled enzyme reaction process to determine triglyceride concentration. Explain in writing how the product(s) of each reaction step are used to determine the triglyceride concentration. (8 pts)

9. Describe the calculation used to assess risk of increased heart disease. Evaluate a patient that has a total cholesterol of 186 mg/dL and a HDL of 39 mg/dL. Show your work. (2 pts)