Experiment 5a

Lipid profile

Estimation of Cholesterol in serum by enzymatic method

Background:

Cholesterol is a steroid with a secondary hydroxyl group in the C position and found in blood, bile and brain tissue. It serves as a precursor to bile acids, steroids and vitamin D. It is synthesized in many types of tissues, but particularly in the liver and intestinal walls. Approximately 75% of cholesterol is newly synthesized and a 25% originates from dietary intake. Measurements of serum cholesterol levels are important in the diagnosis and classifications of hyper-cholesteremias. Elevated cholesterol levels may occur with hypothyroidism, nephritic syndrome, diabetes and various liver diseases. There is a correlation between elevated serum cholesterol level and incidence of coronary artery disease. Normal cholesterol levels are affected by stress, diet, age, gender, hormonal balance and pregnancy. Depressed levels are associated with hyperthyroidism and severe liver diseases.

Principle:

Cholesterol analysis was first reported by Liebermann in 1885 followed by Burchard in 1889. In Leibermann Burchard reaction, cholesterol forms a blue green dye from polymeric unsaturated carbohydrates in an acetic acid/ acetic anhydride/ concentrated sulfuric acid medium. The Abell and Kendall method is specific for cholesterol but is technically complex and requires corrosive agents. In 1974, Allain and Roeschlau were able to combine cholesterol esterase and cholesterol oxidase into a single enzymatic regent for the determination of total cholesterol. Vitro cholesterol reagent combines the use of three enzymes with the peroxidase/phenol/4-aap system of Trinder11 for the measurements of total cholesterol in human serum. The series of reaction involved in the assay system are as follows:

1. Cholesterol esters are enzymatically hydrolysed by cholesterol esterase (CE) to cholesterol and free fatty acids.
2. Free cholesterol is then oxidized by cholesterol oxidase (CHOD) to cholest-4-ene-3-one and H_2O_2.
3. In presence of peroxidise (POD), the formed hydrogen peroxide affects the oxidative coupling of phenol and 4-aminoantipyrine(4-AAP) to form a red coloured quinoneimine dye.

$$\text{Cholesterol esters} + H_2O \rightarrow \text{cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \rightarrow \text{cholest4-ene-3 one} + H_2O_2$$

$$2\ H_2O_2 + \text{4-AAP} + \text{Phenol} \rightarrow \text{quinoneimine dye} + 4\ H_2O$$

The intensity of the colour produced is directly proportional to cholesterol concentration. It is determined by measuring the increase in absorbance at 500-550nm.

Reagents:

R1: Cholesterol standard 200 mg/dl

R2: Pipes buffer, pH-6.9, 90 mmol/l
Phenol 26 mmol/l
Cholesterol oxidase 500 µ/l
Cholesterol esterase 500 µ/l
Peroxidase 1250 µ/l
4-Aminoantipyirine 0.4 mmol/l

Procedure:

1. Prepare the test tubes as shown below:

	Blank	**Standard**	**Sample**
R2 (Reagent)	1.0 ml	1.0 ml	1.0 ml
dH_2O	10 µl		
R1 (Standard)	-	10.0 µl	-
Serum	-	-	10.0µl
Absorbance			

2. Mix and incubate for 5 minutes at 37ºC or 10 minutes at 20-25ºC. Measure the absorbance of specimen and standard against reagent blank at 545. The colour is stable for 60 minutes.

Calculation: Calculate the total Cholesterol concentration by using the following formulae

Total Cholesterol concentration
= (Absorbance of specimen/Absorbance of standard) x [Standard]
(Unit conversion: mg/dl x 0.0259 = ___ mmol/L)

Expected value:
Risk classification Total Cholesterol

Desirable	<200 mg/dl (5.2 mmol/l)
Borderline high	200-239 mg/dl (5.2-6.2 mmol/l)
High	>240 mg/dl (6.2 mmol/l)

Experiment 5b

Lipid profile

Estimation of High Density Lipoprotein (HDL) in serum by enzymatic method.

Background:

Plasma Lipoproteins are spherical particles containing varying amounts of cholesterol, triglycerides, phospholipids, and proteins. The phospholipids, free cholesterol, and protein constitute the outer surface of the lipoprotein particle, while the inner core contains mostly esterified cholesterol and triglycerides. The particles serve to solubilize and transport cholesterol and triglycerides in the blood stream. The relative proportions of protein and lipid determine the density of these lipoproteins and provide a basis on which to begin their classification. These classes are chylomicron, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Numerous clinical studies have shown that the different lipoprotein classes have very distinct and varied effects on coronary heart disease risk. The principle role of HDL in lipid metabolism is the uptake and transport of the cholesterol from peripheral tissues to the liver through a process known as reverse cholesterol transport (a proposed cardio-protective mechanism). Low HDL cholesterol levels are associated with an increased risk of coronary heart disease and coronary artery disease. Hence the determination of serum HDL cholesterol is a useful tool for identifying high-risk patients.

Principle:

The procedure for separation of the different lipoprotein density classes is density adjusted ultracentrifugation, which is not a practical technique for routine laboratory use. Widely used alternatives are based on poly-anion precipitation methods. Poly-anions such as heparins, dextran sulfate, and phosphotungstate, bind to positively charged Apoprotein B- containing lipoproteins which are then cross-linked by the use of divalent cation (manganese, magnesium or calcium) to form a precipitate. The HDL precipitating method utilizes the well-established precipitating properties of phosphotungstic acid to precipitate non-HDL cholesterol. This precipitation technique is frequently used method for HDL procedures. The remaining cholesterol in the supernatant, HDL cholesterol can then be measured using cholesterol reagent kit.

Reagents:
R1: HDL - Cholesterol standard 50.0 mg/dl
R2 (Precipitating reagent):
Phosphotungstic acid 40.0 g/l
$MgCl_2$ 100.0 g/l

Reagent Preparation and Stability
All reagents are stable up to the expiry date given on label when stored at 4ºC and protected from light.

Procedure:
HDL Separation
1. Dispense 500µl of specimen at room temperature in to a test tube.
2. Add 50µl of precipitating reagent (R2). Mix well.
3. Incubate the test tube at room temperature for 10 minutes prior to centrifugation.
4. Centrifuge for 10 minutes at full speed (at least 1000xg).
5. Separate the supernatant containing HDL from the precipitate.

Prepare the test tubes as shown below:

	Blank	**Standard**	**Plasma**
R2 (cholesterol reagent)	1 ml	1 ml	1 ml
dH_2O	100 µl	-	-
R1(Standard)		10 µl	
Plasma	-	-	10 µl
Absorbance			

Mix and incubate for 5 minutes at 37ºC or 10 minutes at 20-25ºC. Measure the absorbance of specimen and standard against reagent blank. The colour is stable for 60 minutes at 545 nm.

Calculation: Calculate the HDL-Cholesterol concentration by using the following formulae

HDL-cholesterol concentration
= (Absorbance of specimen/Absorbance of standard) x [Standard]
(Unit conversion: mg/dl x 0.0259 = ___ mmol/L)

Expected value:

Males

Good prognosis	> 55 mg/dl (> 1.4 mmol/l)
Standard risk	35 -55 mg/dl (0.9-1.4 mmol/l)
Risk indicator	< 35 mg/dl (< 0.9 mmol/l)

Females

Good prognosis	> 65 mg/dl (> 1.7 mmol/l)
Standard risk	45 -65 mg/dl (1.2-1.7 mmol/l)
Risk indicator	< 45 mg/dl (< 1.2 mmol/l)

Experiment 5 c
Lipid profile
Estimation of triglycerides in serum by enzymatic method

Background:

Triglycerides are esters of the trihydric alcohol glycerol with 3 long chain fatty acids. They are the main lipids present in human plasma; the others are cholesterol, phospholipids, and non esterified fatty acids. Triglycerides are synthesized in the intestinal mucosa by the esterification of glycerol and free fatty acids. Triglycerides are synthesized in the intestinal mucosa by the esterification of free fatty acids. They are then released into the mesenteric lymphatics and distributed to most tissues for storage. Triglycerides are the main storage lipids in human, where they constitute about 95% of adipose tissue lipids. Elevated levels of triglycerides have been associated with high risk in severe atheroscelorosis. High triglycerides levels and hyperlipidemia in general can be inherited trait or can be secondary to disorders including diabetes mellitus, biliary obstruction and metabolic disorders associated with endocrine disturbances.

Principle:

Triglycerides are generally determined by a combination of hydrolysis to glycerol and free fatty acids and measurements of the amount of glycerol released. The most commonly used methods involve alkaline hydrolysis and either chemical or enzymatic measurement of glycerol. Chemical means of analysis generally rely on measurement of the product of perodate oxidation of glycerol. Eggstein and kreutz developed an enzymatic method for measuring glycerol released from the triglycerides by alkaline hydrolysis. This method is based on the coupled reaction sequence catalyzed glycerol kinase, pyruvate kinase, and lactate dehyrogenase. A method for complete enzymatic hydrolysis to triglycerides avoiding the need for serum pre-treatment was described by Bucolo and david, using a combination of lipase and at least one proteolytic enzyme. Certain esterases could be combined with a lipase to achieve complete triglycerides hydrolysis. Both methods employed a coupled enzymatic reaction sequence to measure glycerol. Triglycerides reagents combine the use of lipoproteinlipase, glycerol kinase and glycerol phosphate oxidase with the peroxidase/4-chlorophenol/4-aminoantipyrine system of Trinder8 for the measurement of triglycerides in human serum. The series of reactions involved in the assay system are as follows:

1. Triglycerides are hydrolyzed by lipoprotein lipase (LPL) to glycerol and fatty acids.
2. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK).

3. The oxidation of glycerol-3 phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2).

Reagents:

R1	Triglycerides standard	200mg/dl
R2	Pipes buffer, pH 7.8	50mmol/l
	P-Cholophenol	2.0mmol/l
	Lipoprotein lipase	1500U/l
	Glycerolkinase	800U/l
	Glycerol phosphate oxidase	4000U/l
	Peroxidase	1500U/l
	4- Aminoantipyrine	0.4mmol/l
	ATP	0.3mmol/l
	Mg2+	40mmol/l
	Sodium cholate	0.2mmol/l

Procedure:

1. Prepare the test tubes as shown below:

	BLANK	**STANDARD**	**Sample**
R2	1.0ml	1.0ml	1.0ml
dH_2O	10µl		
R1 (Reagent)	-	10µl	-
Serum	-	-	10 µl
Absorbance		-	-

2. Mix and incubate for 5 minutes at 37ºC or 10 minutes at 20-25ºC.
3. Measure the absorbance of specimen and standard against reagent blank at 545.
4. The colour is stable for 60 minutes.

Calculation:

Triglycerides concentration = (Absorbance of specimen/Absorbance of standard) x [Standard]
(Unit conversion: mg/dl x 0.0114 = ___ mmol/L)

Expected value:

Males	40-160 mg/dl (0.45-1.82 mmol/l)
Females	35-135 mg/dl (0.40-1.54 mmol/l)

For the recognition of the risk factor of hypertriglyceridemia, the limits are:

Suspicious	>150 mg/dl (1.71 mmol/l)
Elevated	>200 mg/dl (2.28 mmol/l)

Experiment 5d
Lipid profile
Estimation of Low Density Lipoprotein (LDL) in serum (by calculation)

Background:

Low-density lipoprotein (LDL cholesterol, LDL-C) is one type of lipoprotein that carries cholesterol in the blood. Most often, this test involves using a formula to calculate the amount of LDL-C in blood based on results of a lipid profile. LDL-C is considered to be undesirable and is often called "bad" cholesterol because it deposits excess cholesterol in blood vessel walls and contributes to hardening of the arteries and heart disease. The LDL-C test can help determine an individual's risk of heart disease and help guide decisions about what treatment may be best if the person is at borderline or high risk. The results are considered along with other known risk factors of heart disease to develop a plan of treatment and follow up. Treatment options may involve lifestyle changes such as diet and exercise or lipid-lowering medications.The results of a standard lipid profile, which consists of total cholesterol, HDL-C, and triglycerides, are usually used to calculate the amount of LDL-C in the blood. The results are entered into a formula that calculates the amount of cholesterol present. In most cases, the formula provides a good estimate of the LDL-C, but it becomes less accurate with increased triglyceride levels when, for example, a person has not fasted before having blood drawn. In this situation, the only way to accurately determine LDL-C is to measure it directly. Direct measurement of LDL-C is less affected by triglycerides and can be used when an individual is not fasting or has significantly elevated triglycerides (above 400 mg/dL).

Expected value:

Elevated levels of LDL cholesterol can indicate risk for heart disease, so your LDL-C result is evaluated with respect to the upper limits that are desired, if there has no other risk factors, LDL-C level can be evaluated as,

- <100 mg/dl (2.59 mmol/l) — Optimal
- 100-129 mg/dl (2.59-3.34 mmol/l) — Near optimal, above optimal
- 130-159 mg/dl (3.37-4.12 mmol/l) — Borderline high
- 160-189 mg/dl (4.15-4.90 mmol/l) — High
- >189 mg/dl (4.90 mmol/l) — Very high

Major risk factors (see below) change the desired goals for LDL levels. Treatment (with diet or drugs such as statins) for high LDL-C aims to lower LDL cholesterol to a target value based on risk of heart disease. Your target value is:

- <100 mg/dl (2.59 mmol/l) if you have heart disease or diabetes.*
- <130 mg/dl (3.37 mmol/l) if you have 2 or more risk factors (intermediate risk for heart disease).
- <160 mg/dl (4.14 mmol/l) if you have 0 or 1 risk factor (low risk for heart disease).

Calculation:

Someone must fast for 9 hours prior to the estimation of LDL cholesterol.

Calculate the LDL-cholesterol concentration by using the following formula:

LDL-cholesterol concentration (mg/dl) = Total cholesterol- HDL- (triglycerides/5)
= Total cholesterol- (HDL + triglycerides/5)

LDL-cholesterol (mmol/l) = Total cholesterol – HDL- (triglycerides/2.181)
= Total cholesterol - (HDL+ triglycerides/2.181)

Note: This formula is usually valid for triglycerides concentration up to 400 mg/dl (4.5mmol/l).