# Department of Biochemistry & Microbiology
# Chemistry of Biomolecules Lab ( CHE 203 L)
*Experiment 3.*
## Estimation of Cholesterol 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 hypercholesteremias. 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 hydrolyzed 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.

   Cholesterol esters + $H_2O$ → cholesterol + fatty acids
   Cholesterol+$O_2$→ cholest4-ene-3 one +$H_2O_2$
   2 $H_2O_2$+4-AAP+Phenol → 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** | **Plasma** |
|---|---|---|---|
| **R2 (Reagent)** | 1.0 ml | 1.0 ml | 1.0 ml |
| **$dH_2O$** | 10 µl | | |
| **R1 (Standard)** | - | 10.0 µl | - |
| **Plasma** | - | - | 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.

**Total Cholesterol concentration**= (Absorbance of specimen/Absorbance of standard) x [Standard]

(Unit conversion: mg/dl x 0.0259 = ___ mmol/L)

**Expected value:**

**Risk classification of total cholesterol**

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