CHAPTER - III

MATERIALS AND METHODS
The present study on “The role of thyroid hormone on oxidative stress during cholangrene carcinogenesis” is conducted on a group of male albino mice which are divided into a normal control group and different experimental groups.

**Division of Groups:**

Target population of male albino mice for the present study.

- Normal control
- **Experimental Group**
  - Group I
  - Group II: Castor oil control.
  - Group III: Thyroxine control
  - Group IV: Thyroxine + Castor oil
  - Group V: 3-methylcholanthrene (3 MC)
  - Group VI: 3-methylcholanthrene (3 MC) + Thyroxine.
The experiment is conducted on 120 randomly selected healthy male albino mice weighing between 50 to 65 gms. Before the experimental procedure started, all the animals are acclimatized in the animal room for four weeks and fed on standard animal diet. As per plan of the study the targeted number of animals are randomly divided into different groups as follows:

**Group I (Normal control group):**

20 healthy male albino mice without any sign of deficiencies are randomly selected for normal control group and maintained throughout the whole period of experiment in the same condition.

**Experimental group:**

Animals selected for the experimental groups are subdivided into five sub-groups as:

**Group II (Castor oil control group):**

20 healthy animals are randomly selected for this group and each animal of the group is exposed to single dose of 0.25 ml (250μl) of castor oil by intraperitoneal injection.

**Group III (Thyroxine control group):**

This group consists of 20 healthy albino mice each of which is fed daily with 50μl of 10μg thyroxine solution.

**Group IV (Thyroxine and Castor oil treated group):**

20 animals from the acclimatized general pool are intraperitoneally injected with 0.25 ml (250μl) of Castor Oil alone and daily fed...
with 50μl of 10μg thyroxine solution as in the thyroxine control group.

**Group V (3-methylcholanthrene [3MC] treated group):**

This group consists of 20 randomly selected animals from the general normal healthy pool of already acclimatized animal for the study. A single dose of 0.5 mg of 3-methylcholanthrene in 0.25 ml (250μl) of castor oil is administered intraperitoneally into each animal of this group. During the whole period of experiment this group has received normal standard diet.

**Group VI (Thyroxine supplemented 3-MC treated group):**

20 animals selected from the acclimatized general pool are intraperitoneally administered with a single dose of 0.5 mg 3-methylcholanthrene in 0.25 ml (250μl) of castor oil with simultaneous daily oral administration of 10μg thyroxine in 50μl solution in the thyroxine control group.

**Identification marking of the animals:**

All the animals of different groups are kept in properly labelled cages and individual animals of a group are marked for identification by cutting a small 'V' shaped wedge with specific identity into the border of the ears according to the serial number of the group recorded in an identification sheet.

**Maintenance of behavioural and physical record:**

Throughout the period of experiment all the animals of different
groups are regularly observed for any change in general behaviour and dietary habit. On the day of sample collection the weight of the animals are recorded and a general examination with palpation of the abdomen and thorax are performed before actual collection of sample.

Collection of blood sample:

Whole blood:

With all aseptic and antiseptic measures 1 ml of blood is drawn from the caudal vein of the animal. 0.5 ml of blood is immediately transferred to an EDTA (Ethylene-diamine-tetracetate) vial and thoroughly mixed by gentle rotation in a 8cm diameter circle. The remaining 0.5 ml blood is transferred to a microcentrifuge tube for separation of serum.

Separation of serum:

The blood is allowed to clot in the centrifuge tube for 30 to 45 minutes at room temperature and then the clot is gently removed by a disposable polypropylene tube. The tube containing the serum is then centrifuged for 5 minutes at 3000 rpm. The supernatant serum is then transferred into a dry labelled and stoppered microcentrifuge tube and stored at 2° to 8° for estimation of the proposed parameters.

Frequency of blood sample collection:

Before initiation of the experimental part blood samples are collected from the whole general pool of acclimatized animals to get a normal base line on the day "zero" of the experimental period.
Subsequently, blood samples are collected from each of the individual group along with the normal control group on 10th, 15th, 20th, 25th, 30th, 45th, 60th, 75th, 90th and 120th days of treatment.

Collection of tissue samples:

The male albino mice weighing between 50-65 gm of normal control and different test groups are sacrificed on 30th, 60th, 90th and 120th day by the administration of high doses of chloroform and were dissected to collect the liver and stomach portion for the experiment. The tissue are dried over a filter paper and immediately weighed and recorded. The tissue homogenate are prepared in deionized water with the help of homogenizer.

Laparotomy of animals:

At the end of the 120 days of treatment the animals of each group are sacrificed by the procedure of stunting and a ‘T’ incision is made from pubic symphysis to the mandible for exposing the abdominal, thoracic and cervical regions. The viscera and the lymph glands are examined, photographed for record and preserved in formalin for future reference.

Parameters for biochemical evaluation:

The collected samples of blood and serum from each of the groups of different days of experiment are utilized for estimation of —

(A) Oxidative stress markers:

1. Lipid peroxidation (LPO)
2. Protein peroxidation (PPO)
3. Catalase (CAT)

(B) Metabolic markers:
1. Glucose
2. Cholesterol
3. Total protein

(C) Hormones
1. Triiodothyronine (T3)
2. Thyroxine (T4)
3. Thyroid stimulating hormone (TSH)

(D) Cancer markers:
1. Carcinoembryonic antigen (CEA)
2. Alkaline phosphatase (ALP)

Methods of evaluation:

All the biochemical estimations are done using computer assisted semiautomatic ‘BTS 320 photometer’. The results obtained are statistically analysed and compared between different groups of the study by applying standard statistical procedures to evaluate the changes among different groups in the study.

Method of Estimation:

Estimation of lipid peroxide (LPO) in whole blood:

Whole blood lipid peroxide is estimated by the method of Ohkawa et al. (1979).
Principle:

Lipid peroxides are converted to malondialdehyde by reacting with the thiobarbituric acid to produce a chromogen giving maximum absorbance at 530 nm and is expressed as thiobarbituric acid reactive substance (MDA) in whole blood.

Reagents:

1) Thiobarbituric acid reagent (TBA):

   670 mg of analar grade thiobarbituric acid is dissolved in 100 ml of deionised water with gentle heating till the solution becomes clear. It is stored in an amber coloured bottle at room temperature and is stable for two weeks.

2) 10% Trichloroacetic Acid (TCA):

   10 gm of trichloroacetic acid is dissolved in 100 ml of deionized water.

Procedure:

1) 10 μl of whole blood is treated with 600 μl of 10% TCA solution in a 1.5 ml centrifuge tube and mixed properly. After 5 minutes it is centrifuged at 5000 rpm for 10 minutes.

2) 200 μl of the supernatant is transferred to a 2 ml tube and 150 μl of the TBA reagent is added. 100 μl of deionised water is added and mixed.

3) A blank tube is prepared by adding 200 μl of TCA reagent, 150 μl of TBA reagent followed by 100 μl of deionised water.

4) Both the sample and the blank tubes are placed in a boiling
water bath for 10 minutes and allowed to equilibrate with room
temperature.

5) Absorbance of the blank and sample are measured at 530 nm
with deionised water as baseline and using a cuvette with 1cm light
path.

Calculation:

The whole blood lipid peroxide is expressed as nanomoles of
thiobarbituric acid reactive substance (TBARS) producing
malondialdehyde (MDA) per ml of whole blood. The content of MDA
is calculated by using the molar extinction coefficient for MDA at 530
nm.

\[
\text{Lipid peroxide as MDA in n mol/ ml} = D_F \times \frac{V \times A}{M_E}
\]

\[
= \frac{0.61}{0.01 \times 0.20} \times \frac{0.45 (U - B)}{0.152}
\]

\[
= 305 \times 2.96 \times (U - B)
\]

\[
= 902.80 \times (U - B)
\]

Where,  \(D_F\) = Dilution factor

\(V\) = Total volume of all the reactants.

\(A\) = (Absorbance of unknown) — (Absorbance of blank)

\(= \ U - B\)

\(M_E\) = Molar extinction coefficient.

[118]
Procedure for estimation of LPO in tissue samples:

Tissues from liver and stomach are prepared for estimation of lipid peroxide as follows -

A measured amount of tissue is taken from the sacrificed albino mice and homogenate is prepared by using homogenizer in a concentration of 10 mg tissue/ml of deionized water.

0.5 ml of ethanol is taken in a centrifuge tube. To it 0.5 ml of diethyl ether is added and mixed thoroughly by vigorous shaking. After shaking, 0.2 ml of tissue homogenate is added and again shacked for few minutes. Then the mixture is centrifuged at 5000 rpm for 10 minutes for the extraction of liquids from the tissue homogenate.

Supernatant portion is transferred to a glass test tube and precipitate is discarded. Glass test tube containing supernatant is placed in water bath at 80°C for 10 minutes to evaporate the ether portion. After complete removal of ether, the remaining supernatant is taken as sample solution.

450 μl of the TBA reagent is taken in a test tube. To it 900 μl of deionized water is added and mixed and then 50 μl of sample solution is added and mixed.

A blank tube is prepared by adding 450 μl of TBA, 900 μl of deionized water and 50 μl of ethanol.

Both sample and the blank tubes are placed in a boiling water bath for 10 minutes and allowed to equillibrate with room temperature.

Absorbance of the blank and sample are measured at 530 nm with deionized water as baseline and using a microcuvette with 1 cm light path.
Calculation:

Tissue lipid peroxide is expressed as nanomoles of MDA (malondialdehyde) per mg of tissue. The MDA content is calculated first as n mole of MDA/ml of homogenate by using the molar extinction coefficient for MDA at 530 nm (Ohkawa et al., 1979) and then it is converted to n mol of MDA/mg of tissue.

Lipid peroxide as MDA in n mol/ml.

\[
\text{Lipid peroxide as MDA in n mol/ml.}
\]

\[
= \text{DF} \times \frac{V \times A}{M_e}
\]

\[
= \frac{0.70}{0.20 \times 0.05} \times \frac{1.40 \times (U - B)}{0.152}
\]

\[
= 70 \times 9.21 \times (U - B)
\]

\[
= 644.74 \times (U - B)
\]

Where, \( V \) = Total volume of all the reactants.

\( \text{DF} \) = Dilution factor for homogenate

\( A \) = Absorbance of unknown (U - B)

\( B \) = Absorbance of blank

\( U \) = Absorbance of unknown

\( M_e \) = Molar extinction coefficient.

Estimation of protein peroxide (PPO) in serum:

Protein peroxide content in serum is estimated by the method of Ohkawa et al. (1979) as in the estimation of lipid peroxides.
Reagents

1. Thiobarbituric Acid (TBA)
   670 mg of analar grade TBA is dissolved in 100 ml of deionized water with gentle heating till the solution becomes clear. It is stored in an amber coloured bottle at room temperature and is stable for two weeks.

2. Trichloroacetic Acid (TCA) 10%
   10 gm of TCA is dissolved in 100 ml of deionized water and stored in an amber coloured bottle.

3. Sodium hydroxide solution (0.5 N)
   2 gm of NaOH is dissolved in 100 ml of deionized water.

Procedure

1. 10 μl of serum is treated with 600 μl of 10% TCA solution in a 1.5 ml of centrifuge tube and mixed properly. After 5 minutes it is centrifuged at 5000 rpm for 10 minutes.

2. Supernatant is discarded. Precipitate is washed two times with distilled water and then dissolved in 0.6 ml (600 μl) of 0.5 N NaOH solution. This sample solution is used for estimation of protein peroxide in blood.

3. 200 μl of the sample solution is transferred to a 2 ml tube and 150 μl of the TBA reagent is added. 100 μl of deionised water is added and mixed.

4. A blank tube is prepared by adding 200 μl of 0.5 N NaOH reagent, 150μl of TBA reagent followed by 100 μl of deionised water.

5. Both the sample and the blank tubes are placed in a boiling water bath for 10 minutes and allowed to equillibrate with room temperature.

6. Absorbance of the blank and sample is measured at 530 nm with deionised water as baseline and using a microcuvette with 1 cm light path.
Calculation:

The blood protein peroxide is expressed as nanomoles of thiobarbituric acid reactive substance (TBARS) producing malondialdehyde (MDA) per ml of blood. The content of MDA is calculated by using the molar extinction coefficient for MDA at 530 nm.

Protein peroxide as MDA in n mol/ml

\[
\text{Protein peroxide as MDA in n mol/ml} = D_F \times \frac{V \times A}{M_E} = \frac{0.61}{0.01 \times 0.20} \times \frac{0.45 \times A(U - B)}{0.152} = 305 \times 2.96 \times (U - B) = 902.80 \times (U - B)
\]

Where,

- \( V \) = Total volume of all the reactants
- \( D_F \) = Dilution factor for sample
- \( A \) = Absorbance (U — B)
- \( B \) = Absorbance of blank
- \( U \) = Absorbance of unknown
- \( M_E \) = Molar extinction coefficient.

Procedure for estimation of PPO in tissue samples:

Tissues from liver and stomach are prepared for estimation of protein peroxide as follows -
A measured amount of tissue is taken from the sacrificed albino mice and homogenate is prepared by using homogenizer in a concentration of 10 mg tissue/ml of deionized water.

0.5 ml of tissue homogenate is taken in a centrifuge tube and to it 0.5 ml of 10% TCA is added and mixed. The mixture is centrifuged at 5000 rpm for 5 minutes.

Supernatant portion is discarded. Precipitate is washed with deionised water twice repeatedly and then dissolved in 1 ml of 0.5 N NaOH solution. This sample solution is used for the estimation of protein peroxides.

450 μl of the TBA reagent is taken in a test tube and to it 900 μl of deionised water is added and mixed. Then 50 μl of sample solution is added and mixed.

A blank tube is prepared by adding 450 μl of TBA reagent to 900 μl of deionised water followed by 50 μl of 0.5 N NaOH solution.

Both the tubes containing sample and blank are placed in a boiling water bath for 10 minutes and allowed to equilibrate with room temperature.

Absorbance of the blank and sample is measured at 530 nm with deionized water as baseline and using a microcuvette with 1 cm light path.

**Calculations**

Tissue protein peroxide is expressed as nanomoles of MDA (malondialdehyde) per mg of tissue. The MDA content is calculated first as n mol of MDA/ml of homogenate by using the molar extinction coefficient for MDA at 530 nm (Ohkawa et al., 1979) and then it is converted to n mole of MDA/mg of tissue.
Protein peroxide as MDA in n mol/ml.

\[ VX = \frac{V \times A}{M_e} \times \frac{0.152}{1.4 \times A(U - B)} \times 0.05 \times 0.05 \]

Where,

- \( V \) = Total volume of all the reactants
- \( D_F \) = Dilution factor for sample
- \( A \) = Absorbance \((U — B)\)
- \( B \) = Absorbance of blank
- \( U \) = Absorbance of unknown
- \( M_e \) = Molar extinction coefficient.

**Estimation of catalase (CAT) activity in whole blood:**

Catalase activity in whole blood is estimated by the method of Aebi (1983) adopting UV-assay.

**Principle:**

In the ultraviolet range \( \text{H}_2\text{O}_2 \) shows a continued increase in absorption with decreasing wavelength. Decomposition of \( \text{H}_2\text{O}_2 \) by catalase can be followed by the decrease in extinction at 240 nm and the difference in extinction per unit time is a measure of catalase activity.

**Reagents:**

1) Phosphate buffer (50 mM; pH 7.0) : 6.81 gm \( \text{KH}_2\text{PO}_4 \) is dissolved in distilled water and volume is made up to 1000 ml to prepare solution - A. 8.9 gm of \( \text{Na}_2\text{HPO}_4.\text{H}_2\text{O} \) is dissolved in distilled [124]
water and the volume is made up to 1000 ml to prepare solution-B.

Solution-A and solution-B are mixed in the proportion of 1:1.55 and the pH is checked with a pH meter. It is stored at 2° to 8°C.

2) Standard Hydrogen peroxide (30mM):

0.34 ml of 30% \( \text{H}_2\text{O}_2 \) is diluted with phosphate buffer to 100 ml. This solution is prepared afresh every day.

Sample: — Anticoagulate (EDTA) venous blood.

Procedure:

1) 10\( \mu \text{l} \) of EDTA whole blood is added to 1000 \( \mu \text{l} \) of cold phosphate buffer in a cuvette and allowed to equilibrate for 10 minutes after proper mixing.

2) Absorbance is measured at 240 nm with 1 cm light path at 20°C in a spectrophotometer at 10 second intervals up to 60 seconds.

3) On completion of 60 seconds, if no change of absorbance is noted, 500 \( \mu \text{l} \) of 30mM \( \text{H}_2\text{O}_2 \) solution is added and mixed and changes of absorbance is noted at 10 second intervals up to 60 seconds.

Calculation:

Catalase activity in whole blood = \( 0.153 \times \log \frac{E_1}{E_2} \times 3.4 \times 1000 \) u/l

where 0.153 = Rate constant at 15 seconds.

\( E_1 \) = Initial extinction.

\( E_2 \) = Change of extinction in 15 seconds.

3.4 = Absolute constant for erythrocytic catalase.

1000 = Dilution factor.

Estimation of glucose:

Glucose in blood serum is estimated following Trinder's (1969) method of glucose oxidase-peroxidase using 4-amino antipyrine and phenol as the chromogenic reagent—producing maximum absorbance at 546 nm.
Principle:

Glucose is oxidised by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of the enzyme peroxidase (POD) oxidizes phenol which combines with 4-aminoantipyrine to produce a pink colored quinoneimine dye. The intensity of the colour is proportional to the glucose concentration in the sample.

\[
\text{D-glucose } + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{GOD}} \text{D-gluconic acid } + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine + phenol} \xrightarrow{\text{POD}} \text{quinoneimine dye} + \text{Phenol} + \text{H}_2\text{O}
\]

Reagents:

1. Enzyme Reagent
2. Buffer Solution
3. Glucose standard (100 mg/dl)

The reagents are procured as a ready to use set marked by Crest Biosystems.

Reagent preparation:

The enzyme reagent is reconstituted with the buffer reagent just before use and stored at 2-8°C where it is stable up to four weeks. The stored reagent is equilibrated to room temperature before use.

Procedure:

The tubes are marked as blank, standard and samples. Reagents and samples are added and processed according to the scheme as tabulated below.
Table No. III. 1. - Showing the procedure of estimation of glucose

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank</th>
<th>Sample</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme reagent</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.01 ml</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Standard</td>
<td>—</td>
<td>—</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>—</td>
<td>0.01 ml</td>
<td>—</td>
</tr>
</tbody>
</table>

Incubated for 15 mins at 37°C. Absorbance were taken at 546 nm setting ‘0’ by distilled water.

The contents of all the tubes are mixed well and is incubated at 37°C in a thermostat controlled water bath for 15 minutes. After incubation the absorbance of blank, standard and test samples are measured against the distilled water baseline in a pre-programmed computer assisted photometer at 546 nm.

Calculation:

Glucose concentration in mg/dl of sample

\[
\text{Glucose concentration in mg/dl of sample} = \frac{\text{Absorbance of test} - \text{Absorbance of Blank}}{\text{Absorbance of standard} - \text{Absorbance of Blank}} \times \text{strength of standard}
\]

Estimation of total cholesterol:

Total cholesterol was determined by reagent kit of Dr. Reddy's dependent on enzymetic method with end point suitable colorimetric estimation.

Method:

The method used for estimation of serum total cholesterol was adaption of the cholesterol oxidase - peroxidase method which is the
Allian et al. (1974) modification of original Rishmond (1972) and Flegg (1973) enzymatic method.

**Principle:**

Esterified cholesterol of serum is hydrolyzed by cholesterol esterase enzyme to form free cholesterol and fatty acids. The liberated free cholesterol is then oxidized by enzyme cholesterol oxidase to form cholesterol and hydrogen peroxide. Hydrogen peroxide further reacts with phenol and 4-amino antipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of cholesterol present in the sample.

The cholesterol reaction which takes place in three stages are shown below:

1. **Cholesterol esters + H$_2$O** $\xrightarrow{\text{Cholesterol esterase}}$ **Cholesterol + Fatty acids**

2. **Cholesterol + O$_2$** $\xrightarrow{\text{Cholesterol Oxidase}}$ **Cholesterol + H$_2$O$_2$**

3. **H$_2$O$_2$ + 4Amino antipyrine + Phenol** $\xrightarrow{\text{Peroxidase}}$ **Red Quinoneimine dye + H$_2$O**

**Reagents:**

1. Enzyme reagent.
2. Diluent / Buffer.
3. Cholesterol standard, 200 mg/dl.

**Preparation of working enzyme reagent:**

The enzyme reagent is dissolved with diluent as per quantity specified on the vial.
Storage:
All reagents are stable at 2 - 8°C upto expiry date mentioned on the label. The reconstituted enzyme is stable up to four weeks at 2-8°C.

Precautions:
Following precautionary measures are taken during the whole procedure.
1. Freezing of the reagents are avoided.
2. Reagents are protected from exposure to sunlight.

Specimen:
Non haemolysed serum samples are used.

Procedure:
Three separate test tubes are taken for Blank, Sample and Standard and marked accordingly. 1000 μl of working enzyme reagent is pipetted in all the three test tubes. Then 10 μl of distilled water, serum and cholesterol standard are added in the test tubes marked as blank, sample and standard respectively as shown in the following table III.2. Reagents are then mixed in a cyclomixer and incubated for 15 minutes at 37°C. After setting the photometer at zero, the optical densities (O. D.) of Blank, Standard and Serum are read at 546 nm against distilled water using the preprogrammed location.

Table III. 2. : Showing the procedure of total cholesterol estimation.

<table>
<thead>
<tr>
<th>Reagents added</th>
<th>Blank</th>
<th>Sample</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working enzyme reagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
<tr>
<td>Serum</td>
<td>—</td>
<td>10 μl</td>
<td>—</td>
</tr>
<tr>
<td>Cholesterol standard (200 mg/ dl)</td>
<td>—</td>
<td>—</td>
<td>10 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 μl</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Incubated for 15 mins at 37°C. Absorbance are taken at 546 nm setting 'O' by distilled water.
Calculation:

Values are calculated with the help of the following formula:

\[
\text{Cholesterol in mg/dl} = \frac{T - B}{S - B} \times \frac{V_t}{V_o} \times S_{100} \times 0.01 \times S_y \times 100
\]

\[= \frac{T - B}{S - B} \times 0.01 \times 200 \times 0.01 \times 0.01 \times 100\]

\[= \frac{T - B}{S - B} \times 200\]

\(T = \) O. D. of Sample
\(S = \) O. D. of Standard
\(B = \) Blank
\(V_o = \) Initial Volume of sample
\(V_i = \) Intermediate Volume
\(V_t = \) Volume taken for the test
\(S_y = \) Volume of standard used.
\(S_{100} = \) Concentration of Standard in percent.

Estimation of total protein:

Total protein content of serum is estimated by following the method of Lowry et al. (1951).

Principle:

Protein reacts with the Folin Ciocalteau reagent to give a coloured complex. The colour is due to the reaction of the alkaline copper with the protein as in the biuret test and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Reagents:

1. Alkaline Sodium carbonate solution:
   
   \((2\% \text{ Na}_2\text{CO}_3 \text{ in } 0.1 \text{ N NaOH})\)
400 mg of NaOH is mixed with 100 ml of distilled water to prepare 0.1 N NaOH solution. To it 2 gm of Na₂CO₃ is added and mixed. Alkaline Na₂CO₃ solution is filtered with a filter paper and stored in an amber coloured bottle at room temperature.

2. (A) Stock Copper Sulphate Solution (1%)

500 mg of copper sulphate is dissolved in 50 ml of distilled water.

(B) Stock Sodium Potassium tartarate solution (2%): 1 gm of Na-K- Tartarate is mixed with 50 ml of distilled water. Reagent (2) is prepared by mixing equal volume of reagent 2 (A) and 2 (b) on the day of use.

3. Alkaline solution:

It is prepared on the day of use by mixing 5 ml of reagent (1) and 100 μl of reagent (2).

4. Folin-Ciocalteau reagent:

This is prepared by mixing 0.5 ml FolinCiocalteau reagent (manufactured by Qualigen fine chemicals) with 1 ml of distilled water.

5. Standard protein solution (stock) 0.1 %

Standard protein solution is prepared by dissolving 10 mg of bovine albumin in 10 ml of 0.9% NaCl solution.

6. Trichloroacetic acid (TCA) (10%)

10 gm of TCA is dissolved in 100 ml of distilled water and stored in an amber coloured bottle.

7. Sodium hydroxide solution (0.5 N)

2 gm of NaOH is mixed with 100 ml of distilled water.
**Procedure:**

1. 10 μl of serum is treated with 600 μl of 10% TCA solution in a 1.5 ml centrifuge tube and mixed properly. After 5 minutes it is centrifuged at 5000 rpm for 10 minutes.

2. Supernatant is discarded. Precipitate is washed two times with distilled water and then dissolved in 0.6 ml of 0.5 N NaOH solution. This sample solution is used for estimation of total protein in blood.

3. In a test tube 1.5 ml of alkaline solution is taken and to it 50 μl of sample solution is added and mixed. Tube is kept at room temperature for 20 minutes.

4. A blank tube is prepared by adding 1.5 ml alkaline solution followed by 50 μl of 0.5 N NaOH solution and kept at room temperature for 20 minutes.

5. A standard tube is prepared by adding 1.5 ml of alkaline solution followed by 50 μl of standard protein solution and kept at room temperature.

6. After 20 minutes, 150 μl of Folin reagent is added in all the tubes (Sample tube, standard tube, blank tube) and mixed and kept again for 30 minutes at room temperature.

7. Absorbance of the blank, standard and sample are measured at 670 nm with deionized water as baseline.

**Calculation:**

\[
\text{Total protein in sample} = \frac{U - B}{S - B} \times 0.1 \times 2 \text{ gm/dl of blood.}
\]

Here, 
U = Absorbance of unknown 
B = Absorbance of blank 
S = Absorbance of standard. 
0.1 = Concentration of standard protein
Estimation of serum triiodothyronine (T₃)

Method:

For estimation of T₃, a microwell solid phase sandwiched enzyme immunoassay method is adopted by using the set of reagents supplied in the form of a kit prepared by Syntron BioResearch, Inc. and marketed as Microwell T₃ E1A, catalog# 3810-A-96.

Principle:

Triiodothyronine (T₃) is estimated by a solid phase enzyme immunoassay utilizing the principle of competitive binding. T₃ in the sample competes with a T₃ analog immobilized of the solid phase microwell surface for a T₃-specific antibody conjugated to horseradish peroxidase. The conjugate binding to the T₃ analog coated solid phase is inversely proportional to the T₃ concentration in sample. The unbound T₃ in sample and conjugate are removed by washing and on addition of colour reagent in the form of 8-Anilino-1-naphthalenesulfonic acid (ANSA) and hydrogen peroxide (H₂O₂) as substrate for the bound horseradish peroxidase. A colour change takes place which is inversely proportional to the amount of T₃ in sample.

Reagents:

2. Enzyme Conjugate: Anti-T₃ monoclonal antibody conjugated with horseradish peroxidase in a protein stabilizing buffer.
3. **Set of reference standards:**
   Buffered protein solutions with 0 ng, 80 ng, 200 ng, 500 ng and 1000 ng of T3 per deciliters of the solution.

4. **Control serum:** Human serum.

5. **Diluent buffer:** Buffered protein solution.

6. **Washing buffer:** Phosphate buffered saline.

7. **Substrate reagent:** 0.02% hydrogen peroxide in 0.05 M acetate buffer.

8. **Colour reagent:** 3, 3', 5, 5'- Tetramethylbenzidine in stabilizing buffer.

9. **Stop solution:** 1 N hydrochloric acid.

**Sample - Serum.**

**Procedure:**

1. The microwell tubes and all the reagents are brought to room temperature prior to starting the test procedure.

2. 30 ml of working wash buffer solution is made by mixing 3 ml of washing Buffer with 27 ml of distilled water.

3. Appropriate number of coated microwells needed for the test batch is determined and a data sheet is marked with appropriate information.

4. Required number of microwells are placed in a well holder.

5. 50 μl of each reference standard, control and test sample is dispensed into the appropriate wells.

6. 100 μl of enzyme conjugate is added into each well.

[134]
7. The wells are gently rocked for 20 sec. for proper mixing of contents.

8. After proper mixing the wells are incubated at 37°C for 60 minutes.

9. After incubation for 60 minute the content of the wells are discarded by decandation into a sink by flicking.

10. The microwells are rinsed and washed five times with the diluted washing buffer.

11. The wells are dried by firmly tapping the inverted wells on a paper towel to remove the excess washing solution.

12. 50 μl of substrate reagent is added to each well and gently rocked for 20 seconds.

13. 50 μl of colour reagent is added to each well and again rocked gently for 20 sec for proper mixing.

14. After mixing the wells are incubated at 37°C for 15 minute.

15. After incubation 50 μl of stop solution is added to each well and mixed by gentle rocking for 30 sec.

16. Absorbance of each well is measured at 450 nm against zero set with distilled water within 20 minutes of adding the stop solution.

**Calculation of Results:**

a) After calculating the average absorbance value \( (A_{450}) \) for each reference standard, control and test sample it is divided by absorbance of the 0ng/dl and multiplied by 100 to obtain \( \%\ A/A_0 \) for each sample by using the formula.

\[
\frac{A_{\text{sample or Standard}}}{A_0} \times 100 = \%\ A/A_0
\]

[135]
b) A standard curve is prepared by plotting the \( \% A/A_0 \) on Y axis and concentration of standard on X axis.

c) Concentration of the samples are determined by obtaining the \( \% A/A_0 \) from absorbance and comparing the results corresponding with the prepared calibration curve shown in table - III.3. and fig. III.1 and III. 2.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>( S_0 ) 0.0</th>
<th>( S_1 ) 80</th>
<th>( S_2 ) 200</th>
<th>( S_3 ) 500</th>
<th>( S_4 ) 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changes in Absorbance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>1.925</td>
<td>1.621</td>
<td>1.195</td>
<td>0.612</td>
<td>0.333</td>
</tr>
<tr>
<td>Set 2</td>
<td>1.928</td>
<td>1.618</td>
<td>1.194</td>
<td>0.611</td>
<td>0.330</td>
</tr>
<tr>
<td>Set 3</td>
<td>1.924</td>
<td>1.617</td>
<td>1.196</td>
<td>0.615</td>
<td>0.339</td>
</tr>
<tr>
<td>Set 4</td>
<td>1.924</td>
<td>1.619</td>
<td>1.192</td>
<td>0.610</td>
<td>0.331</td>
</tr>
<tr>
<td>Set 5</td>
<td>1.926</td>
<td>1.617</td>
<td>1.194</td>
<td>0.614</td>
<td>0.339</td>
</tr>
<tr>
<td>Mean</td>
<td>1.925</td>
<td>1.618</td>
<td>1.194</td>
<td>0.612</td>
<td>0.330</td>
</tr>
</tbody>
</table>

Table - III.3.

**Plotting of the calibration curve**

Mean absorbance of each standard is plotted in the Y axis against the corresponding standard concentration of \( T_3 \) (ng/dl) in the X axis to obtain the calibration curve.

The calibration curve (fig. III.1.) obtained is found to fit in the calculated regression curve (fig. III. 2.) and is judged to be reliable.
Fig. III.1: Calibration curve for estimation of concentration of serum T₃.

Fig. III.2: Regression curve for estimation of concentration of serum T₃.
Estimation of serum thyroxine (T₄)

Method:

For estimation of T₄, a microwell solid phase sandwiched enzyme immunoassay method is adopted by using the set of reagent supplied in the form of a kit prepared by Syntron Bioresearch, Inc. and marketed as Microwell T₄ E1A, catalog# 2210-A-96.

Principle:

Thyroxine (T₄) is estimated by a solid phase enzyme immunoassay utilizing the principle of competitive binding. T₄ in the sample competes with a T₄ analog immobilized of the solid phase microwell surface for a T₄-specific antibody conjugated to horseradish peroxidase. The conjugate binding to the T₄ analog coated solid phase is inversely proportional to the T₄ concentration in sample. The unbound T₄ in sample and conjugate are removed by washing and on addition of colour reagent in the form of 8-Anilino-1-naphthalenesulfonic acid (ANS-A) and hydrogen peroxide (H₂O₂) as substrate for the bound horseradish peroxidase. A colour change takes place which is inversely proportional to the amount of T₄ in sample.

Reagents:

2. Enzyme conjugate: Anti-T₄ monoclonal antibody conjugated with horseradish peroxidase in a protein stabilizing buffer.
3. Set of reference standards: Buffered protein solutions with 0.0µg,
2.0 μg, 4.0μg, 8.0μg, 16.0 μg and 24.0 μg per deciliter of the solution.

5. Diluent buffer : Buffered protein solution.
7. Substrate reagent : 0.02% hydrogen peroxide in 0.05 M acetate buffer.
8. Colour reagent : 3, 3', 5, 5'- Tetramethylbenzidine in stabilizing buffer.
9. Stop solution : 1 N hydrochloric acid

Sample - Serum.

Procedure :
1. The microwell tubes and all the reagents are brought to room temperature prior to starting the test procedure.
2. 30 ml of working wash buffer solution is made by mixing 3 ml of washing buffer with 27 ml of distilled water.
3. Appropriate number of coated microwells needed for the test batch is determined and a data sheet is marked with appropriate information.
4. Required number of microwells are placed in a well holder.
5. 50 μl of each reference standard, control and test sample is dispensed into the appropriate wells.
6. 100 μl of enzyme conjugate is added into each well.
7. The wells are gently rocked for 20 sec. for proper mixing of contents.
8. After proper mixing the wells are incubated at 37°C for 60 minutes.

9. After incubation for 60 minute the contents of the wells are discarded by decandation into a sink by flicking.

10. The microwells are rinsed and washed five times with the diluted washing buffer.

11. The wells are dried by firmly tapping the inverted wells on a paper towel to remove the excess washing solution.

12. 50 µl of substrate reagent is added to each well and gently rocked for 20 seconds.

13. 50 µl of colour reagents is added to each well and again rocked gently for 20 seconds for proper mixing.

14. After mixing, the wells are incubated at 37°C for 15 minutes.

15. After incubation 50 µl of stop solution is added to each well and mixed by gentle rocking for 30 seconds.

16. Absorbance of each well is measured at 450 nm against zero set with distilled water within 20 minutes of adding the stop solution.

**Calculation of Results :**

a) After calculating the average absorbance value (A_{450}) for each reference standard, control and test sample it is divided by absorbance of the 0µg/dl and multiplied by 100 to obtain % A/A_0 for each sample by using the formula

\[
\frac{A_{\text{sample or Standard}}}{A_0} \times 100 = \% \frac{A}{A_0}
\]  

[139]
b) A standard curve is prepared by plotting the $%A/A_0$ Y axis and concentration of standard on X axis.

c) Concentration of the samples are determined by obtaining the $%A/A_0$ from absorbance and comparing the result corresponding with the prepared calibration curve shown in table - III.4. and fig. III.3 and III. 4.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Conc. of $T_4$ (µg/dl)</th>
<th>$S_0$ 0.0</th>
<th>$S_1$ 2.0</th>
<th>$S_2$ 4.0</th>
<th>$S_3$ 8.0</th>
<th>$S_4$ 16.0</th>
<th>$S_5$ 24.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changes in Absorbance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>1.530</td>
<td>1.248</td>
<td>0.950</td>
<td>0.571</td>
<td>0.134</td>
<td>0.062</td>
<td></td>
</tr>
<tr>
<td>Set 2</td>
<td>1.534</td>
<td>1.248</td>
<td>0.953</td>
<td>0.570</td>
<td>0.135</td>
<td>0.061</td>
<td></td>
</tr>
<tr>
<td>Set 3</td>
<td>1.528</td>
<td>1.252</td>
<td>0.949</td>
<td>0.570</td>
<td>0.134</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>Set 4</td>
<td>1.529</td>
<td>1.254</td>
<td>0.948</td>
<td>0.568</td>
<td>0.138</td>
<td>0.061</td>
<td></td>
</tr>
<tr>
<td>Set 5</td>
<td>1.531</td>
<td>1.250</td>
<td>0.952</td>
<td>0.573</td>
<td>0.136</td>
<td>0.058</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.530</td>
<td>1.250</td>
<td>0.950</td>
<td>0.570</td>
<td>0.135</td>
<td>0.061</td>
<td></td>
</tr>
</tbody>
</table>

Table - III.4.

Plotting of the calibration curve

Mean absorbance of each standard is plotted in the Y axis against the corresponding standard concentration of $T_4$ (µg/dl) in the X axis to obtain the calibration curve.

The calibration curve (fig. III.3.) obtained is found to fit in the calculated regression curve (fig. III. 4.) and is judged to be reliable.
Fig. III.3: Calibration curve for estimation of concentration of serum T₄.

Fig. III.4: Regression curve for estimation of concentration of serum T₄.

\[ y = -6E-05x^2 + 0.0058x^2 - 0.1636x + 1.5355 \]

\[ R^2 = 0.9995 \]
Estimation of serum thyroid stimulating hormone (TSH)

Method:

For estimation of TSH, a microwell solid phase sandwiched enzyme immunoassay method is adopted by using the set of reagent supplied in the form of a kit prepared by Syntron Bioresearch, Inc. and marketed as Microwell TSH E1A, catalog# 2211-96.

Principle:

TSH is estimated by a solid-phase enzyme immunoassay based on “sandwiched” principle. Two separate antibodies directed against distinct antigenic determinants of the TSH molecule in sample react simultaneously where one antibody is immobilized on the microwell solid surface and the other antibody is conjugated to horseradish peroxidase enzyme which results in formation of an Ab-Ag-Ab-enzyme complex on the microwell surface. After removal of the unbound conjugate by washing and addition of colour development reagent, the bound enzyme produce a colour change which is proportional to the TSH concentration in sample. The concentration of TSH is calculated against a standard curve obtained with TSH reference standards.

Reagents:

1. Coated Microwells: Polystyrene wells coated with anti-TSH antibody.
2. Enzyme conjugate: Anti-TSH antibody conjugated with horseradish peroxidase in a buffer with protein stabilizer.
3. Set of reference standards: Buffered protein solutions with
0.0μIU, 1.0μIU, 2.5μIU, 5.0μIU, 10.0μIU, 20.0μIU, and 40.0μIU per ml of solution.


5. Diluent buffer: Buffered protein solution.


7. Substrate reagent: 0.02% hydrogen peroxide in 0.05 M acetate buffer.

8. Colour reagent: 3, 3', 5, 5' - Tetramethylbenzidine in stabilizing buffer.

9. Stop solution: 1 N hydrochloric acid

Sample - Serum.

Procedure:

1. The microwell tubes and all the reagents are brought to room temperature prior to starting the test procedure.

2. 30 ml of working wash buffer solution is made by mixing 3 ml of washing buffer with 27 ml of distilled water.

3. Appropriate number of coated microwells needed for the test batch is determined and a data sheet is marked with appropriate information.

4. Required number of microwells are placed in a well holder.

5. 50 μl of each reference standard, control and test sample is dispensed into the appropriate wells.

6. 100 μl of enzyme conjugate is added into each well.

7. The wells are gently rocked for 20 seconds for proper mixing of
8. After proper mixing the wells are incubated for 30 minutes.
9. After incubation for 30 minute the content of the wells are discarded by decandation into a sink by flicking.
10. The microwells are rinsed and washed five times with the diluted washing buffer.
11. The wells are dried by firmly tapping the inverted wells on a paper towel to remove the excess washing solution.
12. 50 µl of substrate reagent is added to each well and gently rocked for 20 seconds.
13. 50 µl of colour reagent is added to each well and again rocked gently for 20 seconds for proper mixing.
14. After mixing the wells are incubated at 37°C for 15 minute.
15. After incubation 50 µl of stop solution is added to each well and mixed by gentle rocking for 30 seconds.
16. Absorbance of each well is measured at 450 nm against zero set with distilled water within 20 minutes of adding the stop solution.

Calculation of Results:

1. Average absorbance value ($A_{450}$) is determined for standards, control and samples.
2. A standard calibration curve is constructed by plotting the mean absorbance obtained for each reference standard against the corresponding concentration in µIU/ml on a graph paper with absorbance value on “Y” axis and the concentration on “X” axis.
3. Using the absorbance value of each sample, the corresponding
Fig. III.5: Calibration curve for estimation of concentration of serum TSH.

Fig. III.6: Regression curve for estimation of concentration of serum TSH.
concentration of TSH in μIU/ml is determined from the standard calibration curve shown in table - III.5. and fig. III.5 and III. 6.

<table>
<thead>
<tr>
<th>Tube No. Conc. of TSH (μIU/ml)</th>
<th>S₀</th>
<th>S₁</th>
<th>S₂</th>
<th>S₃</th>
<th>S₄</th>
<th>S₅</th>
<th>S₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changes in Absorbance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>0.046</td>
<td>0.126</td>
<td>0.220</td>
<td>0.434</td>
<td>0.810</td>
<td>1.528</td>
<td>2.21</td>
</tr>
<tr>
<td>Set 2</td>
<td>0.049</td>
<td>0.123</td>
<td>0.220</td>
<td>0.436</td>
<td>0.812</td>
<td>1.525</td>
<td>2.21</td>
</tr>
<tr>
<td>Set 3</td>
<td>0.045</td>
<td>0.120</td>
<td>0.221</td>
<td>0.432</td>
<td>0.810</td>
<td>1.524</td>
<td>2.26</td>
</tr>
<tr>
<td>Set 4</td>
<td>0.045</td>
<td>0.122</td>
<td>0.224</td>
<td>0.436</td>
<td>0.811</td>
<td>1.524</td>
<td>2.25</td>
</tr>
<tr>
<td>Set 5</td>
<td>0.047</td>
<td>0.124</td>
<td>0.222</td>
<td>0.439</td>
<td>0.809</td>
<td>1.526</td>
<td>2.22</td>
</tr>
<tr>
<td>Mean</td>
<td>0.046</td>
<td>0.123</td>
<td>0.221</td>
<td>0.435</td>
<td>0.810</td>
<td>1.525</td>
<td>2.23</td>
</tr>
</tbody>
</table>

Table - III.5.

Plotting of the calibration curve

Mean absorbance of each standard is plotted in the Y axis against the corresponding standard concentration of TSH (μIU/ml) in the X axis to obtain the calibration curve.

The calibration curve (fig. III.5.) obtained is found to fit in the calculated regression curve (fig. III. 6.) and is judged to be reliable.

Estimation of serum carcinoembryonic antigen (CEA)

Serum carcinoembryonic antigen (CEA) is estimated by solid phase sandwiched enzyme — linked immunoabsorbant assay (ELISA) using reagents prepared by United Biotech Inc. California USA.
Principle:

For the solid phase enzyme linked immunoabsorbant assay of carcinoembryonic antigen in serum micro titre wells are coated with specific anti CEA antibodies. The samples, standard and controls containing CEA are incubated with enzyme conjugate of horseradish peroxidase and anti-CEA antibodies. The antibody coating of the well is unlabelled and the conjugated antibodies are enzyme labelled. On incubation, the antigen (CEA) forms a bridge between the unlabelled antibody on the well and the enzyme labelled antibody in the buffer medium by binding with antibodyies and forming a sandwiched with an antigen core. After incubation, the unbound enzyme conjugated or labelled antibodies are washed off. On addition of hydrogen peroxide as substrate and tetramethylbenzidine as the chromogen, the bound enzyme conjugates develops a colour which is proportional to the CEA (antigen) concentration with maximum absorption at 450 nm which is determined by spectrophotometric principle.

Reagents and materials:

1) Anti CEA antibodies: Coated in polystyrene 250 µl microtitre wells.
2) Enzyme conjugate: Anti - CEA antibodies conjugated to horseradish peroxidase.
3) Sample diluent: Buffers for dilution of samples, standards and controls.
4) CEA reference standards: 5, 10, 25, 50, 250 ng CEA per ml of sample diluent.
5) Substrate solution: Buffer solution containing hydrogen peroxide.
6) Chromogen solution: Tetramethylbenzidine in buffer solution.
7) Developer: 1 (N) H₂SO₄ solution.

Reagent preparation:
In this kit prepared by United Biotech Inc., California, all reagents are supplied in the ready to use form.

Storage and Stability:
1) The reagents set are stored at 2° to 8° C in a refrigerator.
2) The microwells are stored and sealed in a dry bag with the supplied desiccant.
3) The reagents are stable up to the expiry date mentioned on the label when stored and handled as directed.
4) Before use it is always noted that the chromogen solution is colourless.
5) Care is taken not to expose the reagents to strong light during storage and use.

Sample Preparation:
After clot formation and retraction, serum is separated by centrifugation at room temperature and stored as labelled vials at 2°C to 8°C till assay within 8 hours. CEA activity remains unaltered up to six months in frozen condition if thawing is avoided.

Procedure for assay:
1) All samples and reagents are brought to room temperature and
Shaken gently.

2) All reagents and samples are kept in ready position before starting the assay and once the test procedure begins it is performed without any interruption.

3) Desired number of coated wells are secured in the well holder and marked accordingly for identification.

4) 25 μl of standards, controls or serum samples are dispensed into the appropriately marked wells.

5) 100 μl of enzyme conjugate is dispensed into each well.

6) Wells are incubated for 60 minutes at room temperature.

7) Each of the wells are rinsed five times with gently running tap water and the water is removed by gently shaking the wells in inverted position.

8) 100 μl of substrate solution and 100 μl of chromogen solution are dispensed into each well and incubated at room temperature for 30 minutes.

9) The reaction is stopped by adding 50 μl of 1 (N) H₂SO₄ to each well.

10) The absorbances of standards, controls and samples are read at 450 nm against distilled water in a reading spectrophotometer after stabilization for 5 minutes.

Calculation of Results:

Concentration of each reference standards are plotted against absorbance and a calibration curve is prepared based on the observation in the table III.6. The calibration curve fig. III. 7. obtained is found to fit
Fig. III.7: Calibration curve for estimation of concentration of serum CEA.
in the calculated regression curve fig. III. 8. and is judged to be reliable.

CEA values of samples are obtained by comparing the absorbance against the calibration curve.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Conc. of CEA (ng/ml)</th>
<th>( S_0 )</th>
<th>( S_1 )</th>
<th>( S_2 )</th>
<th>( S_3 )</th>
<th>( S_4 )</th>
<th>( S_5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changes in Absorbance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>0.018</td>
<td>0.073</td>
<td>0.141</td>
<td>0.310</td>
<td>0.613</td>
<td>2.861</td>
<td></td>
</tr>
<tr>
<td>Set 2</td>
<td>0.021</td>
<td>0.071</td>
<td>0.140</td>
<td>0.312</td>
<td>0.610</td>
<td>2.863</td>
<td></td>
</tr>
<tr>
<td>Set 3</td>
<td>0.017</td>
<td>0.071</td>
<td>0.140</td>
<td>0.312</td>
<td>0.609</td>
<td>2.867</td>
<td></td>
</tr>
<tr>
<td>Set 4</td>
<td>0.017</td>
<td>0.069</td>
<td>0.143</td>
<td>0.309</td>
<td>0.609</td>
<td>2.864</td>
<td></td>
</tr>
<tr>
<td>Set 5</td>
<td>0.019</td>
<td>0.068</td>
<td>0.138</td>
<td>0.309</td>
<td>0.611</td>
<td>2.861</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.018</td>
<td>0.070</td>
<td>0.140</td>
<td>0.310</td>
<td>0.610</td>
<td>2.863</td>
<td></td>
</tr>
</tbody>
</table>

Table: III.6.

**Estimation of alkaline phosphatase (ALP) activity:**

**Method:**

Alkaline phosphatase (ALP) is determined by the Clonital reagent kit, CAT No. KC 118, 24030 - Carvico, Italy, intended for the quantitative determination of ALP (Orthophosphoric-Monoester Phospho - hydrolase, alkaline Optimum) in serum/plasma. Thus ALP kit is based on the recommendation of the Internal Federation of clinical Chemistry (IFCC) and the method is P-Nitrophenyl Phosphate (P-NPP) Kinetic Method of Mayne (1994). This method utilizes P-Nitro Phenylphosphate (P-NPP) as the substrate and the 2-amino - 2-methyl - 1-propanol (AMP) as the buffer.
Fig. III.8: Regression curve for estimation of concentration of serum CEA.

\[ y = 0.0114x + 0.0243 \]

\[ R^2 = 0.9999 \]
Principle:

Serum ALP hydrolyzes P-NPP into yellow coloured P-Nitrophenol (P-NP) at an alkaline pH. The rate of P-NP formation is directly proportional to the ALP activity and is measured in terms of change in absorbance at 405 nm.

\[
P - \text{NPP} + H_2O \xrightarrow{\text{ALP}} P - \text{NP} + \text{Phosphate} \]

\[
\begin{align*}
\text{QN} & \quad \text{O} - \quad \text{P} - \quad \text{O}^- + H_2O \quad \text{QN} & \quad \text{O}^- + \text{HPO}_4^{2-} + H^+ \\
\end{align*}
\]

Reagents: 1. P — Nitrophenylephosphate (P — NPP substitute)

2. Buffer solution.

Preparation of working peagent

The enzyme reagent is prepared by reconstituting one vial (10ml) of P — NPP substrate with 10 ml to buffer solution.

Storage and Stability

All reagents are stable at 2°C - 8°C till the expiry date mentioned on the label. Reconstituted reagent is stable for atleast one month at 2°C - 8°C.

Precaution:

The absorbance of reconstituted reagents increases slowly on
storage at 2°C - 8°C., however, this does not affect its performance. It is discarded if absorbance of working reagent exceeds 0.8 against distilled water.

Specimen:

Non-haemolyzed serum is used for estimation of ALP.

Procedure:

500 µl of reconstituted reagent is taken in a measured dry microcentrifuge tube and added 10 µl of sample serum. Then the reagents and sample serum are mixed and immediately the change of absorbance is measured at 405 nm against a distilled water base line at intervals of 60 to 180 second for estimation of change of absorbance per second.

Calculation

Serum ALP activity in u/l = $\Delta_{405} \times F$

where, $\Delta$ = Change of absorbance per minute at 405 nm.

$F$ = Kinetic factor for the set of reagents supplied with the kit which depends on molar extinction coefficient of paranitrophenol at 405 nm, reagent volume and light path. For the set of reagents used it is 2742.
STATISTICAL ANALYSIS:

All the data obtained during the period of investigation are statistically analysed after Croxton (1953). The mean, the standard deviation of mean, the standard error of mean and coefficient of variation (%) for each set of data are calculated and compared between different sets of data by applying standard statistical procedure to evaluate the changes among different groups in the study.

The level of significance between two sets of data are calculated according to student “t” test. Probability i.e. p value at 5 percent or lower for two sets of data are taken as significant.

Formulae used for statistical analysis.

1. $\bar{x} = \frac{\sum x}{n}$

2. $Df = n - 1$

3. $SD = \sqrt{\frac{\sum (x-\bar{x})^2}{n-1}}$

4. $SEM = \frac{SD}{\sqrt{n}}$

5. $CV\% = \frac{SD}{\bar{x}} \times 100$

6. $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(SEM_1)^2 + (SEM_2)^2}}$
BIBLIOGRAPHY


Croxton, F. E., 1953: Elementary statistics with application in medicine and biological science. Dover, New Delhi.


Syntron Bioreresearch, Inc. An enzyme immunoassay For Quantitative Determination of Thyroxine Hormone in serum or plasma. Catalog No. 2210-A

Syntron Bioreresearch, Inc. An enzyme Immunoassay For Quanatitative Determination of Thyroid Stimulation Hormone in serum or plasma. Catalog. No. 2211

Syntron Bioreresearch, Inc. An enzyme Inmunoassay : For Quantitative Determination of Triidothyronine in serum or plasma. Catalog No. 3810-A


* Original not consulted