CHAPTER – III

MATERIALS AND METHODS
The present study on the effect of curcumin on the primary antioxidant status and mixed function oxygenase enzyme system during cholangrene induced carcinogenesis is conducted under the following scheme:

![Schematic representation of different groups.](image)

The study is conducted on 120 randomly selected healthy male albino mice weighing between 25 to 30 grams. Before the experimental
procedure is 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):** 10 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 groups:** Animals selected for the experimental groups are further subdivided into five sub groups as:

**Group II (Castor oil control group):** 10 healthy animals are randomly selected for the group and each animal of the group is exposed to single dose of 0.25ml (250 µl) of castor oil by intraperitoneal injection.

**Group III (Curcumin treated group):** This group consists of 10 healthy albino mice each of which is fed daily with 10mg curcumin powder.

**Group IV (3-Methyl Cholanthrene [3MC] treated group):** This group consists of 10 randomly selected animals from the general normally healthy pool of already acclimatized animal for the study. A single dose of 0.5mg of 3-methylcholanthrene in 0.25ml (250 µl) of castor oil is administered intraperitoneally to each animal of these groups. During the whole period of experiment these group received normal standard diet.

**Group V (Curcumin supplemented 3MC treated group):** 10 animals selected from the acclimatized general pool are intraperitoneally
administered with 0.5mg of 3-methylcholanthrene in 0.25ml (250 μl) castor oil with simultaneous daily supplementation of 10mg curcumin as in the curcumin treated group.

Identification marking of the animals

All the animals of different groups are kept in properly labeled 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 records

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 samples

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-tetra-acetate) vial and thoroughly mixed by gentle rotation in an 8 cm diameter circle. The remaining 0.5 ml of blood is transferred to a micro centrifuge 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 stick. 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 centrifuge tube and stored at 2° to 8° C for estimation of the proposed parameters.

Frequency of 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 groups along with the normal control group on 10th, 15th, 20th, 25th, 30th, 45th, 60th, 75th, 90th and 120th day of treatment.

Parameters for biochemical evaluation

The collected samples of blood and serum from each of the groups of different days of experiment are utilised for estimations of:

(a) Antioxidant enzyme: Activities of the following antioxidant enzymes are estimated in blood and tissues:

(i) Superoxide dismutase (SOD) and Glutathione peroxidase (GPx): The activities of superoxide dismutase and glutathione peroxidase (GPx) are estimated in whole blood.
(ii) **Catalase (CAT):** The activity of catalase enzyme is estimated in whole blood and liver, kidney and stomach tissues.

(b) **Mixed function oxygenase (MFO) enzymes:** Activities of the following MFO enzymes are estimated in liver, kidney and stomach tissues.

   (i) **Aryl hydrocarbon hydroxylase (AHH).**
   
   (ii) **Cytochrome P450.**
   
   (iii) **Xanthine oxidase (XOD).**

(c) **Lipid Peroxide (LPO):** Lipid peroxide is estimated in blood and in three different tissues – liver, kidney and stomach.

(d) **Alfa-fetoprotein (AFP):** Alfa-fetoprotein in serum is estimated as cancer marker.

**Collection of tissues**

The mice are an anaesthetized by diethyl ether and dissected to collect the liver, kidney and stomach tissues. The tissues are dried over a filter paper and immediately weighted and recorded. The tissue homogenate is prepared in deionised water with the help of homogeniser.

Tissues are collected from normal control as well as experimental mice on the desired days i.e. 30th day, 60th day, 90th day and 120th day of treatment.
Methods of evaluation

All the biochemical estimations are done by using computer assisted semiautomatic ‘BTS320 photo-meter’. 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.

Estimation of Superoxide dismutase activity

Superoxide dismutase (SOD) activity in whole blood is estimated by using the Ransod set of reagent kit marketed by the Randox Laboratories Ltd., following the method of Marklund and Marklund (1974).

Assay principle

Superoxide dismutase (SOD) accelerates the dismutation of the toxic superoxide radical (O$_2^-$), produced during the oxidative process. Dismutation of superoxide radical (O$_2^-$) produces molecular oxygen (O$_2$) and hydrogen peroxide (H$_2$O$_2$). In this method superoxide radical is generated by catalytic activity of xanthine oxidase (XOD) on xanthine. The generated superoxide (O$_2^-$) radical reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye with maximum absorbance at 505 nm. In presence of superoxide dismutase the generated superoxides are rapidly dismuted and the formation of the red formazan dye is inhibited. The degree of inhibition of the formazan dye formation is a measure of superoxide dismutase activity.
Reagents

1) **Mixed substrate:** Supplied as powder in sealed vacuum packed vials to be reconstituted by addition of specific volume of buffer for reconstitution as mentioned in the label. This reagent contains xanthine and I.N.T.

2) **Buffers for reconstitution:** Supplied ready for use and stable up to the expiry date mentioned on the label when stored at 2°C to 8°C. It contains CAPS 50 m mol/litre and EDTA 0.94 m mol/litre with pH 10.2.

3) **Xanthine oxidase:** Supplied in vacuum-sealed vials as lyophilized powder to be reconstituted with deionised water.

4) **SOD standard:** Supplied as lyophilized powder in vacuum sealed vials for reconstitution.
Preparation of working reagents

1) **Mixed substrate:** One vial of mixed substrate is reconstituted with 20 ml of buffer for reconstitution. On reconstitution it is of pH 10.2 and contains:

- Xanthine 0.05 m mol/litre
- I.N.T. 0.025 m mol/litre
- CAPS 50 m mol/litre
- EDTA 0.94 m mol/litre

The reconstituted mixed substrate-working reagent is stable for 10 days when stored at 2° to 8°C.

2) **Xanthine oxidase:** One vial of xanthine oxidase is reconstituted with 10 ml of deionised water. On reconstitution it contains 80U of xanthine oxidase per litre of reconstituted working reagent. The reconstituted working reagent is stable for 15 days when stored at 2° to 8°C.

3) **SOD stock standard:** One vial of lyophilized powder supplied as SOD standard is reconstituted with 10 ml of deionised water to give a stock standard solution of SOD with a concentration as mentioned on the label of the vial. The stock standard solution is stable up to 15 days after reconstitution when stored at 2° to 8°C.

4) **Diluent phosphate buffers for preparation of working SOD standards:** 54 mg of Sodium dihydrogen phosphate (NaH$_2$PO$_4$, 2H$_2$O) and 86 mg of Disodium hydrogen phosphate (Na$_2$HPO$_4$) is
dissolved in 100 ml of deionised water to prepare a 10 mM, pH 7.0 phosphate buffers. This is stable for 7 days when stored at 2° to 8°c.

5) **Preparation of SOD working standards:** A series of SOD working standards are prepared by diluting the SOD stock standards with diluent phosphate buffer as tabulated below to produce a standard calibration curve.

<table>
<thead>
<tr>
<th>Standard no.</th>
<th>Vol. stand. Taken (µl)</th>
<th>Vol. diluent phosphate buffer added (µl)</th>
<th>Concentration of working standard (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7 (Stock)</td>
<td>1000</td>
<td>000</td>
<td>X/1 = 5.300</td>
</tr>
<tr>
<td>S6</td>
<td>500 µl of S7</td>
<td>500</td>
<td>X/2 = 2.600</td>
</tr>
<tr>
<td>S5</td>
<td>500 µl of S6</td>
<td>500</td>
<td>X/4 = 1.325</td>
</tr>
<tr>
<td>S4</td>
<td>500 µl of S5</td>
<td>500</td>
<td>X/8 = 0.662</td>
</tr>
<tr>
<td>S3</td>
<td>500 µl of S4</td>
<td>500</td>
<td>X/16 = 0.331</td>
</tr>
<tr>
<td>S2</td>
<td>500 µl of S3</td>
<td>500</td>
<td>X/32 = 0.165</td>
</tr>
<tr>
<td>S1</td>
<td>500 µl of S2</td>
<td>500</td>
<td>X/64 = 0.082</td>
</tr>
<tr>
<td>S0</td>
<td>500 µl of deionised water</td>
<td>500</td>
<td>X/α = 0.00 (Blank)</td>
</tr>
</tbody>
</table>

The SOD activity of the lyophilised standard powder supplied with each batch of reagent set is indicated on the label and the concentration of
the working standards are calculated accordingly for each batch of reagents. The working standards are stable for 48 hours at 2° to 8°c.

**Preparation of calibration curve**

1. A calibration curve is prepared with the set of SOD working standards of different concentrations obtained and numbered as S₀, S₁, S₂, S₃, S₄, S₅, S₆, and S₇.
2. All reagents and standards are brought to room temperature before proceeding with the procedure.
3. A set of eight disposable 1.5 ml polystyrene tubes are marked as S₀, S₁, S₂, S₃, S₄, S₅, S₆, and S₇ corresponding to different concentrations of SOD standards.
4. 250µl of mixed substrate reagent is transferred into each of the numbered tubes.
5. 10 µl of SOD working standard are transferred to correspondingly numbered tubes and mixed well by a cyclo mixer.
6. The whole set of tubes are allowed to equilibrate at 37°c in an incubator.
7. The spectrophotometer is set to record absorbance at 505 nm with a light path of 1 cm at 37°c against deionised water in two fixed times of initial absorbance after 30 seconds as A₁ and final absorbance after 3 minutes as A₂.
8. After attainment of equilibrium at 37°c, 50µl of xanthine oxidase reagent is added to a tube and mixed well. The tube is immediately presented to the spectrophotometer for measurement of absorbance at the pre-set mode.
9. The procedure is repeated five times with each standard and the corresponding absorbance is recorded.

Calculations

(i) Change of absorbance per minute is determined by the formula:

\[
\frac{A_2 - A_1}{3} = \Delta A/\text{min.},
\]

Where \( A_1 \) is the initial and \( A_2 \) is the final absorbance.

(ii) Percentage inhibition is calculated for each set of absorbance recorded by taking the rate of change in the uninhibited reaction in the Reagent Blank (\( S_0 \)) as 100% and the rate of changes of absorbance in the standards are converted to percentage of the Reagent Blank rate and the percentages of Blank rate are subtracted from 100% to obtain the percentage inhibition as:

\[
100 - \frac{(\Delta A \text{ std/min} \times 100)}{(\Delta A \text{ Blank/min})} = \% \text{ inhibition of standard.}
\]

Plotting of the calibration curve

Percentage inhibition of each standard is plotted in the Y-axis against the standard concentrations in SOD unit/ml in the X-axis to obtain the calibration curve (fig.III.2). The logarithmic straight line (exponential curve \( X_y = ab^x \)) is used when the series is increasing or decreasing by a constant percentage rather than a constant absolute amount. In this case, the data plotted on a semi-logarithmic scale gives a straight-line graph.
The calibration curve obtained is found to fit in the calculated regression curve (fig.III.3) and is judged to be reliable.

The values of % inhibition obtained by using working SOD standards prepared from a stock SOD standard of 5.30 Unit/ml is presented in the Table. III. 2.

Table. III. 2.

<table>
<thead>
<tr>
<th>Tube No.</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>
<th>S_6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of SOD in Unit/ml</td>
<td>0.000</td>
<td>0.165</td>
<td>0.331</td>
<td>0.662</td>
<td>1.325</td>
<td>2.600</td>
<td>5.300</td>
</tr>
<tr>
<td>(Change in % inhibition)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>0</td>
<td>13</td>
<td>27</td>
<td>43</td>
<td>53</td>
<td>65</td>
<td>83</td>
</tr>
<tr>
<td>Set 2</td>
<td>0</td>
<td>14</td>
<td>29</td>
<td>45</td>
<td>55</td>
<td>71</td>
<td>82</td>
</tr>
<tr>
<td>Set 3</td>
<td>0</td>
<td>13</td>
<td>27</td>
<td>41</td>
<td>57</td>
<td>69</td>
<td>80</td>
</tr>
<tr>
<td>Set 4</td>
<td>0</td>
<td>15</td>
<td>26</td>
<td>42</td>
<td>53</td>
<td>72</td>
<td>81</td>
</tr>
<tr>
<td>Set 5</td>
<td>0</td>
<td>13</td>
<td>28</td>
<td>38</td>
<td>55</td>
<td>64</td>
<td>79</td>
</tr>
<tr>
<td>Mean</td>
<td>0.00</td>
<td>13.60</td>
<td>27.40</td>
<td>41.80</td>
<td>54.60</td>
<td>68.20</td>
<td>81.00</td>
</tr>
</tbody>
</table>

Sample preparation and estimation

1) 10 µl of EDTA whole blood sample is diluted with 500 µl of ice-cold deionized water and allowed to stabilize at room temperature.

2) 250 µl of mixed substrate reagent is taken in marked polystyrene tubes and 10 µl of the diluted sample is added and mixed.

3) 50 µl of xanthine oxidase reagent is added, mixed and immediately sipped into the spectrophotometer for measurement of absorbance.
Fig. III. 2. Calibration curve for estimation of SOD activity.
Fig. III. 3. Regression curve for estimation of SOD activity.

$y = 13.55x - 13.257$
at the pre-set mode identical with the mode used for the calibration curve.

4) The change of absorbance and percentage inhibition of the samples are calculated by the same procedure as for the standards.

5) The calibration curve is utilized to convert percent inhibition of samples to unit of SOD in the diluted sample used.

6) SOD Unit /ml of diluted sample obtained from the calibration curve is converted to the SOD Unit /ml of whole blood in the samples by the calculation:

\[
\text{SOD Unit /ml of whole blood} = \text{SOD Unit /ml from the curve} \times \text{dilution factor.}
\]

**Estimation of Glutathione peroxidase activity**

Glutathione peroxidase is estimated in whole blood by following the method of Paglia and Valentine (1967) using Ransel set of reagent kit marketed by Randox Laboratories Ltd.

**Principle**

Glutathione peroxidase catalyses the oxidation of reduced glutathione (GSH) by cumene hydroperoxide (ROOH) to oxidized glutathione (GSSG) with formation of cumene hydroxide (ROH) and water. In presence of the enzyme glutathione reductase (GR) and the coenzyme nicotinamide adenine dinucleotide phosphate in its reduced form
(NADPH), the oxidized glutathione (GSSG) formed due to catalysis by glutathione peroxidase (GPx) is immediately reconverted to the reduced form (GSH) with a concomitant oxidation of NADPH to the oxidized form of the coenzyme (NADP\(^+\)). The reduced form of the coenzyme (NADP) absorbs maximally at 340 nm and there is decrease in absorbance with formation of NADP\(^+\), which is proportional to the rate of oxidized glutathione (GSSG) formed due to initial glutathione peroxidase activity. A unit of glutathione peroxidase activity is taken as the amount of enzyme, which consumes 1 \(\mu\) mol of NADPH per minute.

**Reaction principle**

\[
\begin{align*}
\text{GPx} & \quad 2\text{GSH} + \text{ROOH} \quad \rightarrow \quad \text{ROH} + \text{GSSG} + \text{H}_2\text{O} \\
\text{GR} & \quad \text{GSSG} + \text{NADPH} + \text{H}^+ \quad \rightarrow \quad \text{NADP}^+ + 2\text{GSH}
\end{align*}
\]

**Reagents**

1) **Ransel reagent**: Obtained as lyophilized powder in vacuum-sealed vials to be reconstituted before use. Vacuum-sealed contents are stable up to expiry date mentioned on labels and stored at 2\(^0\) to 8\(^0\) C.

2) **Buffer for reconstitution of Ransel reagent**: Contents supplied as ready for use. Stable up to expiry date when stored at 2\(^0\) to 8\(^0\) C.

3) **Cumene hydroperoxide**: Supplied as concentrate to be diluted before use. Concentrate stable up to labeled expiry date when stored at 2\(^0\) to 8\(^0\) C.
4) **Sample diluent:** Supplied as concentrate powder to be reconstituted before use. Stable up to mentioned expiry date when stored at 2°C to 8°C.

5) **Drabkin’s reagent:** Obtained as concentrate to be diluted before use. Concentrate stable up to labelled expiry date on storage at 2°C to 8°C.

6) **Distilled water:** Deionised water is used within a month of preparation and stored at room temperature.

**Reconstitution and preparation of working reagents**

Before reconstitution and preparation of working reagents all stock reagents and solutions are allowed to equilibrate with room temperature.

1) **Ransel reagent:** After equilibration with room temperature the vacuum-sealed vial containing the lyophilized powder is opened carefully to sudden inflow of air jet. Appropriate volume of buffer for reconstitution as marked on the Ransel reagent vial is then transferred to the vial with a transfer pipette and mixed properly by gentle swirling movement to prevent formation of foam after replacing the vial cap. The reconstituted reagent is used either within two hours or stored at 4°C for next subsequent use. It is stable for eight hours at 15°C to 25°C and up to 48 hours at 2°C to 8°C.

2) **Cumene hydroperoxide solution:** 10 ml of deionised water is taken in an amber coloured leakproof stoppered reagent bottle of 25 ml capacity. 10μl of the supplied concentrate of cumene
hydroperoxide is measured with a positive displacement glass capillary pipette and dispersed into the deionised water and mixed thoroughly in the leakproof capped container vigorously for five minutes. It is then allowed to stabilize for ten minutes before use. This working solution is prepared fresh for daily use and the unused portion is discarded after the day’s work.

3) **Sample diluent:** Concentrate powder supplied in one vial is reconstituted with appropriate amount of deionised water as specified on the label of each vial. The reconstituted sample diluent is stable for 4 weeks when stored at 2\(^0\) to 8\(^0\) C or for three days at 15\(^0\) to 25\(^0\) C.

4) **Working Drabkin’s reagent:** The 20 ml concentrate dispensed in one vial is transported volumetrically to a 500 ml volumetric flask and the final volume is adjusted at the 500 ml mark with deionised water. Mixed properly and transferred to amber coloured capped reagent bottle for storage. The working reagent is stable for six months or labelled expiry date, which one is earlier, when stored at 15\(^0\) to 25\(^0\) C.

**Reagent storage**

All reagents are stored at 2\(^0\) to 8\(^0\) C and brought to room temperature before use. Proportionately large volume reagents relative to daily work load are stored as batches of aliquots to avoid repeated change in storage and working temperature.
Composition of working reagents

The working reagents have the contents and concentration as tabulated below:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Contents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ransel reagent</td>
<td>Glutathione, Glutathione Reductase, NADPH</td>
<td>4 m mol/litre, ≥ 0.5 u/litre, 0.28 m mol/litre</td>
</tr>
<tr>
<td>2. Reconstituted Buffer</td>
<td>Phosphate, Buffer EDTA</td>
<td>0.05 mol/litre, pH 7.2, 4.3 m mol/litre</td>
</tr>
<tr>
<td>3. Cumene Hydroperoxide solution</td>
<td>Cumene Hydroperoxide</td>
<td>0.18 m mol/litre</td>
</tr>
<tr>
<td>4. Sample diluent</td>
<td>Reductant, Phosphate Buffer</td>
<td>Not specified, 0.05 mol/litre, pH 7.2</td>
</tr>
<tr>
<td>5. Drabkin’s reagent</td>
<td>Potassium ferricyanide, Sodium cyanide, Potassium dihydrogen Phosphate</td>
<td>200 mg/litre, 50 mg/litre, 140 mg/litre</td>
</tr>
</tbody>
</table>

Sample preparation

1) 500 µl of sample diluent is taken in a 1500 µl hinged cap disposable polypropylene tube.
2) 10 µl of whole blood sample is added and mixed properly in a vortex mixer.
3) After mixing the tube is incubated at 37°C for five minutes.
4) 500 µl of Drabkin's working reagent is added and remixed in a vortex mixer.

5) A reagent blank is prepared similarly by using only 10 µl of redistilled water from a sample collection vial.

6) The prepared samples are assayed within 20 minutes of adding Drabkin's reagent.

Procedure

1) 500 µl disposable-capped polypropylene tubes are marked for reagent blank and samples.
2) 250 µl of Ransel reagent is taken in each tube.
3) 10 µl of prepared blank is added to the tube marked for reagent blank and mixed by vortex.
4) 10 µl of diluted and prepared sample is added to the tube marked for sample and mixed by vortex.
5) The photometer is set ready for measuring absorbance at 340 nm at 60-second intervals against a baseline set at zero with deionised water and light path of 1 cm with a sip-in volume of 200 µl.
6) 10 µl of working cumene hydroperoxide solution is added to a marked reagent blank or prepared sample tube and vortexed.
7) Absorbance is measured at 60 seconds interval to 180 seconds.
8) Reagent blank values are subtracted from that of the sample and change of absorbance is determined and results are calculated.

Calculation of result

Changes of absorbance per minute = ΔA/min.
\[ \Delta A/min = A_S - A_B \]
\[ = (A_{S60} - A_{S180}) - (A_{B60} - A_{B180}) \]

Where, \( A_S \) = Change of absorbance in sample.
\( A_B \) = Change of absorbance in blank
\( A_{S60} \) = Absorbance of sample at 60 seconds.
\( A_{S180} \) = Absorbance of sample at 180 seconds.
\( A_{B60} \) = Absorbance of blank at 60 seconds.
\( A_{B180} \) = Absorbance of blank at 180 seconds

\[
GPx \text{ in } \mu/ml \text{ of whole blood} = \frac{\Delta A_{340} \times TRV \times D_F}{LPcm \times \mu \text{ mol Ab} \times Vs \text{ ml}}
\]
\[ = \Delta A_{340} \times \frac{0.270 \times 1010}{1 \times 5.587 \times 0.01}
\]
\[ = \Delta A_{340} \times 4880.9 \]

Where, \( \Delta A \) = Average change of absorbance per minute.
\( TRV \) = Total reaction volume
\( D_F \) = Dilution factor
\( LPcm \) = Light path in cm
\( \mu \text{ mol Ab} \) = Micro molar absorptivity of NADPH
\( Vs \text{ ml} \) = Sample volume in ml.

**Standardization**

1) **Preparation of glutathione peroxidase standards:** Ransel control (cat no. SC 692) available as lyophilized powder in sealed vials is reconstituted with appropriate volumes of buffer for reconstitution to
give a stock standard solution of concentration equivalent to 1000 Unit /mol of glutathione peroxidase.

2) The prepared stock standard is diluted with sample diluent to give working standards of different concentrations covering from 0.0 Unit/ml to 1000 Unit /ml as tabulated below (Table III.3).

<table>
<thead>
<tr>
<th>Standard/tube no.</th>
<th>S₀ (Blank)</th>
<th>S₁</th>
<th>S₂</th>
<th>S₃</th>
<th>S₄</th>
<th>S₅</th>
<th>S₆</th>
<th>S₇</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock standard (100 Unit /ml) in µl</td>
<td>0</td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>600</td>
<td>800</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Sample diluent in µl</td>
<td>1000</td>
<td>900</td>
<td>800</td>
<td>600</td>
<td>400</td>
<td>200</td>
<td>0000</td>
<td>0000</td>
</tr>
<tr>
<td>Diluted standard in µl</td>
<td>0</td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>600</td>
<td>800</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

3) The diluted standards are treated exactly as samples for estimation of GPx.

<table>
<thead>
<tr>
<th>Reagent/tube no.</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>GPx reagent (µl)</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Working standard(µl)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cumene HP</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
4) The procedure is repeated five times to obtain a set of readings and the mean change of absorbance with different concentrations of GPx is obtained as follows (Table. III.5)

<table>
<thead>
<tr>
<th>Conc. of GPx in Unit /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>Change in absorbance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>0.000</td>
<td>0.074</td>
<td>0.142</td>
<td>0.296</td>
<td>0.592</td>
<td>0.776</td>
<td>0.982</td>
</tr>
<tr>
<td>Set 2</td>
<td>0.000</td>
<td>0.078</td>
<td>0.141</td>
<td>0.292</td>
<td>0.594</td>
<td>0.772</td>
<td>0.984</td>
</tr>
<tr>
<td>Set 3</td>
<td>0.000</td>
<td>0.076</td>
<td>0.140</td>
<td>0.290</td>
<td>0.590</td>
<td>0.774</td>
<td>0.980</td>
</tr>
<tr>
<td>Set 4</td>
<td>0.000</td>
<td>0.072</td>
<td>0.142</td>
<td>0.296</td>
<td>0.592</td>
<td>0.772</td>
<td>0.981</td>
</tr>
<tr>
<td>Set 5</td>
<td>0.000</td>
<td>0.074</td>
<td>0.145</td>
<td>0.294</td>
<td>0.593</td>
<td>0.775</td>
<td>0.982</td>
</tr>
<tr>
<td>Mean</td>
<td>0.000</td>
<td>0.075</td>
<td>0.142</td>
<td>0.294</td>
<td>0.592</td>
<td>0.774</td>
<td>0.982</td>
</tr>
</tbody>
</table>

5) A calibration curve (Fig. III. 4) is prepared by plotting changes in absorbance against concentration of GPx (Table. III. 5.) in a linear graph. The calibration curve obtained is found to fit in the calculated regression curve (Fig. III. 5.) and is judged to be reliable.
Fig. III. 4. Calibration curve for estimation of GPx activity.
Fig. III. 5. Regression curve for estimation of GPx activity.

The regression equation is:

\[ y = 0.342x - 0.0553 \]
Estimation of Catalase activity

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

Principle

In the ultraviolet range $H_2O_2$ shows a continued increase in absorption with decreasing wavelength. Decomposition of $H_2O_2$ 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

Phosphate buffer (50 mM; pH 7.0): 6.81 gm $KH_2PO_4$ is dissolved in deionised water and volume is made up to 1000 ml to prepare solution-A. 8.9 gm of $Na_2HPO_4 \cdot 2H_2O$ is dissolved in deionised 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°C to 8°C.

Standard Hydrogen peroxide (30mM): 0.34 ml of 30% $H_2O_2$ is diluted with phosphate buffer to 100 ml. This solution is prepared afresh every day.

Sample for blood: Anticoagulated (EDTA) venous blood.
Sample for tissue: Measured amount of tissue is collected from the mice and washed with 0.9% NaCl to remove blood. Tissue homogenate is prepared by using a homogeniser in a concentration of 10mg tissue per ml of deionised water. 1 ml of tissue homogenate is taken in a centrifuge tube and centrifuged for 10 minutes at 5000 rpm. Supernatant portion is used to estimate enzyme activity.

Procedure

1) 10 µl of sample is added to 1000 µl 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 µl of 30 mM H2O2 solution is added and mixed and changes of absorbance is noted at 10-second intervals up to 60 seconds.

Calculation

\[
\text{Catalase activity in whole blood} = 0.153 \times \log \frac{E_1}{E_2} \times 3.4 \times 1000 \text{ Unit/litre.}
\]

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 Aryl hydrocarbon hydroxylase activity

Principle

Aryl hydrocarbon hydroxylase activity is determined by the method of Nebert and Gelboin (1968) based on the principle that when benzo [a] pyrene is used as substrate with NADPH as the co-substrate, there is proportional change in absorbance in 396 nm with changes in enzyme activity with production of 3-hydroxybenzo [a] pyrene and the enzyme activity can be expressed in terms of substrate converted or product produced per minute.

Reagents
1. Tris-chloride buffer (pH 7.5)
2. NADPH Solution (18 μM/ dl)
3. MgCl₂6H₂O
4. Benzo [a] pyrene (200 mM/dl)
5. Acetone
6. NaCl Solution (0.95%)

Preparation of reagents

1. Tris Chloride buffer (pH=7.5)
   10 gm of Tris is dissolved in 1000 ml of deionised water and pH is finally adjusted by addition of 0.1N HCl and checked with a digital pH meter. The buffer is then stored in an amber coloured bottle.

2. NADPH Solution (18 μM/dl)
   149 mg of NADPH is dissolved in 100 ml of deionised water and stored in 4°C in a refrigerator.

3. MgCl₂ Solution
   120 mg of MgCl₂.6H₂O is dissolved in 100 ml of deionised water and stored in a dark bottle.

4. Benzo [a] Pyrene Solution (substrate)
   5 mg of Benzo [a] pyrene is dissolved in 100 ml methanol and stored in a dark bottle.

5. Acetone
   Analar grade acetone is used.

6. NaCl Solution (0.9%)
   900 mg of NaCl is dissolved in 100 ml of deionised water to prepare 0.9% solution and the solution is stored in a dark bottle.

Preparation of sample

1. Measured amount of tissue is collected from the mice and washed with 0.9% NaCl solution
2. Tissue homogenate is prepared with the help of homogenizer in a concentration of 10 mg tissue per ml of deionized water.

3. Before centrifugation, the tissue homogenate is diluted with deionized water in the ration of 1: 50.

4. 1 ml of diluted homogenate is taken in a centrifuge tube and centrifuged in an ultracentrifuge (at 40,000 rpm) for 20 minutes and the supernatant is used as sample for the estimation of enzyme activity.

Procedure

1. In a test tube 0.6 ml of Tris-Chloride buffer, 0.15 ml of MgCl₂ solution, 0.2 ml of NADPH solution and 0.1 ml of Benzo [a] pyrene solution are added and mixed. To it 50 µl of sample is added and mixed. The mixture is shaken at 37°C for 30 minutes in air. The reaction is stopped by the addition of 0.1 ml of cold acetone.

2. A control tube is prepared by adding all the above reagents and sample with addition of 0.1 ml of cold acetone prior to incubation and treated identically as the sample.

3. A blank tube is prepared with all the reagents used for sample where the 50 µl of sample homogenate is replaced by 50µl of Tris-Chloride buffer.

4. Absorbance of sample and control tube is recorded at 396 nm against the blank set as zero.

Calculation

\[ V_R = \frac{[A_U - A_C] \times D_F}{M_{E396}} \]
\[ A_R \times \frac{50}{1 \times 0.05} \times \frac{1.2}{0.15} = A_R \times 8000 \text{ Unit/litre of homogenate} \]

Where, \( A_U \) = Absorbance of unknown
\( A_C \) = Absorbance of Control
\( D_F \) = Dilution factor for homogenate
\( V_R \) = Total volume of all reactants
\( M_{E396} \) = Molar extinction coefficient at 396 nm
\( A_R \) = Change of absorbance during the reaction.

The final results of Aryl hydrocarbon hydroxylase (AHH) activity are expressed as Unit/mg.

**Estimation of Cytochrome P-450 activity**

Activity of Cytochrome P-450 in the tissue of mice is estimated by the method of Omura and Sato (1964).

**Principle**

Cytochrome P-450, the terminal oxidase of MFO system is haemoprotein in nature and it is a reducible pigment and only the reduced form could bind carbon monoxide (CO). The CO compound of the reduced pigment has an intense absorption band at 450 nm and thus can be readily detected in dithionite treated state by differential spectrophotometry. The CO absorption spectrum of reduced P-450 shows no peaks other than at 450 nm.
Reagents

1. **NaCl Solution (0.9%)**
   It is prepared by dissolving 900 mg of NaCl in 100ml of deionized water.

2. **Sodium dithionite (Na₂S₂O₄)**
   60 mg of Na₂S₂O₄ is dissolved in 2 ml of deionized water just before use and the reagent is prepared freshly every day.

3. **Carbon monoxide (CO)**
   It is prepared by the reaction of oxalic acid and conc. sulphuric acid.
   In a 50 ml pyrex flask fitted with all glass gas tight stopper with a bent delivery tube about 20gm of oxalic acid crystals are taken and about 5 ml of conc. H₂SO₄ is added and stoppered. The flask is then heated by using a dry electric hot bath mantle with thermostat control and the evolved gas is purified by passing it through an all glass gas tight sodalime column. The purified gas containing only pure carbon monoxide (CO) is used as the reagent.

Procedure

1. Measured amount of tissue is collected from the mice and washed with 0.9 % NaCl solution to remove blood.

2. Tissue homogenate is prepared by using a glass homogenizer in a concentration of 10 mg tissue per ml of deionized water.

3. 2 ml of tissue homogenate is taken in a centrifuge tube and centrifuged for about 30 minutes in an ultracentrifuge.

4. Precipitate is discarded. 1.5 ml of supernatant is taken in a test tube and to it 200μl of freshly prepared sodium dithionite solution
(concentration = 30 mg/ml of deionized water) is added and mixed and kept at room temperature for 5 minutes.

5. After 5 minutes, above solution is centrifuged for 10 minutes at 5000 rpm.

6. Precipitate is discarded. Supernatant portion is transferred to another test tube. Absorbance of this sample solution is recorded at 450 nm by using deionized water baseline.

7. After recording the absorbance of sample solution, carbon monoxide gas is carefully bubbled through the sample for about 30 seconds.

8. After passing carbon monoxide gas through samples solution, absorbance of the sample is again recorded at 450 nm.

9. The difference between the absorbance of sample solution before and after passing of carbon monoxide gas is used for the measurement of Cytochrome P-450 activity.

**Calculation**

Cytochrome P-450 activity is expressed as n mole/mg tissue. The activity of P-450 is calculated first as n mole of P-450/litre of homogenate by using the molar extinction difference of 91 cm⁻¹ mM⁻¹ for the pigment (Omura and Sato, 1964) and then it is converted to n mole of P-450/mg tissue.

**Factor for calculation**

\[ \text{nM of P-450} = x \times 18681/\text{litre of homogenate}. \]

Here, \( x \) = absorbance of the sample.
Estimation of Xanthine oxidase activity

Xanthine oxidase activity level in tissue is estimated with modification of the method of Fried and Fried (1974) based on the principle of oxidation of the yellow tetrazolium salt by the liberated hydrogen peroxide to a violet formazan dye with maximum absorbance at 530 nm which is proportional to the xanthine oxidase activity in the sample.

Reactions

\[
\text{Xanthine} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{XOD}} \text{Uric acid} + \text{H}_2\text{O}_2
\]
\[
\text{Xanthine} + \text{Tetrazolium salt} \xrightarrow{\text{XOD}} \text{Uric acid} + \text{Formazan}
\]

Reagents

1. Disodium hydrogen Phosphate (\(\text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O}\))
2. Gelatine
3. Ethylenediaminetetra acetate (EDTA)
4. Phenazine methosulphate (PMS)
5. Nitro-BT-tetrazolium salt (NBT)
6. Hypoxanthine
7. Xanthine
8. Standard Xanthine oxidase (XOD)
9. Sodium hydroxide (0.1 N)
10. Hydrochloric acid (0.1 N)
Preparation of reagents

1. **Phosphate buffer (0.1 M; pH 7.8)**
   26.8 gm of Na$_2$HPO$_4$.7H$_2$O is dissolved in 800 ml of deionised water and pH is finally adjusted by addition of 0.1 N HCl and checked with a digital pH meter.

2. **Ethylenediaminetetra – acetate, EDTA (10 mM):**
   3.8 gm of EDTA – Na$_4$.H$_2$O is dissolved in 800 ml. of deionised water and pH is finally adjusted to 7.8 by addition of 0.1 N HCl.

3. **Gelatine (1% W/V)**
   1 gm of gelatine is dissolved in 100 ml. of boiling phosphate buffer (1) and allowed to cool.

4. **Phenazine methosulphate, PMS (0.2 mg/ml)**
   10 mg of PMS is dissolved in 50 ml. buffer solution (1) and stored in a dark bottle.

5. **Nitro-BT-tetrazolium salt (4mg/ml):**
   200 mg of NBT is dissolved in 50 ml of phosphate buffer (1) and stored in a dark bottle.

6. **Xanthine solution (1mM):**
   19.6 mg of xanthine is dissolved in 20 ml. of 0.01 N NaOH to prepare stock solution (5mM). This stock solution is diluted with Phosphate buffer (reagent 1) in the ratio of 1 : 5 to prepare working solution (1mM).
Working solution of Xanthine is prepared freshly before each experiment.

7. **Stock Xanthine oxidase (XOD) standard:**
   One vial of lyophilized XOD powder supplied as XOD standard is reconstituted with 10 ml of phosphate buffer (reagent 1). On reconstitution it contains 80 Unit of xanthine oxidase per litre of reconstituted working reagent. The reconstituted working reagent is stable for 15 days when stored at +2°C to +8°C.

8. **Reagent mixture:**
   Reagent mixture is prepared by adding the following reagents at the time of use.
   
   Phosphate buffer (1) = 6 parts  
   EDTA solution (2) = 5 parts  
   Gelatine solution (3) = 5 parts  
   PMS solution (4) = 1 part  
   NBT solution (5) = 3 parts  
   
   20 parts

**Preparation of XOD working standards**

A series of XOD working standards are prepared by diluting the XOD stock standard with phosphate buffer (reagent-1) as tabulated in Table III.6. to prepare a standard XOD calibration curve.
Table. III. 6.: Series of working standards for preparation of standard calibration curve.

<table>
<thead>
<tr>
<th>Standard No.</th>
<th>Vol. of standard taken (µl)</th>
<th>Vol. of phosphate buffer added (µl)</th>
<th>Concentration of working standard (Unit/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₅</td>
<td>100 µl of stock</td>
<td>1000 µl</td>
<td>80/10 = 8 U/L</td>
</tr>
<tr>
<td>S₄</td>
<td>500 µl of S₅</td>
<td>500 µl</td>
<td>8/2 = 4 U/L</td>
</tr>
<tr>
<td>S₃</td>
<td>500 µl of S₄</td>
<td>500 µl</td>
<td>4/2 = 2 U/L</td>
</tr>
<tr>
<td>S₂</td>
<td>500 µl of S₃</td>
<td>500 µl</td>
<td>2/2 = 1 U/L</td>
</tr>
<tr>
<td>S₁</td>
<td>500 µl S₂</td>
<td>500 µl</td>
<td>1/2 = 0.5 U/L</td>
</tr>
</tbody>
</table>

Preparation of calibration curve of XOD activity

1. A calibration curve is prepared with the sets of XOD working standards of different concentrations obtained and numbered as S₁, S₂, S₃, S₄ and S₅.
2. A set of five test tubes are taken and marked as S₁, S₂, S₃, S₄ and S₅.
3. In all test tubes, 1.8 ml of reagent mixture (reagent-8), 0.5 ml of phosphate buffer and 0.5 ml of Xanthine solution are added and mixed.
4. 0.2 ml of Xanthine oxidase (XOD) working standards are transferred to corresponding numbered tubes (S₁, S₂, S₃, S₄ and S₅) and mixed well by a cyclomixer.
5. A blank tube is prepared by adding all the above reagents except working standard of XOD. In place of working standard of XOD, 0.2 ml of Phosphate buffer is added and mixed.
6. The whole set of tubes are allowed to equilibrate at 37°C in an incubator for 10 minutes.

7. After 10 minutes, absorbance of all the tubes is recorded at 540 nm against blank as zero.

8. The procedure is repeated five times with each standard and the corresponding absorbances are recorded.

Table. III. 7.

<table>
<thead>
<tr>
<th>Std. XOD (U/litre)</th>
<th>Absorbances</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Set-1</td>
<td>0.000</td>
</tr>
<tr>
<td>Set-2</td>
<td>0.000</td>
</tr>
<tr>
<td>Set-3</td>
<td>0.000</td>
</tr>
<tr>
<td>Set-4</td>
<td>0.000</td>
</tr>
<tr>
<td>Set-5</td>
<td>0.000</td>
</tr>
<tr>
<td>Mean</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Plotting of the calibration curve

Mean absorbance of each standard is plotted in the Y-axis against the corresponding standard concentration of XOD (Unit/litre) in the X-axis to obtain the calibration curve (Fig.III. 6).

The Calibration curve (Fig.III. 6) obtained is found to fit in the calculated regression curve (Fig.III. 7) and is judged to be reliable.
Fig. III. 6. Calibration curve for estimation of XOD activity.
Fig. III. 7. Regression curve for estimation of XOD activity.
Preparation of sample

1. Measured amount of tissue is collected from the mice and washed with 0.9% NaCl solution.
2. Homogenate is prepared with the help of homogeniser in a concentration of 5 mg tissue/ml of deionized water.
3. 1 ml of tissue homogenate is taken in a centrifuge tube and centrifuged at 5000 rpm for 10 minutes. Supernatant portion is used as sample for the estimation of enzyme activity.

Estimation of XOD activity in sample

1. In a test tube, 1.8 ml of reagent mixture, 0.5 ml of phosphate buffer and 0.5 ml of xanthine solution are added and mixed. To it 0.2 ml of the sample is added and mixed.
2. A blank tube is prepared by adding all the above reagents except sample solution. In place of sample, 0.2 ml of deionized water is added and mixed.
3. Both sample and blank tubes are allowed to equilibrate at 37°C in an incubator for 10 minutes.
4. Absorbance of sample tube is recorded at 540 nm against blank tube as zero.

Calculation

The calibration curve of XOD is utilized to know the XOD activity in the diluted sample. XOD activity in Unit /litre of diluted sample obtained from the calibration curve (fig III.6).
Estimation of Lipid peroxide

Lipid peroxide is estimated in blood and tissue following the method of Ohkawa et al. (1979).

Principle

Lipid peroxides are converted to malondialdehyde which react 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 deionised water.

Procedure for blood

10 µl of whole blood is treated with 300 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.

1. 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.

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

3. Both the sample and the blank tubes are placed in a boiling water bath for 10 minutes and allowed to equilibrate with room temperature.
4. Absorbance of the blank and sample are measured at 530 nm with deionised water as baseline and using a cuvette with 1 cm light path.

Procedure for tissue

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

2. 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 checking. After checking, 0.2 ml of tissue homogenate is added and again checked for few minutes. Then the mixture is centrifuged at 5000 rpm for 10 minutes for the extraction of lipids from the tissue homogenate.

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

4. 450 μl of the TBA reagent is taken in a test tube. To it 900 μl of deionized mater and 50 μl of ethanol.

5. A blank tube is prepared by adding 450 ml of TBA, 900 μl of deionized mater and 50 μl of ethanol.
6. Both sample and the blank tubes are placed in a boiling water bath for 10 minutes and allowed to equilibrate with room temperature.

7. Absorbance of the blank and sample are measured at 530 nm with deionized water as baseline and using a micro curette with 1 cm light path.

Calculation

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

\[
\text{Lipid peroxide as MDA in nmol/dl} = D_F \times \frac{V \times A}{M_E} \]

\[
= \frac{0.31}{0.01 \times 0.02} \times \frac{0.45 (U - B)}{0.152}
\]

\[
= 1550 \times 3.6 \times (U - B)
\]

\[
= 5580 \times (U - B)
\]

Where, \( D_F \) = Dilution factor
\( V \) = Total volume of all the reactants
\( A \) = (Absorbance of unknown) – (Absorbance of blank)
\( M_E \) = Molar extinction coefficient.
Estimation of Alpha-fetoprotein

Alpha-fetoprotein (AFP) is estimated by solid phase sandwiched enzyme-linked immunosorbent assay (ELISA) using reagents prepared by MONOBIND.INC. Coasta Mesa. USA.

Principle

The essential reagents required for an immuno-enzymetric assay include high affinity and specificity antibodies (enzyme and immobilized) with different and distinct epitope recognition, in excess and native antigen. In this procedure the immobilisation takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-AFP antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labelled antibody and a serum containing the native antigen reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation

\[
\text{Enz} \text{Ab} + \text{Ag}_{\text{AFP}} + B_{\text{m}} \text{Ab} \xrightarrow{K_a} \text{Enz} \text{Ab} - \text{Ag}_{\text{AFP}} - B_{\text{m}} \text{Ab}
\]

\[
B_{\text{m}} \text{Ab} = \text{Biotinylated Monoclonal Antibody (excess Quantity)}.
\]

\[
\text{Ag}_{\text{AFP}} = \text{Native Antigen (Variable Quantity)}.
\]

\[
\text{Enz} \text{Ab} = \text{Enzyme labelled Antibody (Excess Quantity)}.
\]
\( \text{EnzAb} - \text{AgAFP} - \text{BtnAb}^{(m)} \) = Antigen-Antibodies Sandwich Complex.

\( K_a \) = Rate Constant of Association.

\( K_a \) = Rate Constant of Dissociation.

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[ \text{EnzAb} + \text{AgAFP} - \text{BtnAb}^{(m)} + \text{Streptavidin c.w.} \rightarrow \text{Immobilized complex} \]

Streptavidin c.w. = Streptavidin immobilized on well.

Immobilized complex = Sandwich complex bound to the well.

After equilibrium is attained, the antibody bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Reagents and materials

1. Alfa-fetoprotein reference standards.
2. Reference AFP antigen at levels of 0, 5, 25, 50, 250 and 500 ng/ml.
3. Enzyme conjugate and Biotinylated monoclonal antibody containing enzyme labelled antibody, biotinylated monoclonal mouse IgG in buffer, dye and preservative.
4. Streptavidin coated microplate.
5. Wash solution concentrate.
7. Substrate B: Containing hydrogen peroxide (H₂O₂) in buffer.
8. Stop solution: Containing 1N HCl.

Storage and stability

1. The reagents sets are stored in 2°C to 8°C in a refrigerator.
2. The micro wells are stored in a sealed dry bag with the supplied dessicant.
3. The reagents are stable until the expiry date mentioned on the label when stored and handled as directed.

I. Reagents preparation

(i) Wash buffer: Content of wash concentrate is diluted to 1000ml with deionised water in a storage container. The solution is stored at room temperature until the expiry date mentioned on the label.
(ii) Working substrate solution: Required amount of reagent is prepared by mixing equal portions of Substrate A and Substrate B.

Sample preparation

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

1. Before proceeding with the assay all samples and reagents are brought to room temperature.

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 numbers of coated wells are secured in the well holder and marked accordingly for identification.

4. 25μl of standard, control and serum samples are dispensed into the appropriately marked wells.

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

6. The microplate is gently swirled for 20-30 minutes for mixing and incubated for 60 minutes at room temperature.

7. The contents of microplate wells are discarded by decantation or by aspiration. The water is removed by blotting the wells with an absorbent paper in inverted position.

8. 100μl wash buffer is added to each well and then decanted. The procedure is repeated for 5 times. The running wash solution is soaked with absorbent paper in an inverted position.

9. 100μl of working substrate solution is dispensed into each well and incubated at room temperature for 15 minutes.

10. The reaction is stopped by adding 50μl of 1N HCl to each well and mixed gently for 15 to 20 seconds.

11. The absorbance 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. 8. The calibration curve (fig. III. 7) obtained is found to fit in the calculated regression curve (fig. III. 8.) and is judged to be reliable.

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

Table. III. 8.

<table>
<thead>
<tr>
<th>Conc. of AFP in ng/ml</th>
<th>S0</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>absorbance</td>
<td></td>
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<td>0.073</td>
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Fig. III. 8. Calibration curve for estimation of concentration of serum AFP.
Fig. III. 9. Regression curve for estimation of concentration of serum AFP.

\[ y = 0.1713x - 0.321 \]
EXTRACTION OF CURCUMIN

Curcumin was prepared from the alcoholic extract of (Curcuma longa) adapting the procedure described by Kandarkar et al. (1997). Mature rhizomes of Curcuma longa are collected from the local market. The rhizome was washed with plenty of tape water and then is allowed to dry in sunlight until the water on the surface is completely evaporated. The washed and dried rhizomes are then grated to small pieces of about 1 cm. The grated pieces are allowed to dry in shade for about a week until they become pulverisable. The shade-dried pieces are then made to a coarse powder by using a grinder. About 100 gm of dried and powdered rhizome is taking in a stopped conical flash of 1 litre capacity and about 500 ml of 98% ethanol is added with frequent shaking. It is now subjected to gentle shaking for a period of 6 hours by using a mechanical shaker and kept overnight. In the next day after a period of shaking for 30 minutes the alcohol extract is separated by filtration and the filtrate is utilized for another round of extraction. The residue is extracted with fresh amount of 500 ml ethanol under a similar extraction set up. At the end of which the filtered residue become almost colourless. The procedure of first and second extraction is repeated using the alcoholic extract obtained after first and second filtration with a fresh batch of about 100 gm rhizome powder. The extraction procedure is repeated with 5 batches of rhizome powder reusing the alcohol extracts. On obtaining a fairly concentrated alcohol extract as it is distilled and the distillate is collected for re-extraction. When the volume in the distillation flask is reduced to 1/10th of the original volume, the distillation process is stopped and the concentrated extract is evaporated by using an evaporating bath in between 65° C to 70° C to finally obtain crystals of curcumin. Under the presence extraction set up one kg raw rhizome gives a yield of 20 - 30 gm curcumin powder.
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 levels 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. \( t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(SEM_1)^2 + (SEM_2)^2}} \)
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