MATERIAL AND METHODS
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The present study was carried out in the Department of Paediatrics, M.L.B. Medical College, Hospital, Jhansi from September, 1993 to August, 1994. The cases included in the study were divided into two groups i.e. study and control groups.

SELECTION OF CASES

Control group (A)

Fifteen normal full term neonates appropriate for gestational age were included in this group. These neonates did not have perinatal asphyxia or evidence of any liver disease.

Study group (B)

Full term neonates with severe birth anoxia were selected for study group. Babies having significant problems viz. significant congenital anomalies, septicemia, history of leaking more than 12 hours or absent membrane, heart disease, renal failure, hepatosplenomegaly, preterm neonates, twins and low birth weight neonates were excluded both from study and control groups.

DIAGNOSIS OF SEVERE BIRTH ANOXIA

Diagnosis of severe birth anoxia was made when Apgar score was $\leq 3$ and $\leq 5$ at 1 and 5 minutes respectively.

After selection of cases detailed history, clinical examination and investigations were recorded in
PREDESIGNED PROFORMA AS GIVEN BELOW:

PREVIOUS OBSTETRIC HISTORY

It was enquired particularly history of previous abortion, jaundiced baby, birth asphyxia, obstructed labour, heart disease, congenital anomaly, blood group incompatibility.

ANTENATAL HISTORY

Any history of pre-eclampsia, eclampsia, leaking, diabetes mellitus, antepartum haemorrhage, convulsions, exposure to radiation, infection, fever with rashes, drug intake, date of last menstrual period or any other ailment during antenatal period were noted.

NATAL HISTORY

History of presentation, mode of delivery, any medication given during delivery and examination suggestive of foetal distress was noted and monitored.

POSTNATAL HISTORY

History of any medication given after delivery and Apgar score at 1 and 5 minute were noted.

EXAMINATION OF BABY

Each baby was subjected to thorough examination especially for any congenital anomalies, convulsions, septicemia, asphyxia, cardiovascular system, central nervous system and any other abnormalities.
Weight of each newborn infant was recorded with electronic weighing scale.

The assessment of gestational age was done by recording the last date of menstrual period and confirmed by physical and neurological developmental score (Modified scoring system for assessment of gestational age in newborn by Meharban Singh et al, 1975).

INVESTIGATIONS

Blood samples were drawn after 48-72 hours of birth and following investigations were done:

Serum bilirubin  Alkaline phosphatase
S.G.O.T.  S.G.P.T.

Other investigations whenever needed.

Collection of blood samples

Five millilitre blood was drawn from peripheral vein of neonate between 48-72 hours after delivery taking all aseptic precautions and taking informed consent from their attendants. Blood was collected in plain vial for liver function tests. Sample was centrifuged on the same day, serum was separated and preserved in deep freezer for liver function tests.

ESTIMATION OF LIVER FUNCTION

The liver function tests - serum bilirubin, SGOT, SGPT and Alkaline phosphatase were estimated by diagnostic chemical kits (SPAN).
1. **SERUM BILIRUBIN**

**Principle**

The basic principle is that bilirubin is diazotized only in presence of its dissolving solvent (Methanol). Thus the red purple coloured azobilirubin produced in presence of methanol originates from both direct and indirect fractions and thus represents total bilirubin concentration.

The intensity of red-purple colour so developed is measured colorimetrically. It is proportional to the concentration of the appropriate fraction of bilirubin. This reaction can be represented as :-

\[
\text{Bilirubin} + \text{Diazotized sulfanilic acid} \xrightarrow{\text{H}^+} \text{Azobilirubin} \xrightarrow{\text{H}^+} \text{Red purple colour} \quad (\lambda \text{ max } 540 \text{ nm})
\]

**Reagents**

- **Reagent 1**: Diaz-o-A
- **Reagent 2**: Diaz-o-B
- **Reagent 3**: Diaz-o blank
- **Reagent 4**: Methanol
- **Reagent 5**: Artificial standard (\( \leq 10\text{mg}\% \) bilirubin).

**Preparation of working solution**

Diaz-o reagent was prepared by mixing 3.3 ml of reagent 1 with 0.1 ml of reagent 2.

**Procedure**

Clean, dried test tubes labelled total\((T_1)\) and Blank \((T_2)\) were arranged. Amount of reagents pipetted
into each tube was as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>$T_1$</th>
<th>$T_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (ml)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Reagent 3: Diazo blank (ml)</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Diazo reagent (ml)</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 4: Methanol (ml)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Contents of the test tubes were thoroughly mixed and the tubes $T_1$ and $T_2$ were kept in dark at room temperature for 30 minutes and optical density was read against distilled water on a colorimeter using yellow green filter.

**Standard**

- Optical density of reagent 5 (Artificial standard $= 10\%$ bilirubin) was noted against distilled water.

**Calculation**

\[
\text{Total serum bilirubin concentration (mg/dl)} = \frac{\text{Optical density of } T_1 - \text{Optical density of } T_2}{\text{Optical density of standard}} \times 10
\]

2. **ALKALINE PHOSPHATASE**

**Principle**

Alkaline phosphatase present in serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with aminoantipyrine in presence of oxidising agent potassium
ferricyanide and forms an orange-red coloured complex, which can be measured colorimetrically. The colour intensity is proportional to the enzyme activity.

The reaction can be represented as:

\[
\text{Phenyl phosphate} \xrightarrow{\text{Alk. Phosphatase}} \text{pH 10.0} \rightarrow \text{Orange-red coloured complex.}
\]

**Reagents**

Reagent 1: Buffered substrate pH 10.0
Reagent 2: Sodium hydroxide, 0.5 N
Reagent 3: Sodium bicarbonate, 0.5 N
Reagent 4: 4-Aminoantipyrine, 6%
Reagent 5: Potassium ferricyanide, 2.4%
Reagent 6: Stock phenyl standard 10 mg%.

**Preparation of working solution**

Solution I: In vial of reagent 1, 3 ml of distilled water was added and mixed well.

Solution II: Vial of reagent 4 was dissolved in 100 ml distilled water.

Solution III: Vial of reagent 5 was dissolved in 100 ml of distilled water.

Working standard: Vial of reagent 6 was diluted with distilled water in the strength of 1:10.

**Procedure**

Clean, dried test tubes were taken and labelled as Blank (B), Standard (S), Control (C) and Test (T). Test tubes were arranged and amount of reagents pippetted into each tube was as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>B</th>
<th>S</th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution I (ml)</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>2.1</td>
<td>2.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
All the test tubes were well mixed and incubated at $37^\circ C$ for 3 minutes.

Working standard (mL)  -   1.0   -   -
Serum (mL)             -   -   -   0.1

Test tubes were well mixed again and incubated at $37^\circ C$ for 15 minutes.

Reagent 2 (mL)         0.8  0.8  0.8  0.8
Serum (mL)             -   -   0.1   -
Reagent 3 (mL)         1.2  1.2  1.2  1.2
Solution II (mL)       1.0  1.0  1.0  1.0
Solution III (mL)      1.0  1.0  1.0  1.0

All the test tubes were well mixed after addition of each reagent and optical density of Blank (B), Standard (S), Control (C) and Test (T) was measured against distilled water using a green filter.

**Calculation**

Serum Alk. phosphatase activity in KA units. = Optical density of test - optical density of control Optical density of standard - optical density of blank.

3. **S.G.O.T.**

**Principle**

SGOT catalyses the following reaction:

$$
\text{Alpha ketoglutarate} + \text{L-aspartate} \rightarrow \text{L-glutamate} + \text{Oxaloacetate}
$$

Oxaloacetate so formed is coupled with 2, 4-dinitrophenyl hydrazine (2, 4 DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline
medium and this is measured colorimetrically.

**Reagents**

- Reagent 1: Buffered aspartate – alpha – KG substrate pH 7.4
- Reagent 2: DNPH colour reagent
- Reagent 3: Sodium hydroxide, 4 N
- Reagent 4: Working pyruvate standard, 2 mM

**Preparation of working solution**

Solution I: 1 ml of reagent 3 was diluted in 10 ml of distilled water.

**Procedure**

As the reaction proceeds with time, more amount of products are formed and since the end products inhibit the enzymes, there is more of inhibition. Because of this problem, it is necessary to standardize the colorimetric method against a standard kinetic method. In this kit, this standardization is done against the standard Karmen Unit Assay (Kinetic).

Clean, dry test tubes were taken and labelled as 1, 2, 3, 4, 5 and T. Test tubes were arranged and amount of reagents pipetted in each tube as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activity (Unit/ml)</td>
<td>0.00</td>
<td>24</td>
<td>61</td>
<td>114</td>
<td>190</td>
</tr>
<tr>
<td>Reagent 1 (ml)</td>
<td>0.5</td>
<td>0.45</td>
<td>0.4</td>
<td>0.35</td>
<td>0.3</td>
</tr>
<tr>
<td>Reagent 4 (ml)</td>
<td></td>
<td>0.45</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Reagent 2 (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

All the test tubes were well mixed and allowed to stand for 20 minutes at room temperature.

Solution I (ml) 5.0 5.0 5.0 5.0 5.0
Test tubes were well mixed by inversion. These were allowed to stand at room temperature for 10 minutes and optic density of all five test tubes was measured against distilled water on colorimeter with a green filter.

Standard graph was plotted taking enzyme activity on X axis and optic density on Y-axis.

Test

Reagent 1 : 0.5 ml (Incubated at 37°C for 5 minutes)

Serum : 0.1 ml (Mixed well and incubate at 37°C for 60 minutes).

Reagent 2 : 0.05 ml (Mixed well and allowed to stand at room temperature for 20 minutes).

Solution I : 5.0 ml

Test tube was mixed well and allowed to stand at room temperature for 10 minutes and the optic density was read against distilled water on colorimeter using a green filter.
Calculation

The optic density of test was marked on the Y axis of the standard curve and extrapolate it to the corresponding enzyme activity on X - axis.

4. S.G.P.T.

SGPT catalyses the following reaction:

\[
\text{Alpha-ketoglyutarate} + \text{L-Alanine} = \text{L-glutamate} + \text{pyruvate}
\]

Pyruvate so formed is coupled with 2,4-Dinitrophenyl hydrazine to give the corresponding hydrazone, which gives brown colour in alkaline medium and this can be measured colorimetrically.

Reagents

Reagent 1: Buffered alkaline-Alpha-KG substrate pH 7.4
Reagent 2: DNPH colour reagent
Reagent 3: Sodium hydroxide, 4 N
Reagent 4: Working pyruvate standard, 2 mM.

Preparation of working solution

Solution I: 1 ml of reagent 3 was diluted in 10 ml of distilled water.

Procedure

As the reaction proceeds with time, more amount of products are formed and since the end products inhibit the enzyme there is more inhibition. Because of this problem, it is necessary to standardize the colorimetric method against a standard kinetic method. In this, kit
standardization is done against the standard Karmen Unit Assay (Kinetic) and this is extrapolated to different amounts of pyruvate.

Clean, dry test tubes were taken and labelled as 1, 2, 3, 4, 5 and T. These test tubes were arranged and reagents pipetted into each tube was as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Test tube number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Enzyme activity (Units/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Reagent 1 (ml)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reagent 4 (ml)</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>0.1</td>
</tr>
<tr>
<td>Reagent 2 (ml)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at room temperature for 20 minutes.

Solution I: 5.0 5.0 5.0 5.0 5.0

Test tubes were mixed well by inversion and allowed to stand at room temperature for 10 minutes. Optical density was measured of all five test tubes against distilled water on colorimeter with a green filter.

A standard graph was plotted by taking enzyme
activity on X axis and optical density on Y axis.

![Graph showing enzyme activity and optical density]

Test Tube:

- Reagent 1: 0.5 ml (incubated at 37°C for 5 minutes)
- Serum: 0.1 ml (mixed well and incubated at 37°C for 30 minutes)
- Reagent 2: 0.5 ml (mixed well and allowed to stand at room temperature for 20 minutes)
- Solution I: 5.0 ml

Test tube was mixed well and stand at room temperature for 10 minutes and read the optical density against distilled water on colorimeter using a green filter.

Calculation

The optical density of test was marked on the Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.