Chapter – III

PROCEDURE

This chapter describes the methodology and procedure adopted during the entire study. Precisely this includes the selection of subjects, criterion Measures selection of variables, design of the study, instrument reliability, tester’s reliability, collection of data, administration of the test, statistical techniques employed for analysis of data and Techniques used

III. a. Selection of Subjects:

20 male “Cross Country” runners who had participated in West Bengal state level “Cross Country” Championship, age ranging between 23-26 years had been selected as subject randomly.

III. b. Criterion Measures:

1. Placebo Trial: Supplementation of single dose 500ml. water (saccharine mixed) at the 30th minute of run.
2. Glucose Supplementation Trial-I (GST-I): A single dose of 500 ml. glucose mixed water was given soon after 30th minute of run. The quantity of the given glucose was 0.6gm / Kg body weight mixed in 500 ml. water.
3. Glucose Supplementation Trial-II (GST-II): Supplementation of intermittent glucose mixed water was given double dose soon after 30th and 50th minutes of run. The quantity of glucose was 0.6gm/kg body weight in 500 ml. water. The 500 ml. glucose mixed water was shared equally twice.

4. Glucose Supplementation Trial-III (GST-III): Supplementation of intermittent glucose mixed water was given triple dose soon after 30th, 50th and 70th minutes of run. The quantity of glucose was 0.6gm/kg body weight in 500 ml. water. The 500 ml. glucose mixed water was shared equally thrice.

III. c. Selection of Variables:

Three variables were selected for the present study. They were:

1. Blood glucose
2. Blood lactic acid

III. d. Design of the Study:

In placebo condition: A mild dose of saccharine (mixed in 500 ml. water) was given to the subjects so as to minimize the psychological effect. They thought that they are provided with glucose, before the work. It is
essential because psychological condition may affect the work performance and blood glucose level. Supplementation (GST-I) of a single dose (glucose mixed water) was given soon after 30th minute of run. The quantity of the given glucose was 0.6gm / Kg body weight mixed in 500 ml. water. Supplementation (GST-II) of intermittent glucose mixed water was given double dose soon after 30th and 50th minutes of run. The quantity of glucose was 0.6gm/kg body weight in 500 ml. water. The 500 ml. glucose mixed water was shared equally twice. Supplementation (GST-III) of intermittent glucose mixed water was given triple does soon after 30th, 50th and 70th, minutes of run. The quantity of glucose was 0.6gm/kg body weight in 500 ml. water. The 500 ml. glucose mixed water was shared equally thrice. There were three days gap between the two days of tests. Subjects were run for 90 minutes continuously at a pace of 400 meters with in two and half minutes for all four trials. They also had maintain a comfortable life style during the entire study periods.

**III. e. Instrument Reliability**

The instrument used for this study was calibrated and standardized one. There were blood glucose, blood lactic acid and blood uric acid tested by latest scientific Autoanalyzers. Those instruments were available in clinical centers
III. f. Tester’s Reliability

To ensure that the investigator was well versed with the technique of conducting the tests, the investigator had a number of practice session in testing procedure, under the guidance of experts from Doctors Diagnostic and Research Centre, Kolkata. Tester reliability was established by test – re-test process where by consistencies of result were obtained by product moment correlation method. The data collected from 20 subjects in test – re-test method was correlated and the value of coefficient of correlation was (+) 0.99.

III.g. Collection of Data:

The blood glucose, blood lactic acid and blood uric acid were measured by glucose, lactic acid, and uric acid scientific autoanalyzer. Data on blood glucose, blood lactic acid and blood uric acid were collected before the commencement of the endurance performance and soon after the 90 minutes of endurance run for all the four trials (Placebo trial, Glucose Supplementation Trial-I, Glucose Supplementation-Trial-II and Glucose Supplementation Trial-III) in Pathological Laboratory at Garia and Shyambazar in Kolkata.
III. h. Administration of the Test

The tests were administrated in four days conducted in the early morning (6.30 am) of all those days. Subjects were well informed about the day and time of tests. They were also advised to have a comfortable life style during entire study period.

In all the four days of the tests the subjects were suppose to run for 90 minutes continuously at a pace of 400 meters (one lap of the standard track) within two and half minutes. There were three days gap between the two days of tests.

The first day of the test was the Placebo trial. In that day, subjects were run in a notion that they were supplied with glucose mixed water but virtually only saccharine mixed 500 ml. water (without any amount of glucose) were supplied at the 30th minute of run.

On the second day of the test (GST-I), the subjects were supplemented single does glucose mixed water (0.6 gm. Glucose/kg body weight in 500 ml. water) at 30th minute of the run.

On the third day of the test (GST-II), the subjects were supplemented to double does glucose mixed water at the 30th and 50th minutes of run. The glucose was supplemented to the subjects in a quantity of 0.6 gm. glucose/kg
body weight in 500 ml. water. During this time, 500ml. of water (glucose mixed) was given to the subjects twice (during the endurance performance).

On the fourth day of the test, the subjects were supplemented triple does glucose mixed water at the 30th, 50th and 70th minutes of run. The glucose was supplemented to the subjects in a quantity of 0.6 gm glucose/Kg body weight in 500ml. water. During this time 500 ml. water (glucose mixed) was given to the runners thrice (during the endurance performance).

### III. i. Statistical Procedure:

Analysis of variance (ANOVA) and ‘t’ test were calculated to measure the significant differences among various measures of blood glucose, blood lactic acid and blood uric acid.

To examine the significance difference between the pre-test and post-test for all the four trials ‘t’ values were calculated on the variables of blood glucose, blood lactic acid and blood uric acid.

Mean and Standard deviations were calculated to give the descriptive picture of blood glucose, blood lactic acid and blood uric acid for pre-test and post-test of four trials. To measure whether there was any relation between the three variables, correlation coefficient (product moment method) values were calculated. The results are given in the later part of the thesis.
III. j. Techniques Used:

**Methodology for estimation of Blood Glucose: GOD-POD**

**(Glucose oxidase – Peroxidase) method :**

Estimation of blood glucose was done following the method of Trinder, P. (1969), Barham, D. & Trinder, P. (1972), and Henry, R.J. (1963).

❖ **TEST PRINCIPLE :-**

The reaction sequence employed in this assay is as follows:

\[
\text{GOD (glucose oxidase)}: \quad \text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \quad \rightarrow\quad \text{Gluconate} + \text{H}_2\text{O}_2
\]

\[
\text{POD}: \quad 2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-Aminoantipyrine} \quad \rightarrow\quad \text{Red Quinonemine} + 4\text{H}_2\text{O}
\]

Glucose is oxidized by glucose oxidase and produces gluconate and hydrogen peroxide. The hydrogen peroxide is oxidatively coupled with 4-amino antipyrine and phenol. The intensity of the coloured complex (quinoneimine) is proportional to the glucose concentration in the sample and can be measured photometrically at 505nm (500-540 nm).
.NORMAL RANGE:

<table>
<thead>
<tr>
<th></th>
<th>mg/dl</th>
<th>mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum, Plasma:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>70 – 110</td>
<td>3.89 – 6.11</td>
</tr>
<tr>
<td>Post Prandial or Meal</td>
<td>&lt; 140</td>
<td>7.78</td>
</tr>
<tr>
<td>CSF</td>
<td>50 – 70</td>
<td>2.78 – 3.89</td>
</tr>
</tbody>
</table>

It is recommended that laboratories should establish their own normal range.

.KIT CONTENTS:

<table>
<thead>
<tr>
<th></th>
<th>Code No KG 1 (5 x 100 ml.)</th>
<th>Code No KG 2 (2 x 500 ml.)</th>
<th>Code No KG 3 (1 x 1000 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1 Enzyme Reagent</td>
<td>5 vials</td>
<td>2 vials</td>
<td>1 vial</td>
</tr>
<tr>
<td>Reagent 2 Buffer solution</td>
<td>1 x 500 ml.</td>
<td>2 x 500 ml.</td>
<td>1 x 1000 ml.</td>
</tr>
<tr>
<td>Reagent 3 Glucose standard (100 mg/dl)</td>
<td>2 x 5 ml.</td>
<td>2 x 5 ml.</td>
<td>2 x 5 ml.</td>
</tr>
<tr>
<td>Glucose standard (300 mg/dl)</td>
<td>2 ml.</td>
<td>2 ml.</td>
<td>2 ml.</td>
</tr>
</tbody>
</table>

.WORKING REAGENT PREPARATION:

Dissolve one vial of Enzyme Reagent (1) in 100 ml. (5 x 100 ml.), in 500 ml. (2 x 500 ml.) and in 1000 ml. (1 x 1000 ml.) of Buffer solution (2). Mix
immediately working reagent is stable for 60 days when stored in dark at 2 – 8°C.

Enzyme Reagent and standards are to be stored at 2 – 8°C and are stable till the expiry date mentioned on the labels. Buffer can be stored at RT. Store the reconstituted reagent at 2 – 8°C.

❖ PROCEDURE:

Pipette into test tubes labelled Blank (B), Standard (S) and Test (T) as follows:-

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
</tr>
<tr>
<td>Glucose Standard (3) (100 mg/dl)</td>
<td>-</td>
<td>10 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Specimen</td>
<td>-</td>
<td>-</td>
<td>0.1 ml.</td>
</tr>
</tbody>
</table>

To make the volume constant 10 ml. of distilled water mixed with the blank solution. All the solutions had to mixed well and incubate at 37°C for 10 minutes or 15 minutes at RT. Read absorbance of standard (S) and Test (T) against Blank (B) at 505nm or with green filter (500-540 nm).
 CALCULATIONS:-

\[
\text{Glucose conc. In mg/dl} = \frac{\text{Abs of } T}{\text{Abs of } S} \times 100
\]

The final colour is stable for 1 hour at R.T. (32°C)

Methodology for estimation of Blood Lactic Acid :
LO-POD (Lactic oxidase – Peroxidase) Enzymatic Colorimetric method


PRINCIPLE OF THE METHOD:

Lactate is oxidized by lactate oxidase (LO) to pyruvate and hydrogen peroxide (H₂O₂) which under the influence of Peroxidase (POD). 4-amino phanazone (4-AP) and 4-chlorophenol form a red quinone compound.

\[
\text{LO (lactate oxidase)} \quad \text{L – Lactate + O₂ + H₂O₂} \quad \rightarrow \text{Pyruvate + H₂O₂}
\]

H₂O₂ + 4-AP + 4-chlorophenol in proportional to the lactate concentration in the sample.
PREPARATION:

Working reagent (WR): Dissolve (→) the contents of one vial R2 enzymes in 10 ml. of R1 Buffer. Cap and mix gently to dissolve contents. The reagent is stable after reconstitution 1 month at 2 – 8°C.

PROCEDURE:

1. Assay conditions

   Wave length ............... 505 nm (490 – 550)
   Cuvette ..................... 1 cm light path
   Temperature............... 25°C - 37°C

2. Adjust the instrument to zero with distilled water.

3. Pipette into a cuvette:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR (ml.)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Standard (Note 1,2) / (ml.)</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Sample (ml.)</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

4. Mix and incubate for 5 min. at 37°C or 10 min. at room temperature (15 – 25°C)

5. Read the absorbance (A) of the sample and standard, against the Blank.

   The colour is stable for at least 30 minutes.

CALCULATIONS:

\[
\frac{(A)_{\text{Sample}} \times 100}{(A)_{\text{Sample}}} \times (\text{standard conc.}) = \text{mg/dl lactate in the sample.}
\]

Conversion factor: mg/dl x 0.111 = m mol/L
Methodology for estimation of Blood Uric Acid  
(URICASE – POD METHOD WITH DHBS)

Estimation of blood uric acid was done following the method of Praful, B.Godkar (1994), Thefeld, C et.al. (1973), Town et. al. (1985), and Trinder, P. (1969).

❖ TEST PRINCIPLE :

Enzymatic conversion of uric acid to allantoin quantitatively produces hydrogen peroxide. Hydrogen Peroxide thus produced is measured though an indicator reaction involving peroxidase.

\[
\text{Uric Acid + O}_2 + 2 \text{H}_2\text{O} \xrightarrow{\text{Uricase}} \text{Allantoin} + \text{CO}_2 + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{DHBS} + 4 \text{AAP} \xrightarrow{\text{Peroxidase}} \text{Red quinoneimine complex} + \text{HCl} + 4\text{H}_2\text{O}.
\]

❖ NORMAL RANGE OF SERUM URIC ACID:

<table>
<thead>
<tr>
<th>Gender</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>2.4 – 5.7 mg/c.c of blood</td>
</tr>
<tr>
<td>Men</td>
<td>3.4 – 7.0 mg/ c.c of blood</td>
</tr>
</tbody>
</table>
KIT CONTENTS:

<table>
<thead>
<tr>
<th></th>
<th>Code No. KUAI (1 x 50 ml.)</th>
<th>Code No. KUA2 (2 x 50 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1 Enzyme Reagent</td>
<td>50 ml.</td>
<td>2 x 50 ml.</td>
</tr>
<tr>
<td>Reagent 2 Uric Acid. Standard (10 mg/dl)</td>
<td>3 ml.</td>
<td>3 ml.</td>
</tr>
</tbody>
</table>

SAMPLE:

Serum, Heparinised plasma or EDTA plasma. Urine, Urine should be diluted 1 : 10 with distilled water before use.

WORKING REAGENT PREPARATION:

All reagents are ready-to-use and are stable at 2 °C – 8°C till the expiry date mentioned on the labels. When opened, care should be taken to avoid contamination.

PROCEDURE:

Pipette into test tubes labeled Blank (B), Standard (S) and Test (T) as follows:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Reagent (1)</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
</tr>
<tr>
<td>Uric Acid Standard (2) (10 mg/dl)</td>
<td>-</td>
<td>0.02 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Specimen</td>
<td>-</td>
<td>-</td>
<td>0.02 ml.</td>
</tr>
</tbody>
</table>
To make the volume same, the black was mixed with 0.02 ml. of distilled water. All the solutions were mixed well and incubated for 10 minute at 37°C (or) for 15 minutes at R.T.

Absorbance was taken to standard (S) and Test (T) against Blank (B) at 520 nm using green filter (505 – 520 nm). The final colour is stable to 30 minutes at R.T.

❖ **CALCULATIONS:**

Serum Uric Acid in mg/dl = \[ \frac{\text{Abs. of T}}{\text{Abs. of S}} \times 10 \]

Urine Uric Acid in mg/24 hrs Urine = \[ \frac{\text{Abs. of T}}{\text{Abs. of S}} \times \frac{V}{10} \]