4. MATERIAL AND METHODS
4.1 CLINICAL STUDIES

4.1.1 DEMOGRAPHIC STUDY:

All the patients with hypertension and/or diabetes-mellitus attending Shukla General Hospital's OPD during December 1990 to March 1993 were enrolled for the demographic study. All the patients were analysed to find out the prevalence of hypertension in diabetes-mellitus, occurrence of cardiac dysfunction among diabetic and non-diabetic hypertensive patients, and a 12 hours fasting blood samples were collected to find out occurrence of hyperlipidaemia among diabetic and non-diabetic hypertensive patients.

4.1.2 EFFECTIVENESS OF VARIOUS ANTIHYPERTENSIVES IN DIABETIC AND NON-DIABETIC HYPERTENSIVE PATIENTS:

4.1.2.1 Patient selection:

The study was a controlled open clinical trial. It was randomised, nonblind and based on a parallel group design. The protocol and proforma was approved by the local ethical committee. The proforma and approval of the ethical committee is given in Annexure-1 and Annexure-2 respectively.

From December 1990 to May 1993, 487 patients with either hypertension or diabetes visited the Shukla hospital's, Bardoli. Out of 487 patients 198 patients were selected for the study.

4.1.2.2 Inclusion criteria:

Patients of either sex with mild to moderate hypertension (defined as mean diastolic blood pressure more than 90 mm Hg but less than 115 mm Hg and systolic blood pressure between 160 mm Hg and 190 mm Hg) were selected for the study. Patients with non-insulin dependent diabetes for more than 2 years associated with or without essential hypertension were also selected. Data was also collected for
a separate group of patients who had severe hypertension but unaware of it and did not receive any treatment. All the patients belonged to the age group of 45 to 70 years. All patients were within -15 to +25% of the ideal body weight.

4.1.2.3 Exclusion criteria:

Patients were excluded from the study if their age was above 70 years; had complicated hypertension or recent myocardial infarction (less than 3 months from the study), bradycardia (heart rate less than 50 beats/min), second or third degree atrioventricular (AV) block, congestive heart failure, recent cerebrovascular events (less than 3 months previously), severe dyslipidaemia (familiar hyperlipidaemias or total cholesterol more than 350 mg/dl), renal or hepatic failure, severe concomitant diseases, or a history of hypersensitivity to any of the test drugs.

4.1.2.4 Treatment schedule:

The patients selected from hospital’s OPD were fully explained about the procedures and written informed consent (Annexure-3) was taken from them. Patients who met eligibility criteria were admitted to Shukla General Hospital, Bardoli for one day, and underwent a physical examination and routine clinical examination for chest X-ray, blood pressure, heart rate and ECG. The selected patients received placebo treatment for 2-3 weeks. For the follow up patients attended the OPD of Shukla Hospital. Patients with diabetes-mellitus were maintained on their usual diet and received the antidiabetic treatment (Glibenclamide 5-20mg/day) for the control of diabetes. At the end of the placebo washout period, if they still met the qualifying criteria, both essential hypertensive and diabetic hypertensive patients were randomised to receive either Atenolol (50 mg/day), Nifedipine (10 mg/day), Enalapril (5 mg/day), or Clonidine (50 mcg/day) for 9 months (Table:4.1).
Table : 4.1

GROUP OF PATIENTS WITH TREATMENT SCHEDULE.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non-insulin dependent diabetes mellitus (NIDDM)</td>
<td>Glibenclamide</td>
<td>5-20 mg/day</td>
</tr>
<tr>
<td>2</td>
<td>Uncontrolled hypertensive patients (UN-HT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Diabetic hypertensive patients (DM-HT)</td>
<td>1. Enalapril</td>
<td>5-20 mg/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Atenolol</td>
<td>50-100 mg/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Nifedipine</td>
<td>10-20 mg/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Clonidine</td>
<td>50-100 mcg/day</td>
</tr>
<tr>
<td>4</td>
<td>Essential hypertensive patients (EH)</td>
<td>1. Enalapril</td>
<td>5-20 mg/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Atenolol</td>
<td>50-100 mg/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Nifedipine</td>
<td>10-20 mg/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Clonidine</td>
<td>50-100 mcg/day</td>
</tr>
</tbody>
</table>

After four weeks of active treatment, patients whose mean diastolic blood pressure was less than 90 mm Hg were instructed to continue taking the same dose. Patients whose diastolic blood pressure was more than 90 mm Hg were instructed to increase their dose for two weeks. After two weeks, if diastolic blood pressure was still more than 90 mm Hg, they were instructed again to increase their dose for the remaining period of the study. The maximum increase in dose allowed was 20 mg/day for Enalapril, 100 mg/day for Atenolol, 20 mg/day for Nifedipine and 100 mcg/day for Clonidine.

4.1.2.5 Data Collection:

During 9 months of the active treatment, patients visited regularly the hospital at least at an interval of one month and the clinical examination along with the adverse effects of drugs were recorded by the investigator. At the end of the placebo wash out period, and after 1, 3, 6 and 9 months of treatment, supine systolic and diastolic blood pressure were measured by sphygmomanometer on the same arm and whenever possible, by the same nurse or physician. Heart rate was assessed by palpation over a period of 1 minute. Before the initiation of treatment, and at the end of 3 and 9-months treatment period, the fasting blood samples were
collected for the estimation of total cholesterol, triglycerides, high density lipoproteins (HDL), blood glucose, urea and creatinine.

4.1.2.6 Life-style monitoring:
No specific dietary prescription was provided to avoid any diet fluctuation. Patients were required to continue their usual diet habits throughout the study. Patients were asked not to make changes in physical exercise or smoking habits during the course of study. Drug compliance was assessed by pill counts.

4.1.3 STATISTICAL ANALYSIS:
The results were analysed by two way analysis of variance followed by Tuckey's test to find out the significant difference among the treatment. The value of probability less than 5% (p < 0.05) was considered as statistically significant.

4.2 ANIMAL STUDIES

4.2.1 EFFECTS OF CHRONIC TREATMENT WITH CLONIDINE IN DIABETIC AND/OR HYPERTENSIVE RATS:

4.2.1.1 Induction of Diabetes:
Healthy female albino rats of Wistar strain, weighing 180-250gm were used in the experiment. Diabetes was induced by a single tail vein injection of streptozotocin (STZ) (45mg/kg) dissolved in 0.1M citrate buffer (pH 4.5). The diabetes was confirmed by measuring urine glucose with the help of Diastix® (Miles India) after 48 hours of STZ injection. 5% glucose solution was given 2 days before and 3 days after the STZ injection to prevent initial hypoglycemic effect of STZ.

4.2.1.2 Induction of Hypertension:
Hypertension in rats was induced by subcutaneous administration of deoxycorticosterone acetate (DOCA) in the dose of 5 mg/kg/day throughout the study period. Animals receiving DOCA were fed with 2% salt solution as drinking water. Animals showed development of hypertension after 10
days of DOCA injection.

4.2.1.3 Treatment Protocol:

The animals exhibiting glucosuria, 48 hours after the withdrawal of glucose feeding i.e. 5 days after STZ injection were considered as diabetic. Control (Non-diabetic) animals received only citrate buffer. Three days after the administration of buffer or STZ, the animals were randomly divided into two sub-groups: hypertensive and non-hypertensive.

Clonidine was administered daily (P.O.) in the dose of 25 mcg/kg. The details of the groups of animals are given in Table: 4.2.

**TABLE : 4.2**

<table>
<thead>
<tr>
<th>Name of the Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>Non-diabetic, Non-hypertensive, Untreated</td>
</tr>
<tr>
<td>II. Control treated</td>
<td>Non-diabetic, Non-hypertensive, Clonidine treated</td>
</tr>
<tr>
<td>III. Diabetic control</td>
<td>Diabetic, Non-hypertensive, Untreated</td>
</tr>
<tr>
<td>IV. Diabetic treated</td>
<td>Diabetic, Non-hypertensive, Clonidine treated</td>
</tr>
<tr>
<td>V. Hypertensive control</td>
<td>Non-diabetic, Hypertensive, Untreated</td>
</tr>
<tr>
<td>VI. Hypertensive treated</td>
<td>Non-diabetic, Hypertensive, Clonidine treated</td>
</tr>
<tr>
<td>VII. Diabetic hypertensive control</td>
<td>Diabetic, Hypertensive, Untreated</td>
</tr>
<tr>
<td>VIII. Diabetic hypertensive</td>
<td>Diabetic, Hypertensive, Clonidine treated</td>
</tr>
</tbody>
</table>

During the six weeks study period, the following physiological parameters were recorded in all the groups of animals:

1. Body weight
2. Water intake
3. Blood pressure
4. Food intake
5. Mortality
6. Heart rate.
All the rats were housed under identical conditions and the standard diet and water was provided ad libitum throughout the study period. The composition of diet provided to animals during the study was as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat powder (coarse)</td>
<td>60%</td>
</tr>
<tr>
<td>Maize powder (coarse)</td>
<td>20%</td>
</tr>
<tr>
<td>Grain powder (coarse)</td>
<td>10%</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>5%</td>
</tr>
<tr>
<td>Milk powder</td>
<td>2.5%</td>
</tr>
<tr>
<td>Iodized salt</td>
<td>2.5%</td>
</tr>
</tbody>
</table>

4.2.1.4. Measurement of Blood Pressure and Heart Rate:

The blood pressure and heart-rate were recorded using HARWARD BLOOD PRESSURE MONITOR (TAIL CUFF METHOD) attached with STUDENT’S OSCILLOGRAPH. The tail was introduced into cuff and initial gain set was established by the use of pulse sensor in order to get minor deflection. The pressure was initially raised up to 200 mmHg and then it was slowly released by a screw attached in the bulb. During this decline in pressure, the point at which the magnitude of the deflection of pulse analyser is increased was considered as systolic blood pressure of rat. Heart rate was also recorded by pulse analyser at particular chart speed.

Blood pressure and Heart rate were measured before the starting of clonidine therapy and at every 10th day up to the completion of treatment.

4.2.1.5 Collection of Blood Samples and Their Analysis:

At the end of treatment with clonidine, the blood samples were collected from all the groups of animals from retino-orbital plexus of the eye. About 4-5 ml of blood samples were collected in centrifuge tubes and allowed to clot for 30 min. at room temperature. Serum was separated by centrifuging the tubes at 3000 rpm for 30 minutes. Supernatant clear serum was separated and transferred to Ependorf tubes. The serum samples were stored at -20°C until the analysis was done. Serum was analysed for glucose, insulin, total cholesterol, HDL-cholesterol, triglycerides, triiodothyronine (T₃), thyroxin (T₄), creatinine, blood urea nitrogen, alkaline phosphatase.
Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) diagnostic assay kits.

4.2.1.6 Recording of Cardiac Function:

After six weeks of the treatment animals were sacrificed by stunning and bleeding to death. Heart was quickly dissected out from the rat and placed in warm aerated Chenoweth-Koelle buffer (37°C) where extraneous tissue was dissected free. The aortic stump was located and tied to a 15 gauge stainless steel aortic perfusion canula. Perfusion was initiated in the retrograde manner through the aorta at 45 cm H$_2$O (30 mmHg) aortic filling pressure. The perfusion fluid was Chenoweth-Koelle buffer maintained at 37±1°C and bubbled with oxygen. A 16 gauge stainless steel canula connected to atrial filling reservoir was then inserted and tied to the pulmonary vein. Left ventricular developed pressure (LVDP) was measured by means of pressure transducer-polygraph system with a 3 cm piece of polyethylene tube attached to a 20 gauge needle. This was inserted through the apex of the heart into left ventricle. Cardiac work was initiated by switching the perfusion system from the retrograde mode to the working heart mode.

In the working heart mode the perfusate entered the left ventricle through the left atrium and was pumped out through aortic pump. Aortic outflow was subjected to an afterload of 45 cm polyethylene tubing. Left ventricular pressure was recorded on polygraph.

The hearts were allowed to stabilise at 10 cm H$_2$O filling pressure for 10 mins. The cardiac function curve was obtained by changing the height of the left atrial filling reservoir from 2.5 to 20 cm in 2.5 cm increment steps. At each point, pressure development was allowed to stabilize before it was recorded. A complete function curve was usually performed in about 30 min. The total time of perfusion of each heart was approximately 45 min.
Composition of Chenoweth-Koelle Buffer was as follows:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>mM/Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>119.80</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>5.63</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>2.88</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>4.50</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>3.80</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.00</td>
</tr>
<tr>
<td>Disodium EDTA</td>
<td>0.03</td>
</tr>
<tr>
<td>Distilled water sufficient to make 1.0 Litre.</td>
<td></td>
</tr>
</tbody>
</table>

After getting the cardiac function curve, the hearts were taken out, blotted with filter paper to remove excess of water and weight of the heart was noted down to calculate the index of hypertrophy as wet heart weight to body weight ratio.

4.2.1.7 Histological Study:

Histological study of heart and liver was carried out to observe the effects of chronic clonidine treatment on degenerative changes induced by diabetes and hypertension on these organs.

Fixation of the tissues:

Left ventricle of the heart and the liver were dissected free and kept in Bouin’s fixative for 18 to 24 hours. Tissues were then washed twice with distilled water and kept in 70% alcohol. A pinch of lithium carbonate was added and tissues were kept in overnight. Lithium carbonate removed all excessive stain and tissues were again kept in fresh 70% alcohol. After that tissues were transferred into 90% alcohol and kept in it overnight. Next morning all tissues were transferred into 100% alcohol and kept for 3 hrs. Then tissues were transferred to xylene and kept till they become transparent.

Microtomy:

Tissues were fixed in melted paraffin in wooden blocks, so that sectioning can be performed. Several sections of 5
um thickness were taken from each tissue and sections with uniform shape and size were selected for histology. Selected sections were fixed on the clear glass slides with the help of egg albumin.

Staining:

Tissues were stained with Haemotoxyllin and Eosin stains. The staining procedure was as follows:

- Slides → Xylene → 100% alcohol → 90% alcohol
  - 20 min → 2-3 dips → 1-2 min

- Distilled water → alcohol → 30% alcohol → 50% alcohol
  - 2-5 min → 2-5 min → 2-5 min

- Haemotoxyllin stain → Water acidic Ammonia → Distilled water
  - 2-4 dips → 2-4 dips → 2 min

- Eosin stain → alcohol → 70% alcohol → 50% alcohol
  - 2-4 min → 2-3 min → 2-3 min

- Alcohol → 100% alcohol → Xylene
  - 2-3 dips → 2-4 dips → 48 hrs.

Perfectly stained slides were mounted with Diphenyl Xylene (DPX) and observed under light microscope. All the histological sections were examined by an individual who was unaware about the section group being examined.

4.2.2 EFFECTS OF NIFEDIPINE TREATMENT IN DIABETIC RATS:

Diabetes was induced by a single tail vein injection of STZ (45 mg/kg) in female albino rats of wistar strain as described earlier in section 4.2.1.1. The rats were divided into four groups: Control, Control treated with nifedipine, Diabetics and Diabetics treated with nifedipine. Nifedipine suspension in 1% carboxy methyl cellulose (CMC) was given orally in the dose of 35 mg/kg/day for a period of six weeks with food and water given adlibitium.

The blood were collected from retino-orbital plexuses before STZ injection and four times during 6 weeks of
nifedipine treatment, at an interval of 10 days. Blood samples were allowed to clot and after half an hour serum was separated by centrifuging the tube at 3000 rpm. Serum was aspirated and stored at -20°C until the biochemical analysis. Serum glucose and insulin levels were measured initially and at an interval of 10 days upto six weeks study period. While cholesterol, triglyceride, tri-iodothyronine (T₃), thyroxin (T₄) and thyrotropin (TSH) levels were analysed from all the groups at the end of six weeks of treatment period. All biochemical parameters were measured by using a standard diagnostic kits.

At the end of six weeks the mean blood pressure and heart rate were recorded using blood pressure monitor (Tail cuff method) attached with a student oscillograph. The index of hypertrophy as wet heart weight to body weight ratio were obtained as described earlier in the section 4.2.1.6.

4.3 ESTIMATION OF BIOCHEMICAL CONSTITUENTS

4.3.1 ESTIMATION OF GLUCOSE (GOD/POD method)

Principle

In the single reagent system, glucose oxidase converts glucose to gluconic acid and hydrogen peroxide. The peroxide in the presence of horseradish peroxidase forms a colored complex of hydroxybenzoate and 4-aminophenazone. The intensity of the color formed is proportional to the glucose level.

Procedure

<table>
<thead>
<tr>
<th>Serum</th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 2: (Glucose standard 100 mg%)</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>--</td>
</tr>
<tr>
<td>Solution 1</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
</tbody>
</table>
The content of tubes was incubated at 37°C for 15 minutes, and colour developed was measured at 510 nm against distilled water.

**Calculation**

Serum Glucose in mg % = \( \frac{O.D.(test) - O.D.(blank)}{O.D.(std.) - O.D.(blank)} \times 100 \)

**4.3.2 ESTIMATION OF INSULIN (Radioimmunoassay)**

**Principle**

When serum containing insulin is added to a tube containing a fixed amount of antibody and a fixed amount of the radio-labeled insulin (\(^{125}\)I-labelled), insulin present in serum and the radio labeled insulin competes for the antibody. The amount of radiolabeled insulin bound to the antibody is inversely proportional to the amount of insulin in serum. A standard curve with known amounts of the test substance can thus be constructed and the amount in the unknown samples can be calculated.

**Procedure**

All reagents were allowed to reach room temperature and mixed thoroughly before the use.

1. Tubes were arranged and labeled as total counts, non specific binding (NSB), standards, controls and unknowns.
2. Insulin standards, controls and unknown (serum samples) were added (100 ul) to the appropriate tubes while in NSB tubes 200 ul of the insulin standard (0 uIU/ml) was added.
3. 100 ul Insulin Antiserum was added to all tubes except NSB and total count tubes.
4. 100 ul of insulin (I\(^{125}\)) reagent was added to each tube.
5. All tubes were vortexed.
6. All tubes were incubated at 2°C-8°C for 16 hours.
7. 1 ml of precipitating reagent was added to all tubes except total count tubes.
8. Again all tubes were vortexed and incubated at room temperature for 10 to 15 minutes.
9. All tubes except the total count tubes were centrifuged for 15 to 20 minutes at 4500 RPM.
10. All tubes except total count tubes were decanted by simultaneous inversion with a sponge rack into a radioactive waste receptacle. Tubes were allowed to drain on absorbent material for 15-20 seconds and blotted to remove droplets adhering to the rim before returning them to the upright position.
11. All tubes were counted on a Gamma counter for one minute.

Calculation
1. The blank count was subtracted from all the other counts to give corrected counts.
2. $\%B/Bo = \frac{Corrected\ count\ of\ sample\ or\ standard}{Corrected\ count\ of\ zero\ standard} \times 100$
3. The standard curve was prepared as $\%B/Bo$ on the logit and uU/ml of insulin on the logarithmic scale of logit-log graph paper.
4. The concentration of insulin in sample was read from the standard curve by extrapolation.

4.3.3 ESTIMATION OF TRI-IODO THYRONINE (T₃)

Principle

It was estimated by RIA test system. The essential reagents required for a solid phase radioimmunoassay include insoluble antibody, radio labeled antigen and negative antigen. Upon mixing insolubilised antibody, radiolabeled antigen and a serum containing the negative antigen, a competition reaction results between the negative antigen and the radiolabeled antigen for a limited number of insolubilised binding sites. The interaction is illustrated by the following reaction.

$^{125}\text{I-}Ag + Ag + Ab \rightleftharpoons AgAb + ^{125}\text{I-}AgAb$

$K_a$
Ab = Monospecific insolubilised antibody (Constant quantity)
Ag = Negative antigen (Variable quantity)
AgAb = Antigen-Antibody complex
Ag$_{125}$IAb = Radiolabeled antigen-antibody complex
Ka = Rate constant of association
K-a = Rate constant of dissociation
= Ka/K-a = Equilibrium constant

After equilibrium is attained, the antibody bound fraction is separated from unbound antigen following simple centrifugation by aspiration of decantation. The radioactivity in the antibody bound fraction is inversely proportional to the negative antigen concentration.

Reagents (Supplied in the kit)
Thyroxin human serum references:
0, 2.5, 5.0, 10.0 and 20.0 ul/dl
Non-specific reagent
Tracer reagent
Antibody reagent
Human serum controls

Procedure
Before proceeding with the assay, all the reagents brought to ambient temperature. Labeled test tubes for each serum references and test serum. 0.05 ml of the appropriate serum reference, control or specimen added into the assigned tubes and 0.05 ml tracer reagent to all. Then properly mixed uniform slurry of antibody reagent dispensed 1.0 ml to each tube. Vortex each tube for 2-3 seconds. Then incubate if it R.T. for 30 minutes. Centrifuged all tubes at 3000 rpm for 10 minutes at 23°C ± 2°C. A clear separation of precipitate and supernatant should be observed and if not, recentrifuge at a higher gravitational force. Aspirated all tubes complete counted for 30 seconds.

Calculations
\[
\% \text{ Bound} = \frac{\text{Sample count} - \text{NSB counts}}{\text{Average total count}} \times 100
\]
By utilizing several different serum references of known antigen concentration, a standard curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.3.4 ESTIMATION OF SERUM THYROXIN (T₄):

Principle

It was estimated by solid phase RIA. The essential requirement for a solid phase radioimmunoassay include insoluble antibody, radiolabeled antigen and negative antigen. It is same as described in T₃ assay.

Reagents (Supplied in the kit)

- Thyroxin human serum references: 0, 2.5, 5.0, 10.0 and 20.0 ul/dl
- Non-specific reagent
- Tracer reagent
- Antibody reagent
- Human serum controls

Procedure

Before proceeding with the assay, all the reagents brought to ambient temperature. Labeled test tubes for each serum references and test serum. 0.05 ml of the appropriate serum reference, control or specimen added into the assigned tubes and 0.05 ml tracer reagent to all. Then properly mixed uniform slurry of antibody reagent dispensed 1.0 ml to each tube. Vortex each tube for 2-3 seconds. Then incubate if it R.T. for 30 minutes. Centrifuged all tubes at 3000 rpm for 10 minutes at 23°C ± 2°C. A clear separation of precipitate and supernatant should be observed and if not, recentrifuge at a higher gravitational force. Aspirated all tubes completely and counted for 30 seconds.

Calculations

% Bound = \frac{\text{Sample count} - \text{NSB counts}}{\text{Average total count}} \times 100

By utilizing several different serum references of known antigen concentration, a standard curve can be
generated from which the antigen concentration of an unknown can be ascertained.

4.3.5 ESTIMATION OF SERUM THYROTROPIN (TSH) :
Principle
The assay is based on a sandwich technique using two monoclonal antibodies. It is performed in two steps.

**Immunological Step**

1. Monoclonal anti-TSH coated tubes
2. TSH present in the sample, standards and control
3. Enzymatic conjugate : Korseadish peroxidase-labeled monoclonal anti-TSH
4. Chromogen substrate : Ortho Phenylene Diamine (O.P.D.)/H₂O₂

The TSH concentration of each sample is determined using a calibration curve.

**Reagents supplied in the kit :**

TSH reagents
R₁ Anti-TSH tubes
R₂-0 to R₂-E TSH standard (between 0 and 40 mIU/l)
R₃ Control (Human serum)
R₄ Anti-TSH conjugate

Color EIA reagents
Color 0 Wash solution
Color 1 Chromogen
Color 2 Color 1 diluent
Color 3 Stopping reagent (18N H₂SO₄)
Procedure

It is recommended to perform the assay in duplicate. The dispensing and incubation times should be identical for all tubes in the same series.

Once started, the test should be completed without interruption.

Immunological Step:

<table>
<thead>
<tr>
<th>Dispense</th>
<th>Anti-TSH tubes (R₁)</th>
<th>IM EIA tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>Control</td>
</tr>
<tr>
<td>Standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R₂-O to R₂-E</td>
<td>200 ml</td>
<td>-</td>
</tr>
<tr>
<td>Control (R₃)</td>
<td>-</td>
<td>200 ml</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Conjugate (R₄)</td>
<td>200 ml</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

Incubate for 2 hours at 18-25°C with continuous shaking at 350 rpm.

Aspirate the liquid from each tube

Rapidly dispense working wash solution (2 ml of diluted color 0) in series of no more than 48 hours. The solution should not remain in the tubes for longer than one minute.

Aspirate immediately

Repeat the washing procedure

Aspirate thoroughly all traces of solution

Enzymatic Step:

<table>
<thead>
<tr>
<th>Color working Solution (Color 1 to Color 4)</th>
<th>300 ml</th>
<th>300 ml</th>
<th>300 ml</th>
<th>300 ml</th>
</tr>
</thead>
</table>

Incubate 30 min at 18-25°C in the dark.

<table>
<thead>
<tr>
<th>Stopping Reagent Color 3</th>
<th>1 ml</th>
<th>1 ml</th>
<th>1 ml</th>
<th>1 ml</th>
</tr>
</thead>
</table>
Shake on a vortex or equivalent
Read at 492 nm standard R2-0, A, B, C, D, E and samples against the reagent blank.

Notes:
The absorbance of the reagent blank should be <=0.2
The absorbance of standard R2-D should be >=1.0
If reading is delayed (2 hours maximum), place the tubes immediately in the dark and store at room temperature.

4.3.6 ESTIMATION OF TOTAL CHOLESTEROL (Wybenga and Pileggi Method):
Principle
Cholesterol reacts with hot solution of Ferric perchlorate, Ethyl acetate and Sulphuric acid (Cholesterol reagent) and gives a lavender colored complex, which is measured colorimetrically.

Procedure
The tubes were arranged and the reagents and the serum samples were added as follows:

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1: Cholesterol Reagent</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Reagent 2: Working Cholesterol (200 mg %)</td>
<td>--</td>
<td>0.05 ml</td>
<td>--</td>
</tr>
<tr>
<td>Serum</td>
<td>--</td>
<td>--</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

Tubes were then mixed well and kept immediately in the boiling water bath exactly for 90 seconds. They were cooled immediately to room temperature under running tap water. The optical density (O.D.) of standard(S) and test(T) was measured against blank(B) on a spectrophotometer at 560 nm.

Calculations
Serum cholesterol in mg/100 ml = \(\frac{\text{O.D. (test)}}{\text{O.D. (std.)}}\) \times 200

4.3.7 ESTIMATION OF HDL-CHOLESTEROL:
Principle
Chylomicrons, VLDL (Very Low Density Lipoproteins) and
LDL fractions in serum or plasma are separated from HDL by precipitating with phosphotungstic acid-magnesium chloride. After centrifugation, the cholesterol in the HDL fraction, which remains in the supernatant is assayed with enzymatic cholesterol method, using cholesterol esterase, cholesterol oxidase, peroxidase and the chromogen, 4-aminoazone/phenol.

General System Parameters

- Reaction Type: Endpoint
- Wavelength: 505 nm (505-530 nm)
- Flowcell Temp.: 30°C
- Incubation: 30 Min. R.T./5 Min. 37°C
- Sample Vol.: 200 ul
- Precipitating reagent Vol.: 200 ul
- Supernatant Vol.: 20 ul
- Reagent Vol.: 1.0 ml
- Standard Conc.: 50 mg/dl
- Zero Setting With: Reagent Blank

4.3.8 ESTIMATION OF TRIGLYCERIDE:

Principle

Triglycerides incubated with lipoprotein lipase are hydrolyzed to free fatty acids and glycerol. Glycerol kinase catalyzes the conversion of glycerol and ATP to glycerol-3-phosphate and ADP. The glycerol-3-phosphate gets oxidized to dihydroxyacetone phosphate by glycerol-3-phosphate oxidase. Hydrogen peroxide (H₂O₂) formed in this reaction with the help of peroxidase, reacts with chromogens 4-aminoantipyrine/3,5, dichloro-2-hydroxybenzenesulfonic acid to give a red coloured complex which is read at 510 nm (500-530 nm).

LP Lipase \( \rightarrow \) Glycerol + Free Fatty Acids

Glycerol Kinase

Glycerol ATP \( \rightarrow \) Glycerol 3-P + ADP

Glycerol-3-P + O₂ \( \rightarrow \) DHAP + H₂O₂
H₂O₂ + 4-aminoantipyrine + DHBS $\rightarrow$ Red coloured compound

**General System Parameters**

- **Reaction Type**: Endpoint
- **Wavelength**: 505 nm (500 - 530)
- **Flowcell Temp.**: 30°C
- **Incubation**: 15 Min. R. T.
- **Sample Vol.**: 10 ul
- **Reagent Vol.**: 1.0 ml
- **Standard Conc.**: 200 mg/dl
- **Zero Setting With**: Reagent Blank.

**4.3.9 ESTIMATION OF LDL-CHOLESTEROL:**

After determining serum cholesterol, triglyceride and HDL cholesterol, serum LDL levels were found with the help of Fiedman's formula.

\[
LDL = \frac{\text{Total Cholesterol} - \text{Triglyceride}}{5} \text{ - HDL}
\]

**4.3.10 ESTIMATION OF CREATININE (Alkaline Picrate method):**

**Principle**

Creatinine forms a coloured complex with picrate in alkaline medium. The rate of formation of the complex is measured.

**Procedure**

- **Wavelength**: 510 nm (490 - 510 nm)
- **Spectrophotometer**: 490 nm
- **Cuvette**: 1 cm light path
- **Temperature**: 25°C.
- **Blank**: Air

<table>
<thead>
<tr>
<th>Pipette into cuvette</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent mixture 5</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Reagent 1 sample</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

Mix and start stopwatch at the same time. After 30 sec, read absorbance A₁ of standard and sample respectively, and exactly 2 min. later, read absorbance A₂ of standard and sample. $A_2 - A_1 = A$ sample or $A$ standard.
Calculation

The concentration (c) of creatinine in serum or plasma:

\[
c = 2.0 \times \frac{\text{A sample}}{\text{A standard}} \quad (\text{mg/100 ml})
\]

4.3.11 ESTIMATION OF UREA (DAM method):

Principle

Urea reacts with not acidic Diacetylmonoxime in presence of Thiosemicarbazide and produces a rose-purple color complex, which is measured colorimetrically.

Reagents (Supplied in the kit)

Reagent 1: Urea reagents
Reagent 2: Diacetylmonoxime (DAM)
Reagent 3: Working urea standard 30 mg%

Preparation of working solution

Solution 1: Dilute 1 ml of reagent 1 to 5 ml with distilled water reagent 2 and reagent 3 are ready for use.

Procedure

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Test (T)</th>
<th>Standard (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>0.02 ml</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 3</td>
<td>-</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
</tr>
</tbody>
</table>

Mix well

| Reagent 2 | 0.5 ml | 0.5 ml | 0.5 ml |

Mix well and keep the tubes in a boiling water bath exactly for 10 minutes. Cool them immediately under running water for 5 min., mix by inversion and measure the color intensity within 10 mins using a green filter against blank.

Calculation

\[
\text{Urea in mg/100 ml} = \frac{\text{O.D test}}{\text{O.D std.}} \times 30
\]
4.3.12 ESTIMATION OF SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT):

Principle and procedure

SGPT catalyses the following reaction.

Alpha keto glutarate + L-Alanine \(\rightarrow\) L-Glutamate + Pyruvate

Pyruvate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2, 4- DNPH ) to give a corresponding hydrazone, which gives brown colour in alkaline medium and this can be measured colorimetrically. The standard curve with different concentrations of standard enzymetic activity is created and from that curve enzymatic activity in serum samples is determined.

4.3.13 ESTIMATION OF SERUM GLUTAMATE OXALOACETATE TRANSAMINASE (SGOT):

Principle

GOT (AST) catalyzes the transfer of the amino group from L-aspartate to alpha - ketoglutarate to yield oxaloacetate and L - glutamate. Malate dehydrogenase (MDH), then converts oxaloacetate and NADH to malate and NAD. The conversion of NADH to NAD decreases the absorance at 340 nm, the rate of which is proportional to the GOT (AST) activity.

\[
\text{L-Asparatate} + \text{a - Ketoglutarate} \xrightarrow{\text{GOT(AST)}} \text{Oxaloacetate} + \text{L - Glutamate}
\]

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L - Malate} + \text{NAD}^+
\]

General System Parameters

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Kinetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>340</td>
</tr>
<tr>
<td>Flowcell Temp.</td>
<td>37°C</td>
</tr>
<tr>
<td>Delay Time</td>
<td>60 Sec.</td>
</tr>
<tr>
<td>No. Of Readings</td>
<td>4</td>
</tr>
<tr>
<td>Interval</td>
<td>30 Sec.</td>
</tr>
<tr>
<td>Sample Vol.</td>
<td>100 ul</td>
</tr>
<tr>
<td>Reagent Vol.</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Pathlength</td>
<td>1 Cm.</td>
</tr>
<tr>
<td>Factor</td>
<td>1749</td>
</tr>
<tr>
<td>Zero Setting With</td>
<td>Distilled Water.</td>
</tr>
</tbody>
</table>

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4.3.14 ESTIMATION OF ALKALINE PHOSPHATASE:

Principle

Alkaline Phosphatase hydrolysis P-nitrophenyl phosphate (PNPP) into P-nitrophenol and phosphate. At the alkaline pH of the buffered medium, P-nitrophenol is yellow. The colour developed by hydrolysis is measured at 405 nm and is proportional to the alkaline phosphatase activity.

\[ \text{P-nitrophenol phosphate} + \text{H}_2\text{O} \xrightarrow{\text{ALKP}} \text{P-nitrophenol} + \text{phosphate} \]

\[ \text{(Colourless in acid/alkali) \xrightarrow{\text{yellow in colour}} } \]

General System Parameters

- Reaction Type: Kinetic
- Wavelength: 405 nm
- Flowcell Temp.: 25°C
- Delay Time: 60 Sec.
- No. Of Readings: 4
- Interval: 30 Sec.
- Sample Vol.: 30 ul
- Reagent Vol.: 1.0 ml
- Pathlength: 1 Cm.
- Factor: 1826
- Zero Setting With: Distilled Water.
### List of Assay Kits

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of Kit</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Glucose in serum (GOD / POD Method)</td>
<td>Span Diagnostics Ltd., Udhana (Surat)</td>
</tr>
<tr>
<td>2.</td>
<td>Insulin (Radio Immunoassay)</td>
<td>B.A.R.C. Bombay</td>
</tr>
<tr>
<td>3.</td>
<td>Tri-iodothyronine (T&lt;sub&gt;3&lt;/sub&gt;) (RIA)</td>
<td>Monobind Pvt. Ltd., California, USA</td>
</tr>
<tr>
<td>4.</td>
<td>Thyroxin (T&lt;sub&gt;4&lt;/sub&gt;) (RIA)</td>
<td>Monobind Pvt. Ltd., California, USA</td>
</tr>
<tr>
<td>5.</td>
<td>Thyrotropin (TSH) (ELISA)</td>
<td>Biomerieux, France</td>
</tr>
<tr>
<td>6.</td>
<td>Cholesterol (Wybenga and Pileggi Method)</td>
<td>Miles India Ltd., Baroda</td>
</tr>
<tr>
<td>7.</td>
<td>HDL Cholesterol (Phodpotungstate method)</td>
<td>Miles India Ltd., Baroda</td>
</tr>
<tr>
<td>8.</td>
<td>Triglycerides (Enzymatic)</td>
<td>Miles India Ltd., Baroda</td>
</tr>
<tr>
<td>9.</td>
<td>Creatinine</td>
<td>Miles India Ltd., Baroda</td>
</tr>
<tr>
<td>10.</td>
<td>Blood Urea (DAM method)</td>
<td>Span Diagnostics Ltd., Udhana (Surat)</td>
</tr>
<tr>
<td>11.</td>
<td>Alkaline Phosphatase</td>
<td>Miles India Ltd., Baroda</td>
</tr>
<tr>
<td>12.</td>
<td>SGOT</td>
<td>Miles India Ltd., Baroda</td>
</tr>
<tr>
<td>13.</td>
<td>SGPT</td>
<td>Miles India Ltd., Baroda</td>
</tr>
<tr>
<td>14.</td>
<td>Glucose in urine (Enzymatic)</td>
<td>Miles India Ltd., Baroda</td>
</tr>
</tbody>
</table>