MATERIAL AND METHODS

CHAPTER-II

MATERIALS AND METHODS

This study is carried out on total 180 subjects, comprising of both the normal subjects and the patients.

Totally thirty normal subjects are studied, including twenty-one males and nine females. Their age ranges from twenty-seven to seventy-six years. These subjects were selected at random from the S.S.G.Hospital and Medical College, Baroda.

The patients are divided into five groups as shown below :

Group	Patients suffering from	Total No.	No.of males	No.of females	Range of age (years)
I	Diabetes mellitus	30	18	12	30-70
II	Diabetics with cardiac diseases	30	16	14	35-70
III	Diabetics with renal diseases	30	18	12	09-55

Group	Patients suffering from	Total No.	No.of males	No.of females	Range of age (years)
IV	Diabetics with hypertension	30	18	12	41-55
V	Diabetics with neuro-muscular disorders	30	18	12	03-70

All these patients were selected from the S.S.G.Hospital, Baroda.

Blood was collected in fluoride and plain bulbs from all the subjects after overnight fasting and the plasma and sera respectively separated by centrifugation.

The samples from all the subjects were analysed for the levels of (I) blood sugar, (ii) protein-bound hexose, (iii) protein-bound sialic acid, (iv) protein-bound fucose and (v) protein-bound hexosamine.

All the methods selected for the present study are standard procedures, giving reproducible results and these methods were feasible to carry out in our laboratory. All chemicals used were of analytical grade from standard companies.

I) **Blood Sugar**

All the plasma samples from fluoride bulb were analysed for blood sugar estimation. Estimation of blood sugar was carried out by the enzymatic Glucose Oxidase-Peroxidase method of Trinder P (1969).
This method is based on the principle that glucose oxidase (GOD) converts glucose to gluconic acid. Hydrogen peroxide formed in this reaction, in presence of peroxidase (POD), oxidatively couples with 4-aminoantipyrine and phenol to produced red quinoneimine chromogen. This chromogen has absorbance maximum at 505 mμ.

The intensity of the coloured complex is directly proportional to the concentration of glucose in the sample.

To GOD/POD working solution, plasma and standard glucose solution are added. The mixture is incubated at room temperature for 30 minutes and later the absorbance is read against reagent blank at 505 nm.

The concentration of glucose is then calculated in terms of milligrams percent.

Serum from plain bulb is utilized for the estimation of protein-bound hexose (PBH), protein-bound sialic acid (PBSA), protein-bound fucose (PBF) and protein-bound hexosamine (PBHA).

ii) **Protein-bound Hexose**

Protein-bound hexose is estimated by the method of Weimer and Moshin (1952).

In this method, the hexose moiety of glycoprotein-conjugates, precipitated by ethanol at room temperature, is determined by the orcinol reaction. Galactose-Mannose mixture is used in the preparation of standard solution.

To proteins precipitated by ethanol and dissolved in sodium hydroxide (0.1 N) to standard solution and to distilled water constituting the blank, the orcinol-sulphuric acid reagent is added. The tubes are capped to avoid evaporation and incubated at 80° for 15 minutes. After cooling, the absorbance is read against reagent blank at 540 mμ.

The amount of PBH is then calculated in terms of milligrams percent and expressed as galactose and mannose.

iii) **Protein-bound Sialic Acid**

The PBSA is estimated by the method of Coburn et al (1953).

Sialic acid when reacted with diphenylamine reagent forms a purple chromogen. Sialic acid from Sigma is used for preparation of standard.

Serum and standard solution are treated with trichloroacetic acid (5%) and kept in boiling water bath for 15 minutes. Filtrates from the respective tubes is then treated with diphenylamine reagent. For non-specific colour development , the filtrates are treated with acid mixture. The tubes are placed in boiling water bath for 30 minutes and then after cooling, the absorbance is read against a reagent blank at 530 mμ.

The amount of PBSA is then calculated in terms of milligrams percent.

iv) **Protein-bound Fucose**

The PBF is estimated by the method of Dishe and Shettles (1948).

Serum containing protein-bound methylpentose when heated with sulphuric acid, followed by the addition of cysteine forms coloured complex. The absorbace is read at two different wavelengths in order to avoid the colour developed by other sugars. Standard solution is prepared by using fucose.

To the proteins precipitated by ethanol and dissolved in sodium hydroxide (0.1N), and the standard solution, ice-cold sulphuric acid mixture is added. These tubes are placed in boiling water bath for 3 minutes and after cooling, cysteine reagent is added. After 60 to 90 minutes, at room temperature, the absorbance is read at 396 and 430 mμ, setting the zero by distilled water.

The amount of fucose is then calculated in terms of milligrams percent.

v) **Protein-bound Hexosamine**

The PBHA is estimated by the method of Remington (1940).

Serum proteins are precipitated by alcohol and the hexosamines are liberated from the glycoproteins by acid hydrolysis. Acetylation in alkaline medium cyclizes the hexosamines to pyrrole derivatives, which couples with para-dimethylaminobenzaldehyde forming coloured complex. Standard solution is prepared by using glucosamine.

Proteins are precipitated by ethanol and hydrolysed by hydrochloric acid. Hydrolysate is then neutralized. To the neutralized hydrolysate and the standard solution, acetylacetone reagent is added and then tubes are placed in boiling water bath for 15 minutes. After cooling, Ehrlich's reagent is added and the final volume adjusted by ethanol. After 30 minutes, the absorbance is read against reagent blank at 530 mμ.

The concentration of PBHA is calculated and expressed in terms of milligrams percent glucosamine.

The data obtained in this study were subjected to statistical analysis by Computer.