MATERIAL
&
METHODS
MATERIAL AND METHODS

The present study was conducted in the Dept. of Biochemistry M.G.M. Medical College and Associated M.Y. Hospital Indore. The subjects were selected from the outpatient Department of Medicine and Central laboratory collection room M Y Hospital, Indore. The study comprised of 319 type II diabetes patients that included 162 patients without nephropathy and 157 diabetics with nephropathy grouped on basis of microalbuminuria, which is the earliest sign of diabetic nephropathy. Hundred and sixty five age matched healthy non diabetic volunteers were taken as control group.

Group A - Normal Healthy Control – (N=165)

Group B – Type II DM patients without nephropathy
(Microalbuminuria<30mg/day) (N= 162)

Group C – Type II DM patients with nephropathy
(Microalbuminuria>30mg/day) (N=157)

Group C patients were further divided into two sub groups as diabetics with microalbuminuria, C-I (urine albumin 30-299 mg/day) (n=30) and diabetics with macroalbuminuria, C-II (urine albumin ≥ 300mg/day) (n=127).

In few samples of the above mentioned groups, urine creatinine and albumin were measured on fasting/random urine samples and the groups were designated as A\(_{(\text{UACR})}\) N= 26; B\(_{(\text{UACR})}\) N= 52; C\(_{(\text{UACR})}\) N= 65.

For diagnosis of diabetes mellitus W.H.O. criteria was used:

Symptoms of diabetes plus

a) Random blood glucose concentration >11.1 mmol/l (200mg/dl) or

b) Fasting plasma glucose >7.0mmol (126mg/dl) or

c) Two-hour plasma glucose >11.1mmol (200mg/dl) during an OGTT.
The inclusion criteria for individuals in control group were:

1. Normal, healthy individuals
2. Non Diabetic with normoglycemia, non alcoholic and non smokers.
3. No past history of chronic infection, hypertension, Diabetes, systemic disorder or any major surgery.
4. They were not taking any kind of treatment or vitamin supplements, which were likely to affect the biochemical parameters observed in the present study.

The inclusion criteria for individuals in study group were:

1. Any new adult patient who fitted in WHO criteria of diabetes with no history of juvenile diabetes.
2. Known type II diabetic patients on regular treatment & follow up.
3. Type II Diabetic patients with microalbuminuria / frank albuminuria were put in the category of DM with nephropathy, while patients without microalbuminuria were taken as DM without nephropathy.

The exclusion criteria for individuals in study group were:

1. Patients on any kind of multivitamin, lipid lowering agents, anti-inflammatory drugs, analgesics, anticoagulants like aspirin or admitted patients.
2. Sample collection during febrile duration was avoided.
3. Pregnant or lactating women were not included in the study.
4. Alcoholics, smokers and individuals with tobacco or drug addiction were not included.
5. Past or present history of chronic illness like tuberculosis, rheumatoid arthritis other autoimmune disorders were excluded.
6. Patients of juvenile and type I DM were excluded from study group.

SAMPLE COLLECTION:

Fasting venous blood sample was drawn from all the subjects in EDTA tube (2ml) and plain tube (5ml), the serum was carefully separated and transferred
to microtubes and stored at – 20°C until analysis. Post prandial venous blood sample was collected 2 hours after meal. For collection of 24 hour urine samples, wide mouth 5 litre containers were provided to the patients and control subjects and instructions regarding urine collection were given. The urine volume was measured and 20 ml sample was preserved for analysis.

STATISTICAL ANALYSIS

The present study was a case control study and the method of sampling used was non random – purposive. For comparing above said parameters in control and study group, we applied unpaired t test for equality of means to see 2-tailed significance values. Correlations were calculated by Pearson’s Correlation Coefficient (two-tailed). The said calculations were made on SPSS software version 19.

Estimation of the following biochemical parameters was done by standard techniques in all the samples.

1. Plasma glucose fasting and post prandial
2. Estimated Average Glucose (eAG, calculated)
3. Serum urea
4. Serum creatinine
5. LIPID PROFILE
   - serum total cholesterol
   - serum triglycerides
   - serum high density lipoprotein cholesterol (HDL-C)
   - serum very low density lipoprotein cholesterol (VLDL-C, calculated)
   - serum low density lipoprotein cholesterol (LDL-C, calculated)
6. Serum high sensitivity C-reactive protein (hs-CRP)
7. Glycosylated hemoglobin (HbA1c) in EDTA whole blood
8. Serum total sialic acid (TSA)
9. Serum ceruloplasmin (Cp)
10. Serum malondialdehyde (MDA)
11. Serum super oxide dismutase (SOD)
12. Serum vitamin C
13. Urine microalbumin
14. Urine creatinine
15. Urine albumin:creatinine ratio (UACR, calculated)
Serum sialic acid, ceruloplasmin, MDA, SOD and vitamin-C were analyzed by manual methods using computerized fully automated Shimadzu pharma spec (UV-1700) double beam UV-VIS spectrophotometer. Glycosylated hemoglobin in whole blood, serum high sensitivity C-reactive protein, plasma glucose fasting and post prandial, serum urea, serum creatinine, Lipid Profile, urine microalbumin/albumin and creatinine were measured using Biosystems A-25 and Vitalab SELECTRA-E computerized fully automated random access clinical chemistry analyzer.

**ESTIMATION OF PLASMA GLUCOSE**

Plasma Glucose was estimated by GOD-POD method.\(^{522}\)

**Principle**

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GOD}} \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

\[
2 \text{H}_2\text{O}_2 + \text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinonemine} + 4\text{H}_2\text{O}
\]

**Sample**

Unhemolysed plasma sample.

Glucose in serum or plasma is stable for 5 days at 2-8\(^0\)C.

**Assay Parameters**

- Analysis Mode: mono.fixed-time
- Reading: monochromatic
- Sample volume (\(\mu l\)): 3
- Reagent volume (\(\mu l\)): 300
- Delay time (Reading 1): 45 sec
- Kinetic interval (Reading 2): 15 min
- Wavelength 1 (nm): 500
- Wavelength 2 (nm): ----
- Linearity limit (mg/dl): 500
Procedure

<table>
<thead>
<tr>
<th>Sample or standard</th>
<th>3μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoreagent</td>
<td>300μl</td>
</tr>
</tbody>
</table>

The reaction mixture is incubated for 15 min. at 37°C in the analyser. Note the results from the monitor. The absorbance remains stable for 30 minutes.

Calculations

\[
\text{Glucose (mg/dl)} = \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{conc. of standard (mg/dl)}
\]

Reference range

Fasting Glucose: 70-110 mg/dl, Post Prandial Glucose: 70-140 mg/dl

ESTIMATION OF SERUM UREA

Photometric determination of urea according to the Urease/Glutamate dehydrogenase method (GLDH- kinetic UV).\(^{523}\)

Sample

Unhemolysed serum sample.

Urea in serum or plasma is stable for 7 days at 2-8°C.

Principle

The estimation of Urea in serum involves the following enzyme catalyzed reactions:

\[
\text{Urea} + \text{H}_2\text{O} \xrightarrow{\text{urease}} 2\text{NH}_4^+ + \text{CO}_2
\]

\[
\text{NH}_4^+ + \text{NADH} + \text{H}^+ + \text{2-oxoglutarate} \xrightarrow{\text{GLDH}} \text{Glutamate} + \text{NAD}^+
\]

The rate of absorbance changing at 340 nm is directly proportional to Urea concentration in the serum.
**Assay Parameters**

- Analysis Mode: mono.fixed-time
- Reading: monochromatic
- Sample volume (μl): 3
- Reagent volume (μl): 300
- Delay time (Reading 1): 45 sec
- Kinetic interval (Reading 2): 90 sec
- Wavelength 1 (nm): 340
- Wavelength 2 (nm): ----
- Linearity limit (urea) (mg/dl): 300
- Linearity limit (BUN) (mg/dl): 140

**Procedure**

<table>
<thead>
<tr>
<th>Sample or standard</th>
<th>3μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoreagent</td>
<td>300μl</td>
</tr>
</tbody>
</table>

Mix, incubate for 45 sec. at 37° C and read absorbance A1 exactly after 90 sec.

**Calculations**

\[ \text{Urea (mg/dl)} = \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{conc. of standard (mg/dl)} \]

**Reference range**

Serum Urea: 15-39 mg/dl

Blood urea nitrogen (BUN): 7-18 (mg/dl)

**ESTIMATION OF CREATININE**

Alkaline picrate method.524

**Sample**

Serum, Urine (dilute urine sample 1/20 with distil water before analysis)

**Principle**

The assay is based on the reaction of creatinine with sodium picrate. Creatinine reacts with alkaline picrate forming a red complex. The intensity of the color formed is proportional to the creatinine concentration in the sample.
Assay Parameters

- Analysis Mode: mono.fixed-time
- Reading: monochromatic
- Sample volume (μl): 30
- Reagent volume (μl): 300
- Delay time (Reading 1): 30 sec
- Kinetic interval (Reading 2): 90 sec
- Wavelength 1 (nm): 505nm
- Wavelength 2 (nm): ----
- Linearity limit: 20 mg/dl

Procedure

<table>
<thead>
<tr>
<th>Sample or standard</th>
<th>30μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoreagent</td>
<td>300μl</td>
</tr>
</tbody>
</table>

Mix, incubate for 30 sec. at 37°C and read absorbance A1 exactly after 90 sec.

Calculations

Creatinine (mg/dl) = \( \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{conc. of standard (mg/dl)} \)

Urine Creatinine (mg/dl) = \( \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{conc. of standard (mg/dl)} \times 20 \)

UACR in mg/gm = \( \frac{\text{Urine albumin (mg/dl)}}{\text{Urine creatinine (gm/dl)}} \)

Reference range

Serum Creatinine: 0.6-1.1 mg/dl
Urine Creatinine: 11-26 mg/kg/day
UACR: < 30 mg/gm

ESTIMATION OF SERUM CHOLESTEROL

Serum Cholesterol was estimated by the CHOD-PAP method.525

Principle

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyses the esters. In the subsequent oxidation by
cholesterol oxidase $\text H_2\text O_2$ is liberated. The colorimetric indicator quinoneimine is generated from 4-aminoantipyrine and phenol by $\text H_2\text O_2$ under the catalytic action of peroxidase.

\[
\text{CHE} \\
\text{Cholesterol ester} + \text H_2\text O \rightarrow \text cholesterol + \text Fatty acid
\]

\[
\text{CHO} \\
\text Cholesterol} + \text O_2 \rightarrow \text Cholesterol-3-one} + \text H_2\text O_2
\]

\[
2\text H_2\text O_2 + 4\text -\text Aminoantipyrine} + \text Phenol \rightarrow \text Quinoneimine} + 4\text H_2\text O
\]

**Sample**

Serum or EDTA plasma

The stability in serum or plasma is 7 days at 2-8°C.

**Assay Parameters**

- Analysis Mode: mono.endpoint
- Reading: bichromatic
- Sample volume (µl): 3
- Reagent volume (µl): 300
- Delay time (Reading 1): ----
- Kinetic interval (Reading 2): 300 sec
- Wavelength 1 (nm): 505 nm
- Wavelength 2 (nm): 670 nm
- Linearity limit: 1000 mg/dl

**Procedure**

<table>
<thead>
<tr>
<th>Sample or standard</th>
<th>3µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoreagent</td>
<td>300µl</td>
</tr>
</tbody>
</table>

Mix, incubate for 15 sec. at 37° C and read absorbance A1 exactly after 300 sec.

**Calculations**

\[
\text{Cholesterol (mg/dl)} = \frac {A \text{ of Sample}} {A \text{ of Standard}} \times \text{conc. of standard (mg/dl)}
\]
Reference Range

Desirable = < 200 mg/dl
Borderline high = 200 – 239 mg/dl
High = > 240 mg/dl

ESTIMATION OF SERUM TRIGLYCERIDES

Serum triglycerides were estimated according to the GPO – POD method.526

Principle

Determination of triglycerides involves enzymatic splitting with lipoprotein lipase; indicator is quinoneimine, which is generated from 4-aminoantipyrine and 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxides.

\[
\text{Lipase} \\
\text{Triglycerides} + \text{H}_2\text{O}_2 \rightarrow \text{Glycerol} + \text{fatty acids}
\]

\[
\text{GK} \\
\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol} 3\text{-phosphate}
\]

\[
\text{GPO} \\
\text{Glycerol} 3\text{-phosphate} + \text{O}_2 \rightarrow \text{Dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
\text{POD} \\
2\text{H}_2\text{O}_2 + \text{Aminoantipyrine} + 4\text{Chlorophenol} \rightarrow \text{Quinoneimine} + 4\text{H}_2\text{O}
\]

Sample

Serum or EDTA plasma

The stability in serum or plasma is 7 days at 2-8\textdegree{}C.

Assay Parameters

- Analysis Mode: endpoint
- Reading: bichromatic
- Sample volume (\(\mu l\)): 3
- Reagent volume (\(\mu l\)): 300
- Delay time (Reading 1): 300 sec
- Kinetic interval (Reading 2): ----
- Wavelength 1 (nm): 505nm
- Wavelength 2 (nm): 670nm
- Linearity limit: 600 mg/dl
**Procedure**

<table>
<thead>
<tr>
<th>Sample or standard</th>
<th>3μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoreagent</td>
<td>300 μl</td>
</tr>
</tbody>
</table>

Mix, incubate for 300 sec. at 37° C and read absorbance A.

**Calculations**

\[
\text{Triglycerides (mg/dl)} = \frac{A \text{ of Sample}}{A \text{ of Standard}} \times \text{conc. of standard (mg/dl)}
\]

**Reference Range**

Normal: upto 150 mg/dl  
Borderline high: 150-199 mg/dl  
High: 200-499 mg/dl  
Very high: >500 mg/dl

**ESTIMATION OF HDL CHOLESTEROL**

Direct. Enzymatic – Liquid.\(^{527}\)

**Principle**

The method depends on the properties of a detergent which solubilizes only the HDL, so that the HDL-C is released to react with the cholesterol esterase, cholesterol oxidase and chromogens to give colour. The non HDL lipoproteins LDL, VLDL and chylomicrons are inhibited from reacting with the enzymes due to absorption of the detergents on their surfaces.

The intensity of the colour formed is proportional to the HDL-C concentration in the sample.

\[
\text{CHE} \\
\text{CHEsterol ester} + \text{H}_2\text{O} \rightarrow \text{CHEsterol} + \text{Fatty acid}
\]

\[
\text{CHO} \\
\text{CHOlesterol} + \text{O}_2 \rightarrow \text{CHolesterol-3-one} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{DSBmT} \rightarrow \text{Quinoneimine} + 4\text{H}_2\text{O}
\]

\[
\text{DSBmT} = \text{N,N-bis (4-sulfobutyl)-m-toluidine}
\]

**Sample**

Serum or EDTA plasma.  
The stability in serum or plasma is 7 days at 2-8° C.
**Assay Parameters**

- Analysis Mode: mono.fixed-time
- Reading: monochromatic
- Sample volume (μl): 7
- Reagent 1 volume (μl): 750
- Delay time (Reading 1): 5 min
- Reagent 2 volume (μl): 250
- Kinetic interval (Reading 2): 5 min
- Wavelength 1 (nm): 600
- Wavelength 2 (nm): ----
- Linearity limit (mg/dl): 200

**Procedure**

<table>
<thead>
<tr>
<th>Sample or standard</th>
<th>7μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1</td>
<td>750μl</td>
</tr>
<tr>
<td>Mix, incubate for 5 min at 37° C and read absorbance A1</td>
<td></td>
</tr>
<tr>
<td>Reagent 2</td>
<td>250 μl</td>
</tr>
</tbody>
</table>

Mix, incubate for 5 min at 37° C and read absorbance A₂. ΔA = A₂ - A₁

**Calculation**

\[
\text{Cholesterol (mg/dl)} = \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{conc. of standard (mg/dl)}
\]

**Reference Range**

Low risk: > 60 mg/dl

High risk: upto 35 mg/dl

**Calculation of LDL and VLDL** By Friedwald Equation

VLDL (mg/dl) = Triglycerides / 5

LDL Cholesterol (mg/dl) = Total Cholesterol − (VLDL + HDL)
ESTIMATION OF HbA1c IN WHOLE BLOOD

Immunoturbidimetric method.529

Principle

After preparing the hemolysate using Tetradecyltrimethylammonium bromide (TTAB) as the detergent, the HbA1c concentration is quantified by a turbidimetric inhibition immunoassay. First, by the addition of the sample to a reagent with antibodies against a specific site of HbA1c, soluble complexes formed by the union of two molecules appear. After the addition to the reaction mixture of a second reagent formed by polyhaptens, the excess anti-HbA1c antibodies form insoluble complexes, antibody-polyhapten, which can be determined turbidimetrically. The estimation of the HbA1c in percent is made by the measure of total hemoglobin concentration by spectrometry.

Reagents

1. Tetradecyltrimethylammonium bromide (9gm/lit)
2. Phosphate buffer (20mmol/L) pH7.4
3. MES buffer (25mmol/L), TRIS buffer (15mmol/L), goat anti-HbA1c antibodies. pH 6.2.
4. MES buffer (25mmol/L), TRIS buffer (15mmol/L), HbA1c polyhapten (≥8μg/ml). pH 6.2.

Sample

Unhemolysed whole blood sample in EDTA.

HbA1c in whole blood is stable for 7 days at 2-8°C

Procedure

Hemolysate preparation
Bring the reagent 1 to room temperature.
Sample | 10μl
---|---
Reagent 1 | 1000 μl

Shake thoroughly. Avoid the formation of foam.

The hemolysate can be used after the solution has changed color from red to brownish-green (approximately 3 minutes). The hemolysate is stable 4 hours at 15-25°C, 24 hours at 2-8°C and 6 months at −20°C. Freeze once only.

Assay parameters

<table>
<thead>
<tr>
<th>Test name</th>
<th>Hb Total</th>
<th>HbA1C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis mode</td>
<td>endpoint</td>
<td>differential</td>
</tr>
<tr>
<td>Reading</td>
<td>bichromatic</td>
<td>monochromatic</td>
</tr>
<tr>
<td>Sample volume (μl)</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>Reagent 1 volume (μl)</td>
<td>170</td>
<td>200</td>
</tr>
<tr>
<td>Reagent 2 volume (μl)</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>Postdilution factor</td>
<td>1.2</td>
<td>2</td>
</tr>
<tr>
<td>Wavelength 1 (nm)</td>
<td>560</td>
<td>340</td>
</tr>
<tr>
<td>Wavelength 2 (nm)</td>
<td>670</td>
<td>-</td>
</tr>
<tr>
<td>Delay time Reading 1</td>
<td>300 s</td>
<td>180 s</td>
</tr>
<tr>
<td>Lag time Reading 2</td>
<td>-</td>
<td>480 s</td>
</tr>
<tr>
<td>Lag time Reagent 2</td>
<td>-</td>
<td>195 s</td>
</tr>
<tr>
<td>Linearity</td>
<td>40gm/dl</td>
<td>2.5gm/dl</td>
</tr>
</tbody>
</table>

Calculations

The Hb_A1c percentage in the sample is calculated using the following general formula. The values are traceable to IFCC Reference Method:

\[
\% \text{Hb}_A1c - \text{IFCC} = \frac{\text{Hb}_A1c (g/dL)}{\text{Hb} (g/dL)} \times 100
\]

The traceable values to Reference Method as described by the US National Glycohemoglobin Standardization Program (NGSP) are calculated using the following general formula:

\[
\% \text{Hb}_A1c - \text{NGSP} = 0.915 \times \% \text{Hb}_A1c - \text{IFCC} + 2.15
\]
### Reference Range

<table>
<thead>
<tr>
<th>Degree of Control</th>
<th>DCCT / NGSP</th>
<th>IFCC</th>
<th>ADA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Diabetic</td>
<td>4.0 - 6.0</td>
<td>2.0 - 4.2</td>
<td>5.0 - 5.5</td>
</tr>
<tr>
<td>Goal</td>
<td>6.0 - 6.5</td>
<td>4.2 - 4.8</td>
<td>5.5 - 6.0</td>
</tr>
<tr>
<td>Good Control</td>
<td>6.5 - 8.0</td>
<td>4.8 - 6.4</td>
<td>&lt; 7.0</td>
</tr>
<tr>
<td>Action suggested</td>
<td>&gt; 8.0</td>
<td>&gt; 6.4</td>
<td>&gt; 8.0</td>
</tr>
</tbody>
</table>

### CALCULATION OF ESTIMATED AVERAGE GLUCOSE (eAG)\(^{530}\)

Estimated average glucose in mg/dl (eAG) = \((28.7 \times Hb_{A1c}) - 46.7\)

### ESTIMATION OF SERUM **hsC-REACTIVE PROTEIN**

Latex -immunoturbidimetric high sensitivity method.\(^{531}\)

#### Principle

Serum C-reactive protein (CRP) causes agglutination of the latex particles coated with anti-human C-reactive protein. The agglutination of the latex particles is proportional to the CRP concentration and can be measured by turbidimetry.

#### Reagents

A. Glycine buffer (0.1mol/L), Sodium azide (0.95gm/L) pH 8.6

B. Suspension of latex particles coated with anti human C-reactive protein Antibodies, Sodium azide (0.95gm/L)

C. CRP hs- standard

#### Sample

Unhemolysed serum sample.

CRP in serum is stable for 7 days at 2-8\(^{0}\)C.

#### Procedure

Calibration curve: Prepare dilution of the hs-CRP Standard using 9g/L saline as diluent. Multiply the concentration of the hs-CRP Standard by the corresponding factor indicated below to obtain the hs-CRP concentration of the dilution.
<table>
<thead>
<tr>
<th>Dilution</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard (µL)</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>Saline (µL)</td>
<td>70</td>
<td>60</td>
<td>40</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Factor</td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Pipette into a cuvette:

| Sample/standard/ water(blank) | 20 µl |
| Working Reagent ( 9 ml reagent A + 1 ml reagent B) | 1.5 ml |

Mix and immediately insert the cuvette into the instrument. Start stopwatch. Record the absorbance at 540 nm and after 10 seconds ($A_1$) and after 5 seconds ($A_2$).

**Calculations**

Calibration curve: Calculate the absorbance difference ($A_{\text{Standard}} - A_{\text{Blank}}$) of each point of the calibration curve and plot the values found against the hs-CRP concentration. hs-CRP concentration in the sample is calculated by interpolation of its absorbance ($A_{\text{Standard}} - A_{\text{Blank}}$) on the calibration curve.

**Reference Range**

Serum hs-CRP in adults – 2.68 to 8.5 mg/L.

**ESTIMATION OF URINE MICROALBUMIN**

Latex immunoturbidimetric method.\(^{532}\)

**Principle**

Albumin in the urine sample causes agglutination of the latex particles coated with anti-human albumin. The agglutination of the particles is proportional to the albumin concentration and can be measured by turbidimetry.

**Reagents**

A. Borate buffer (0.1 mol/L), Sodium azide (0.95 gm/L) pH 10.0
B. Suspension of latex particles coated with anti human albumin antibodies, Sodium azide (0.95gm/L)

C. Albumin- standard

Sample

Urine collected by standard procedures. Urine should be centrifuged before analysis.

Assay Parameters

- Analysis Mode  fixed time monochromatic
- Reading  monochromatic
- Turbidimetry test  yes
- Sample volume (μl)  3
- Reagent volume (μl)  420
- Post dilution factor  1.4
- Delay time (Reading 1)  30 sec
- Kinetic interval (Reading 2)  165 sec
- Wavelength 1 (nm)  535 nm
- Wavelength 2 (nm)  ----
- Linearity limit  130

Procedure

<table>
<thead>
<tr>
<th>Sample or standard</th>
<th>3 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent (1 ml reagent B + 4 ml reagent A)</td>
<td>420 μl</td>
</tr>
</tbody>
</table>

Mix, incubate for 30 sec. at 37° C and read absorbance A exactly after 165 sec.

Calculations

\[
\text{Microalbumin (mg/L)} = \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{conc. of standard (mg/L)}
\]

\[
\text{Microalbumin (mg/day)} = \text{Microalbumin (mg/L)} \times \frac{\text{Urine Vol. in ml(24hrs)}}{1000}
\]
Reference Range

Normal adults: upto 20 mg/L

ESTIMATION OF SERUM TOTAL SIALIC ACID (TSA)

Thiobarbituric acid (TBA) assay of sialic acid.533

Principle

Sialic acid forms β-formylpyruvic acid upon periodate oxidation, which is then coupled with the thiobarbituric acid, resulting in the formation of a coloured complex with a maximum absorption at 549 nm. The excess of periodate is reduced with sodium arsenite and the thiobarbituric acid (TBA) reagent is added to the reactant as soon as the yellow colour liberated due to iodine has disappeared. The colour formation from N-acetyl neuraminic acid (NANA) is increased considerably by carrying out the periodate oxidation in strong acid solution and by extracting the final coloured solution into cyclohexanone.

Since the TBA assay measures only unbound or free sialic acid, the value for total sialic acid concentration is obtained after hydrolysis of the specimen.

Reagents

1. H₂SO₄ (0.1 N)
2. Sodium metaperiodate (0.2 M) in Phosphoric acid (9M)
3. Sodium Sulphate (0.6 M) in Sulphuric acid (0.1 N)
4. Sodium arsenite (10%) in above prepared solution of sodium sulphate.
5. Thiobarburic acid (0.6%)
6. Cyclohexanone

Sample

Unhemolysed serum sample.
Sialic acid in serum is stable for 4 weeks at -20°C
Procedure

Mix 0.2 ml of sample with 1.8 ml of H₂SO₄ (0.1 N) and heat at 80°C to release the bound moiety of sialic acid.

<table>
<thead>
<tr>
<th>S No.</th>
<th>Reagent</th>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distil Water</td>
<td>0.2 ml</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Hydrolysed sample</td>
<td>-</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>3</td>
<td>Sodium metaperiodate</td>
<td>0.1 ml</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Mix and allow to stand at room temperature for 20 min.

<table>
<thead>
<tr>
<th>4</th>
<th>Sodium arsenite</th>
<th>1 ml</th>
<th>1 ml</th>
</tr>
</thead>
</table>

Mix and allow to stand for for 5 min until yellow brown colour disappears

<table>
<thead>
<tr>
<th>5</th>
<th>Thiobarbituric acid</th>
<th>3 ml</th>
<th>3 ml</th>
</tr>
</thead>
</table>

Mix and heat in vigorously boiling water bath for 15 min. Remove the tubes and place in cold water for 5 min

<table>
<thead>
<tr>
<th>6</th>
<th>Cyclohexanone</th>
<th>4.3 ml</th>
<th>4.3 ml</th>
</tr>
</thead>
</table>

Shake vigorously, and then centrifuge for 3 min. Optical density of the top clear organic phase is determined at 532 nm and 549 nm against the blank solution. OD at 532 nm was taken as a corrective measure and OD₅₄⁹ - OD₅₃₂ was finally used

Standards of varying concentrations between 0-15 µg/ml were made in distill water

![Calib. curve equation](image)

**Figure 15. Photographs of TSA standard curve on Shimadzu pharma spec (UV-1700) double beam UV-VIS spectrophotometer**
Calculations

Using standard graph the equation obtained was

\[ \text{ABS} = K_3C^3 + K_2C^2 + K_1C + K_0 \]

where \( K_3, K_2 \) and \( K_0 = 0 \) and \( K_1 = 0.0017 \)

\( r^2 \) for the method was 0.9983

Therefore total serum sialic acid levels were calculated as follows:

\[
\text{Total serum sialic acid (\( \mu \)g/ml)} = \frac{\text{OD}_{\text{sample}}}{0.0017} \times 10 \text{ (dilution factor)}
\]

\[
\text{Total serum sialic acid (mg/dl)} = \frac{\text{OD}_{\text{sample}}}{0.0017}
\]

Reference Range

40 mg/dl to 75 mg/dl

ESTIMATION OF SERUM CERULOPLASMIN

Principle

Ceruloplasmin catalyses the oxidation of PPD at pH 5.4 to yield a coloured product. The rate of formation of the coloured oxidation product is proportional to the serum ceruloplasmin if a correction is made for non enzymatic oxidation of PPD. Therefore simultaneous assays are carried out with and without sodium azide, which inhibits the enzymatic oxidation of PPD. The difference between the results of the two assays is proportional to the ceruloplasmin concentration.

Reagents

1. Sodium acetate (0.2 mol/L)
2. Acetic acid solution (0.2 mol/L)
3. Acetate Buffer solution (0.1 mol/L) Reagent 1- 430 ml, Reagent 2- 70 ml, distilled water- 400 ml. Warm the contents to 37° C in a water bath, adjust the pH to 5.45 by addition of sodium acetate or acetic acid and make up the volume upto 1 liter with distilled water.
4. Sodium azide solution (1.5 mol/L)
Buffered PPD solution (27.6 mmol/L) in acetate buffer solution, adjust pH to 5.45 by dropwise addition of NaOH (1 mol/L) and diluted upto 100 ml with acetate buffer solution.

Sample

Unhemolysed serum sample.

Ceruloplasmin in serum is stable for 4 weeks at -20°C

Procedure

Each sample would be analysed in two tubes as follows

<table>
<thead>
<tr>
<th>S No.</th>
<th>Reagent</th>
<th>Sample Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetate buffer</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>2</td>
<td>Serum</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Place the tubes in water bath at 37°C. A flask containing buffered PPD solution is also placed in the water bath.

<table>
<thead>
<tr>
<th>S No.</th>
<th>Reagent</th>
<th>Sample Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Warm, buffered PPD</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Mix and keep in water bath at 37°C for 5 min. The water bath is covered, to avoid exposure of the tubes to light.

<table>
<thead>
<tr>
<th>S No.</th>
<th>Reagent</th>
<th>Sample Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Sodium azide solution</td>
<td>50µl</td>
<td>_</td>
</tr>
</tbody>
</table>

Mix and keep in water bath at 37°C for 30 min.

<table>
<thead>
<tr>
<th>S No.</th>
<th>Reagent</th>
<th>Sample Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Sodium azide solution</td>
<td>_</td>
<td>50µl</td>
</tr>
</tbody>
</table>

Mix and read Absorbance at 530 nm with blank in the reference cuvette.

Calculations

Ceruloplasmin (g/L) = 0.752 (A_S - A_{SB})

Ceruloplasmin (mg/dl) = 75.2 (A_S - A_{SB})

Where A_S = Absorbance of Sample and A_{SB} = Absorbance of sample blank

Reference Range

20 mg/dl to 40 mg/dl

ESTIMATION OF SERUM MDA

Principle

Auto oxidation of unsaturated fatty acid involves the formation of semi stable peroxides, which then undergoes a series of reactions to form short chain aldehydes like malondialdehyde. One molecule of MDA reacts with two
molecules of TBA with the elimination of water to yield pink crystalline pigment MDA-TBA adducts, with an absorption maximum at 532 nm

**Reagents**

1. Trichloro acetic acid 40%
2. Thiobarbituric acid 0.67%
3. 1,1,3,3 tetra ethoxy propane as standard

**Sample**

Unhemolysed serum sample.

Store serum sample on ice. If not assaying the same day freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.

**Procedure**

<table>
<thead>
<tr>
<th>S No.</th>
<th>Reagent</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Trichloro acetic acid</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>2</td>
<td>Thiobarbituric acid</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>3</td>
<td>Sample</td>
<td>-</td>
<td>1ml</td>
</tr>
<tr>
<td>4</td>
<td>Distil water</td>
<td>1ml</td>
<td>-</td>
</tr>
</tbody>
</table>

Heat in boiling water bath for 10 min. Cool in ice cold water bath for 2 min. Centrifuge at 3000 rpm for 3 min. Take supernatant and read optical density at 532 nm with blank in the reference cuvette.

Standards of varying concentrations between 0.5-10 nmoles/ml were analysed in a similar manner. A standard graph was plotted between optical density and concentration.

![Figure 16. Photographs of MDA standard curve on Shimadzu pharma spec (UV-1700) double beam UV-VIS spectrophotometer](image-url)
Calculations
Using standard graph the equation obtained was
\[ \text{ABS} = K_3 C^3 + K_2 C^2 + K_1 C + K_0 \]
where \( K_3, K_2 \) and \( K_0 = 0 \) and \( K_1 = 0.0075 \)
\( r^2 \) for the method was 0.9987
Therefore serum Malondialdehyde levels were calculated as follows:

\[ \text{serum Malondialdehyde (nmoles/ml)} = \frac{\text{ODsample}}{0.0075} \]

Reference Range
1 to 3.42 nmoles/ml

ESTIMATION OF SERUM SOD

Principle
Superoxide radical generation by photoreduction of riboflavin is combined with nitrite formation from hydroxylamine hydrochloride to detect superoxide radicals. Superoxide radicals are allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite in turn reacts with sulphathilic acid to produce a diazonium compound which subsequently reacts with napthylamine to produce a red azo compound whose absorbance is measured at 543nm. SOD scavenges superoxide radicals produced by photoreduction of riboflavin. Therefore nitrite formation is inversely proportional to the amount of SOD.

Reagents
1. Phosphate Buffer (50mM) pH 7.4
2. L Methionine (20mM)
3. Triton X 100 (1% v/v)
4. Hydroxylamine Hydrochloride (10mM)
5. EDTA (50mM)
6. Riboflavin (50mM)
7. Greiss Reagent – freshly prepared by mixing equal volumes of 0.1% \( \delta \) Napthyl ethylene diamine and 1% sulphathilamide in 5% phosphoric acid.
Sample
Unhemolysed serum sample.
If not assaying the same day freeze at -80°C. Superoxide dismutase in frozen serum is stable for at least one month

Procedure

<table>
<thead>
<tr>
<th>S No.</th>
<th>Reagent</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphate Buffer</td>
<td>1.110 ml</td>
<td>1.110 ml</td>
</tr>
<tr>
<td>2</td>
<td>L- Methionine</td>
<td>0.075 ml</td>
<td>0.075 ml</td>
</tr>
<tr>
<td>3</td>
<td>Triton X 100</td>
<td>0.040 ml</td>
<td>0.040 ml</td>
</tr>
<tr>
<td>4</td>
<td>Hydroxylamine hydrochloride</td>
<td>0.075 ml</td>
<td>0.075 ml</td>
</tr>
<tr>
<td>5</td>
<td>EDTA</td>
<td>0.100 ml</td>
<td>0.100 ml</td>
</tr>
<tr>
<td>6</td>
<td>Sample</td>
<td>-</td>
<td>0.100 ml</td>
</tr>
<tr>
<td>7</td>
<td>Normal saline</td>
<td>0.100 ml</td>
<td>0.100 ml</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 5 min.

8    Riboflavin     80 µl  80 µl

Light exposure in Aluminium foil coated box for 10 min

| 9     | Greiss Reagent | 1 ml | 1 ml |

Mix and read Absorbance at 543 nm with reagent blank in the reference cuvette.

Calculations

Enzyme activity (U/ml) = \( \frac{V_0}{V-1} \)

\( V_0 \) = Absorbance of control (Blank)
\( V \) = Absorbance of sample

One unit enzyme activity is defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

Reference Range
5.14 - 6.88 U/ml
ESTIMATION OF SERUM VITAMIN C

Principle
Ascorbic acid is oxidized by copper to form dehydro ascorbic acid and diketogulonic acid. These products are treated with 2,4-DNPH to form the derivative bis-2,4 DNPH. This compound in strong sulphuric acid undergoes a rearrangement to form a product with an absorption band that is measured at 520 nm.

Reagents
1. Trichloro acetic acid (TCA) 10% and 5% (for standard)
2. DTC solution: Add 0.4 gm thiourea, 0.05 gm CuSO₄·5H₂O and 3.0 gm 2,4-DNPH and bring to a total volume of 100 ml with 9 N H₂SO₄.
3. H₂SO₄ 65%
4. Ascorbic acid

Sample
Unhemolysed serum sample.
Vitamin C is highly sensitive against oxidation. Therefore samples should be stabilized immediately after arrival in the laboratory. For stabilization, the precipitating reagent is added. Plasma or serum, containing the precipitating reagent is stable for 24 h at 2-8°C. After centrifugation the supernatant is stable for 3 month at -20°C.

Procedure
1 ml of plasma is added to 1 ml of ice cold 10% TCA add, mixed thoroughly and centrifuged for 20 min at 3500 rpm.

<table>
<thead>
<tr>
<th>S No.</th>
<th>Reagent</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distil water</td>
<td>1ml</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Supernatant of the precipitated sample</td>
<td>-</td>
<td>1ml</td>
</tr>
<tr>
<td>3</td>
<td>DTC</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

Incubate at 37 for 3 hrs

| 4     | 65% H₂SO₄ – ice cold             | 1.5 ml | 1.5 ml   |

Mix well and stand in dark at room temperature for 30 min. Read optical density at 520nm with blank in the reference cuvette.
Standards of varying concentrations between 0-20 μg/ml were made in 5% TCA and analysed in a similar manner. A standard graph was plotted between optical density and concentration.

Calculations

Using standard graph the equation obtained was

\[ \text{ABS} = K_3C^3 + K_2C^2 + K_1C + K_0 \]

where \( K_3, K_2 \) and \( K_0 = 0 \) and \( K_1 = 0.0346 \)

\( r^2 \) for the method was 0.9984

Therefore Vitamin C level was calculated as follows

\[
\text{Vitamin C (mg/dl)} = \frac{\text{OD}_{\text{sample}} \times 2 \text{ (dilution factor)}}{0.0346} \times \frac{100}{1000}
\]

Reference value

0.6 – 2.0 mg/dl
## Compilation of the parameters studied along with their methods and reference values

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter</th>
<th>Method</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>plasma glucose fasting and post prandial</td>
<td>GOD-POD</td>
<td>fasting: 70-110 mg/dl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Past Prandial: &lt;140 mg/dl</td>
</tr>
<tr>
<td>2</td>
<td>Serum Urea</td>
<td>GLDH- kinetic UV</td>
<td>15-40 mg/dl</td>
</tr>
<tr>
<td>3</td>
<td>Serum Creatinine</td>
<td>Alkaline picrate</td>
<td>Serum Creatinine: 0.6-1.3 mg/dl</td>
</tr>
<tr>
<td>4</td>
<td>Urine Creatinine</td>
<td>Alkaline picrate</td>
<td>Urine Creatinine: 11-26 mg/kg/day</td>
</tr>
<tr>
<td></td>
<td>serum total cholesterol</td>
<td>CHOD-POD</td>
<td>Desirable = &lt; 200 mg/dl</td>
</tr>
<tr>
<td>5</td>
<td>serum triglycerides</td>
<td>GPO – POD</td>
<td>Normal: &lt; 150 mg/dl</td>
</tr>
<tr>
<td>6</td>
<td>high density lipoprotein cholesterol (HDL-C)</td>
<td>Direct Enzymatic</td>
<td>Low risk: &gt; 60 mg/dl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High risk: upto 35 mg/dl</td>
</tr>
<tr>
<td>7</td>
<td>low density lipoprotein cholesterol (LDL-C)</td>
<td>Friedwald Equation</td>
<td>&lt;100 Optimal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100-129 Near Optimal</td>
</tr>
<tr>
<td>8</td>
<td>Total Chol: HDL-C</td>
<td>simple division</td>
<td>Low risk: men &lt; 4, women &lt; 3.8</td>
</tr>
<tr>
<td>9</td>
<td>LDL : HDL</td>
<td>simple division</td>
<td>Low risk: men &lt; 3.6, women &lt; 3.2</td>
</tr>
<tr>
<td>10</td>
<td>Glycosylated hemoglobin (HbA1c) in whole blood</td>
<td>Immunoturbidimetric method.</td>
<td>Degree of Control (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DCCT/NGSP: IFCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non Diabetic 4.0-6.0; 2.0-4.2;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Goal 6.0-6.5; 4.2-4.8;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Good Control 6.5-8.0; 4.8-6.4;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Action suggested &gt; 8.0; &gt; 6.4;</td>
</tr>
<tr>
<td>11</td>
<td>Serum high sensitivity CRP (hs-CRP)</td>
<td>Latex Immunoturbidimetric high sensitivity method.</td>
<td>2.68 to 8.5 mg/L.</td>
</tr>
<tr>
<td>12</td>
<td>Urine Microalbumin</td>
<td>Latex Immunoturbidimetric method</td>
<td>upto 20 mg/day</td>
</tr>
<tr>
<td>13</td>
<td>Urine albumin creatinine ratio (UACR)</td>
<td>calculated</td>
<td>UACR: &lt; 30 mg/gm</td>
</tr>
<tr>
<td>14</td>
<td>Serum total sialic acid (TSA)</td>
<td>Thiobarbituric acid</td>
<td>40 - 75 mg/dl</td>
</tr>
<tr>
<td>15</td>
<td>Serum ceruloplasmin (Cp)</td>
<td>PPD oxidase activity</td>
<td>20 - 40 mg/dl</td>
</tr>
<tr>
<td>16</td>
<td>Serum malondialdehyde (MDA)</td>
<td>Wilbur</td>
<td>1 to 3.42 nmoles/ml</td>
</tr>
<tr>
<td>17</td>
<td>Serum super oxide dismutase (SOD)</td>
<td>Spectrophotometric - nitrite formation</td>
<td>5.14 to 6.88 U/ml</td>
</tr>
<tr>
<td>18</td>
<td>Serum vitamin C</td>
<td>DNPH</td>
<td>0.6 – 2.0 mg/dl</td>
</tr>
</tbody>
</table>