Material & Methods
MATERIAL AND METHODS

4.1 Study population-Human

Present study was conducted during January 2007 to April 2010 on Nephrotic Syndrome on outdoor and indoor patients of Nephrology Department of M.G.M. Medical College and other private hospitals like Bombay hospital, CHL Apollo and clinics like Ideal pathology. Patients are also chosen voluntarily and on the basis of personal contacts, health camps etc. of Indore (M.P.), India. Consent was taken from each person involved in the study. The study was approved by the ethical committee of the D.A.V.V. Indore.

4.2 Test-Disease- Nephrotic Syndrome.

The present study was conducted on 314 Nephrotic Syndrome patients. The diagnosis of Nephrotic Syndrome was made on the basis of clinical history, diagnosis and biopsies evidences. Out of 315 patients, 105 were diagnosed to have primary Nephrotic Syndrome according to the criteria of the International Study of Kidney Disease in Children (ISKDC, 1981).

4.3 Exclusion criteria

The patients suffering from other diseases which may lead to oxidative stress such as diabetes, inflammatory disease, cardiac disease, hepatic impairment and respiratory diseases or other systemic disease as well as smokers and alcoholics were excluded from the study.

4.4 Experimental design

The present study was conducted on 414 subjects. Studied subjects were divided in the following four groups-

A. Control Group: In this group, 99 normal healthy subjects were included, which were free from any illness by clinical examination and taking balance diet with no history of NS and CVD.
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A. Control Group: In this group, 99 normal healthy subjects were included, which were free from any illness by clinical examination and taking balance diet with no history of NS and CVD.
B. Controlled (Pre treated) NS: In this group, 105 Nephrotic syndrome patients with PNS, with no active medical complication were included.

C. Managed (Post treated) NS group: In this group, 105 adult Nephrotic syndrome patients which were in remission after receiving standard oral corticosteroid induction therapy for one month were included.

D. Uncontrolled NS: In these groups 105 patients with complicated nephrotic syndrome were included.

A proper and detailed history of each patient was taken including question like –

- Patients' name
- Age and gender
- Blood pressure (systolic and diastolic)
- Height
- Weight
- Habit of smoking
- Habit of alcohol consumption
- Diet (vegetarian/Non-vegetarian)
- Previous investigation of patient's disorder.

4.5 Biochemical investigations

After 12 hours overnight fasting, 10 ml venous blood was drawn from all subjects. The blood was allowed to clot at room temperature and centrifuged at 5000 rpm for 10 minutes and then the serum was kept frozen at -70°C in aliquots until the time of assay of the parameters. The prospective study was carried out at Biochemistry laboratory of the Government Holkar Science College, Indore (M.P.) and Department of Biochemistry at M.G.M Medical College, Indore (M.P.) India.

Analysis of following parameters were carried out:

- Albumin
- Electrolyte Sodium and Potassium
- Trace element Zinc and Copper
Lipid profile includes Total Cholesterol (TC), Triglyceride (TG), HDL-Cholesterol (HDL-C), LDL-Cholesterol (LDL-C), VLDL-Cholesterol (VLDL-C).

- Lipoprotein (a) [Lp(a)]
- Total Antioxidant Capacity (TAC)
- Homocysteine (tHcy)

### 4.6 Normal level of biochemical parameters under study

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>Parameter</th>
<th>Reference range in Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Albumin</td>
<td>3.5-5 gm/dl</td>
</tr>
<tr>
<td>2</td>
<td>Sodium</td>
<td>134-148mEq/L</td>
</tr>
<tr>
<td>3</td>
<td>Potassium</td>
<td>3.7-5.6 mEq/L</td>
</tr>
<tr>
<td>4</td>
<td>Zinc</td>
<td>70-127μg/L</td>
</tr>
<tr>
<td>5</td>
<td>Copper</td>
<td>70-127μg/L</td>
</tr>
<tr>
<td>6</td>
<td>Total Cholesterol</td>
<td>Up to 200mg/dl</td>
</tr>
<tr>
<td>7</td>
<td>Triglyceride</td>
<td>Up to 190mg/dl</td>
</tr>
<tr>
<td>8</td>
<td>HDL-C</td>
<td>45-65 mg/dl</td>
</tr>
<tr>
<td>9</td>
<td>LDL-C</td>
<td>35-130mg/dl</td>
</tr>
<tr>
<td>10</td>
<td>VLDL-C</td>
<td>5-40mg/dl</td>
</tr>
<tr>
<td>11</td>
<td>Lipoprotein(a)</td>
<td>&lt;25mg/dl,</td>
</tr>
<tr>
<td>12</td>
<td>Total Antioxidant Capacity</td>
<td>1.84-2.24mmol/L</td>
</tr>
<tr>
<td>13</td>
<td>Homocyst(e)ine</td>
<td>5-15 μmol/L</td>
</tr>
</tbody>
</table>
4.7 Methods used in the study

4.7.1 Determination of Serum Albumin

Name of the method: Bromocresol green method (Doumas B.T. et al., 1971)

Principle

Albumin present in serum binds specifically with Bromocresol green at pH 4.1 to form green colored complex, intensity of which can be measured colorimetrically by using 640 nm (or a red filter).

Normal range

3.3– 4.8 g/dl

Preparation of reagents

1. Albumin reagent (ready to use): it is prepared by mixing following chemicals in 900 ml distilled water.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a) succinic acid</td>
<td>8.85 g</td>
</tr>
<tr>
<td>b) bromocresol green</td>
<td>108 mg</td>
</tr>
<tr>
<td>c) sodium azide</td>
<td>100 mg</td>
</tr>
<tr>
<td>d) brij – 35</td>
<td>4.0 ml</td>
</tr>
</tbody>
</table>

pH of this solution is adjusted by using 1N sodium hydroxide to 4.1. Final volume is made to 1 liter by using distilled water.

2. (Note- Brij 35: 4 gms in 10 ml distilled water)

3. Albumin standard (4.0) g/dl: Bovine albumin 4.0 g in 100 ml of normal saline containing 0.1 g/dl sodium azide.

Stability of the reagents

Reagents 1 & 2 are stable at room temperature (25°C ± 5°C) for 1 year.
Reagent 3 is stable at 2 – 8°C for 1 year.

**Procedure**

Mono - step method

Pipette in 3 tubes are labeled as follows –

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin reagent, ml</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Serum, ml</td>
<td>0.05</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Albumin standard, ml</td>
<td>—</td>
<td>0.05</td>
<td>—</td>
</tr>
<tr>
<td>Distilled water, ml</td>
<td>—</td>
<td>—</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Mix thoroughly & keep at room temperature (25°C ± 5°C) for exactly 10 minutes. Measure the intensity of the test & standard by setting blank at 100% T, by using 640 nm (red filter).

**Calculations**

Serum albumin, (g/dl) = O.D. of Test/ O.D. Standard x 4

**Source of error**

Icteric & lipemia sera may interfere with test.

**Procedure limitations**

Linearity of this method is up to 6.0 g/dl

4.7.2 Determination of Serum Sodium and Potassium

Name of method: Flame photometry (Brown D.E., 1956)

Normal range

Serum sodium: 133-148 mEq/l
Serum potassium: 3.8-5.6 mEq/l

Flame photometer

Specimen: serum or heparinized plasma (the specimen need not to be fasting one).

Standards

Mixed standards are prepared by using following 2 stock standards.

Stock standard for sodium: 1000 mEq/l: it is prepared by dissolving 5.85 g of analar grade NaCl in glass distilled water & diluted to 100 ml by using a volumetric flask.

Stock standard for potassium (100mEq/l): it is prepared by dissolving 0.740g of Potassium chloride (AR) in glass distilled water & diluted to 100 ml by using a volumetric flask.

Mixed working standards are prepared as follows:

Sodium/Potassium: 120/2.0 mEq/L: it contains 120 mEq of sodium & 2.0 mEq of Potassium per liter volume of distilled water. It is prepared mixing 12 ml of stock standard 1 & 2.0 ml of stock standard 2, in 86 ml of glass distilled water.

Sodium/Potassium: 140/4.0 mEq/L: it is prepared by mixing 14 ml of stock standard 1, & 4.0 ml of stock standard 2, in 82 ml of glass distilled water.

Sodium/potassium: 160/6.0 mEq/L: it is prepared by mixing 16 ml of stock standard 1, & 6.0 ml of stock standard 2, in 78 ml of glass distilled water.

Flame photometer

A dual channel instrument capable of quick simultaneous estimation of sodium & potassium is used. The equipment is equipped with the facilities incorporated to select calcium in place of sodium & lithium in place of potassium. Simultaneous determination of 2 samples minimizes the sample quantity, cost of operation & operation time.
**Principle**

The solution under test is passed carefully, under controlled conditions as a very fine spray in the air supply to non luminous flame. In the flame the solution evaporated and the salt dissociates to give neutral ions, which emit light of the characteristic wavelength. The flame is simultaneously monitored by both the channels. Each channel consists of a detector which views the flame through a narrow band optical filter. The photo detector outputs are connected to two independent digital displays which are calibrated for direct concentration readouts. Initial calibration is done by using at least three standards of different concentrations.

**Procedure**

Pipette in the tubes labeled as follows:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std: 1</th>
<th>Std: 2</th>
<th>Std: 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glass distilled water, ml</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2. Serum or plasma, ml (heparinized)</td>
<td>0.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3. Std: 120/2.0, ml</td>
<td>—</td>
<td>0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4. Std: 140/4.0, ml</td>
<td>—</td>
<td>—</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>5. Std: 160/6.0, ml</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Mix and transfer to beakers or bulbs for the flame photometric determination.

**Operation of flame photometer**

Put on the main switch.

Put on air compressor & adjust the required air pressure, by adjusting the knob meant for air.

Introduce glass distilled water, through atomizer.

Put on gas & control the flame by adjusting the knob meant for gas.

Adjust the proper filter for the simultaneous determination of sodium & potassium.

Make zero adjustment by introducing distilled water.

Introduce the standard 120/2.0 & by using the knob meant for sodium the digits 120.0 & by using the knob meant for potassium the digits 2.0 are adjusted.
Introduce the standard 140/4. If the standards are accurately prepared the digital display will indicate exact concentration for both sodium & potassium.

Introduce standard, 160/6.0 & confirm accuracy of standardization.

Now introduce the test & records the readings for sodium & potassium.

4.7.3 Determination of Zinc

Method (Akita Abe et al., 1989)

Principle

The zinc produces with the specific complex ant 5 – Br – PAPS [(2 – 5- bromo – 2 – 2- pyridylazo ) – 5 - ( N – propyl – N – sulpho – propylamino) phenol ], a stable colored complex which color intensity is directly proportional to the amount of zinc in the sample. The interferences due to the oligo elements (iron copper, cobalt) present in the sample are eliminated using specific masking agents.

Procedure

Dilute supernatant 1: 100 with physiological solution. Multiply result by dilution factor.

Shake and bring the samples at room temperature (+15/ 25 °C) before using.

Ready to use liquid reagents

<table>
<thead>
<tr>
<th>Reagent (A) Zn Liquid</th>
<th>Buffer complexants</th>
<th>200mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol. 2 x 50 ml</td>
<td>5 – Br – PAPS</td>
<td>&lt; 0.1 %</td>
</tr>
<tr>
<td>Standard (B) Zn Liquid</td>
<td>Zinc</td>
<td>µg/dl</td>
</tr>
<tr>
<td>Vol. =10 ml</td>
<td></td>
<td>30.6 µmol/L</td>
</tr>
</tbody>
</table>

Wavelength : 560 nm
Cuvette : 1 cm light path
Temperature : +25/30/37 C
Reading : Against blank reagent
Assay tipe : End point
Sample/ Reagent / Ratio: 1/20
Mix, incubate for 10 min at room temperature; read sample and standard extinction against blank reagent.

Color is stable at least 15 min at room temperature.

Volumes can be proportionally modified.

This methodology describes the manual procedure to use the kit.

### Calculation

Zinc : \( \mu g/dL = \frac{(E) \text{ Sample Extinction}(E)}{\text{ Standard Extinction} \times 200} \)  
(Standard value)

Standard Concentration: 200 \( \mu g/dL \) (30.6 \( \mu mol /L \))

### Limitations

It is impossible read at 546 nm and at 578 nm.

During pregnancy and during the menstruation, the concentrations can be reach subnormal values.

For concentration higher than 1000 \( \mu g/dL \) repeat the measure on a sample diluted 1; 2 with saline solution and multiply the results by 2.

Do not use hemolyzed sera. Turbid and itteric sera can interfere in the reaction raising the values.

Readings above advised wavelengths are impossible.

Do not use the plasma obtained from EDTA.

### 4.7.4 Estimation of Copper

Name of method ((Ventura S and King E.J., 1951))
Principle

The copper in presence of a buffer at acidic pH is released from ceruloplasmin and albumin. The copper so obtained reacts with Zinc 2-(5-dibromo-2-pyridyla-o-5-diethylaminophenol) Br -PADAP to produce a stable colored complex of which color intensity is directly proportional to the amount of copper in the sample.

Copper in serum is stable at least 3 days in refrigerant (+2/8 C) Remove serum from clot as soon as possible .Shake and bring the samples at room temperature (+ 15/25 C) before using.

Ready to use liquid reagents

<table>
<thead>
<tr>
<th>Reagent (A) Cu Liquid</th>
<th>Buffer complexants</th>
<th>200mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol. = 2 × 50 mL</td>
<td>5 – Br – PADAP</td>
<td>&lt; 0.1 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt; 0.1 %</td>
</tr>
<tr>
<td>Decolourant (B)</td>
<td>EDTA</td>
<td>10 mmol/L</td>
</tr>
<tr>
<td>Liquid – Vol. = 10 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard (B) Zn</td>
<td>Copper</td>
<td>200 μg/dl</td>
</tr>
<tr>
<td>Liquid – Vol. =10 mL</td>
<td></td>
<td>31.48 μmol/L</td>
</tr>
</tbody>
</table>

Store +2/8 C.
Stable until the expiration date reported upon the package.
After the unsealing and the taking of the reagent , it is advised to close up the bottle immediately in order to avoid evaporation , direct light exposure and bacteric contamination.

Reagent preparation and stability

Ready to use liquid reagents. Reagents must be at room temperature before using stable until the date reported on the label.
The reagent is limpid and yellow / orange; do not use if turbid.
Procedure

Wavelength: 595 nm
Cuvette: 1 cm light path
Temperature: +25/30/37 °C
Reading: Against blank reagent
Assay type: End point
Sample/Reagent/Ratio: 1/10

Pipetting in tubes:

<table>
<thead>
<tr>
<th></th>
<th>BLANK</th>
<th>SAMPLE</th>
<th>STANDARD</th>
<th>µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent (A)</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>µL</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>100</td>
<td></td>
<td></td>
<td>µL</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td>100</td>
<td></td>
<td>µL</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td>100</td>
<td>µL</td>
</tr>
</tbody>
</table>

Mix, incubate for 10 min at room temperature; read sample and Standard extinction against blank reagent.

Color is stable at least 15 min at room temperature.

Volumes can be proportionally modified.

This methodology describes the manual procedure to use the kit.

Calculation

Copper: µg/dL = (E) Sample / (E) Standard × 200

Standard Concentration: 200 µg/dL (31.48 µmol/L)

4.7.5 Homocysteine

Name of method: Elisa 96 Test Kit

The presence of elevated total homocysteine levels in plasma or serum has been connected to the increased risk of thrombosis, neural tube defects and atherosclerotic vascular disease. With the steadily growing demand for testing, laboratories require fast, reliable determinations that are cost-effective to process. Bio-Rad supplies two kits for homocysteine analysis: Homocysteine by HPLC and Homocysteine by EIA. Bio-Rad's expertise in both HPLC and immunoassay technology assures you of quality results and efficient workflow.
Homocysteine by EIA Kit

Bio-Rad offers an easy-to-use immunoassay for assessment of homocyst(e)ine levels in serum or plasma. The Homocysteine EIA kit features these unique advantages:

Highly specific enzymatic sample pretreatment
Convenient microplate format
Standardized immunoassay procedure
Parallel sample processing
Wide dynamic range
Excellent correlation with reference HPLC method
4.7.6 Determination of serum Lipoprotein (a) [(Lp(a))]  

**Principle of Procedure**

Solid phase capture sandwich ELISA assay using a micro well format.

Store all components at 2-8°C with the exception of the standard, which should be stored frozen.

**Sample and Standard Dilutions:**

Dilute each serum or plasma specimen to be tested 1:400 with the Lp(a) specimen diluent provided. (Serum specimens with high Lp(a) levels should be diluted more than 1:400 for accurate Lp(a) determination.)

*Note: A pre-dilution using PBS (phosphate buffer) may be done followed by a final dilution in specimen diluents to bring the serum or plasma final dilution to 1:400.

Construct a standard curve as follows:

a) Perform a series of at least four, twofold dilutions of the 1:400 standards. Use the specimen diluent alone as a blank or zero control.

b) Use the declared value on the vial of Lp(a) standard to calculate the values on the standard curve.

**Materials Supplied:**
Goat Anti-Human Lp(a) coated microwell strips 12x8 with plastic frame
Lp(a)N Conjugate – 12mL
Lp(a) standard (diluted 1:400) – 1 mL
TMB/peroxide substrate color developer –12mL
Lp(a) specimen diluent – 60mL
Sulfuric acid termination reagent (0.5N) –12mL
15 X Wash buffer concentrate – 60mL
Dynamic Range:
3 µg/dL – 405 µg/dL
Reproducibility
C.V. 4%-8% depending upon the region of the standard curve.
Assay Procedure
Allow each reagent to reach room temperature before use
Add 100µL of diluted specimen or standard to each microwell
Incubate at room temperature for 60 minutes
Decant and wash each microwell five times with wash buffer (dilute buffer 1:15
with reagent grade water)
Add 100µL of anti-human Apo B-100 conjugate to each well
Incubate at room temperature for 60 minutes
Decant and wash as in step 3
Add 100µL of TMB/peroxide substrate and incubate at room temperature for 3
minutes
Terminate the reaction with 100µL of 0.5N sulfuric acid
Set zero the micro well reader at 450nm using the specimen diluents zero control
well
Determine the optical density (O.D.) of the remaining wells
Construct a standard curve using the O.D. values obtained for each of the standards
Interpolate the unknowns from the standard curve.

4.7.7 Total Antioxidant Capacity

Name of method  D.Koracevic et al., (2001) method
Protocol

The assay measures the capacity of the biological fluids to inhibit the production of thiobarbituric acid reactive substances (TBARS) from sodium benzoate under the influence of the free oxygen radicals derived from Fenton’s reaction. A solution of 1mmol/litre uric acid was used as standard (D.Koracevic et.al; 2001).

Principle

A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton type reaction, leading to the formation of hydroxyl radicals(OH) These reactive oxygen species(ROS) degrade benzoate, resulting in the release of TBARS. Antioxidants from the added sample of human fluids cause suppression of the production of TBARS. This reaction can be measured spectrophotometrically at 532 nm and the inhibition of color development is defined as the serum antioxidant activity.

Reagents

Sodium Phosphate buffer: 100mmol/lit. (pH 7.4)
Sodium Benzoate: 100mmol/lit.
NaOH: 50mmol/lit.
EDTA: 2mmol/lit in Phosphate buffer
Fe (NH₄)₂SO₄: 2mmol/lit.
Fe-EDTA Complex: (Freshly prepared by mixing equal volumes of solution of EDTA and Fe (NH₄)₂SO₄, left to stand 60 min. at room temp.)
H₂O₂: 10mmol/lit.
Acetic Acid: 20%
Thiobarbituric Acid (TBA): 0.8% (Wt/Vol.) in 50 mmol. /lit. NaOH
Uric Acid: 1mmol/lit. (in 5mmol/lit NaOH)

Analytical procedure

Each sample (A₁) should have its own control (A₀, sample blank) in which Fe-EDTA mixture and H₂O₂ should be added after 20% acetic acid. For each series
of analysis a negative control (K₁ and K₀) should be prepared (at least in triplicate), containing the same reagents A₁ and A₀, except that serum (or other human fluid) is replaced with phosphate buffer. Standards containing 1mmol/litre uric acid (UA₁ and UA₀) are used for calibrations.

Pipette into tubes (in milliliters):

<table>
<thead>
<tr>
<th></th>
<th>A₁</th>
<th>A₀</th>
<th>K₁</th>
<th>K₀</th>
<th>U₁₀</th>
<th>U₀₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.49</td>
<td>0.49</td>
<td>0.50</td>
<td>0.50</td>
<td>0.49</td>
<td>0.49</td>
</tr>
<tr>
<td>Na benzoate</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Acetic acid</td>
<td></td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Incubate for 60 minutes at 37°C, then add:-

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>TBA</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Incubate for 10 minutes at 100°C (in a boiling water bath), and then cool in an ice bath. Measure the absorbance at 532 nm, against deionised water.

**Antioxidant Activity (AOA)** = (CUA) \((K - A) / (K - UA)\)

Where, K is absorbance of negative control & K = K₁ - K₀

A is absorbance of control & A = A₁ - A₀

UA is absorbance of standard uric acid & UA = UA₁ - UA₀

CUA is concentration of uric acid in mmol / liter
4.7.8 Cholesterol

Name of Method: Zak’s (1957) method.

Principle: Cholesterol dissolved in glacial acetic acid when treated with ferric chloride and conc. H₂SO₄ gives red color. This is used to estimate total cholesterol content by measuring optical density at 560 nm.

Reagents required

Conc. H₂SO₄
Glacial acetic acid
0.9 % NaCl: 900 mg of NaCl in 100 ml of d/w.
FeCl₃ solution: 0.05 gm of FeCl₃.6H₂O in 100 ml of glacial acetic acid.
Standard cholesterol:
Stock cholesterol: 100 mg in 100 ml glacial acetic acid.
Working cholesterol: Dilute 4 ml stock to 100 ml with ferric chloride-acetic acid reagent.

Procedure

In a glass stoppered centrifuge tube take 0.1 ml of serum.
Add 10 ml of ferric chloride-acetic acid reagent.
Mix well and stand for 10 minutes.
Centrifuge.
Transfer 5 ml of the clear supernatant fluid to a glass stoppered centrifuge tube.
Take 3 tubes and mark them as T, B and S.
Blank (B) - 5 ml of ferric chloride-acetic acid reagent and 3 ml of conc. H₂SO₄.
Standard (S) - 0.1 ml of physiological saline and 10 ml of working standard of cholesterol, use 5 ml of this as standard and 3 ml of conc. H₂SO₄.
Test (T) - 5 ml of supernatant fluid and 3 ml of conc. H₂SO₄.
Stand for 20-30 minutes.
Read the optical density of test and standard against the blank using a yellow filter or at 560 nm.
Calculation:

Cholesterol (mg per 100 ml of serum) = \( \frac{T}{S} \times \frac{100}{0.05} \times 0.2 \)

Result

The serum cholesterol is found to be _____________ mg %.

Precautions

Glass wares should be dry.
Conc. H₂SO₄ should be poured from burette.
Fresh FeCl₃ should be used.

4.7.9 Determination of Serum HDL-Cholesterol

Name of the Method: Phosphotungstate method (Weibe et al., 1985)

Normal range: 30-65 mg/dl

Principle

In the presence of phosphotungstic acid and magnesium chloride, LDL, VLDL and chylomicrons are precipitated. Centrifugation leaves only the HDL in the supernatant. Cholesterol in the HDL fraction can be tested by the usual methods for cholesterol.

Reagents

Phosphotungstic acid reagent (PTA) - 2.25 g of phosphotungstic acid in 8 ml of 1N NaOH and 42 ml D/W
Magnesium Chloride reagent- 20.34 gm magnesium chloride in 50 ml D/W
Cholesterol reagent 1 and 2 as stated in previous method
Cholesterol standard- 100 mg/dl in acetic acid.
**Reaction parameters**

- **Reaction type**: End point
- **Wavelength**: 505 nm (492-550 nm)
- **Flow cell temperature**: 30°C
- **Incubation**: 5 min at 37°C
- **Sample volume**: 0.05 mL (50μl)
- **Reagent volume**: 1.0 mL
- **Std. conc.**: 50 mg/dL
- **Zero setting**: Reagent blank

**Procedure**

<table>
<thead>
<tr>
<th>Pipette into centrifuge tube</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL reagent (precipitating reagent)</td>
<td>300 μl</td>
<td>300 μl</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>300 μl</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>300 μl</td>
</tr>
</tbody>
</table>

The content were mixed well and let to stand for 10 minutes at 37°C end then centrifuged for 10 minutes at 1000 rpm. The HDL cholesterol concentration of the supernatant was determined within one hour after centrifugation.

<table>
<thead>
<tr>
<th>Pipette into tube</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol Reagent</td>
<td>1 mL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 μL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard supernatant</td>
<td>-</td>
<td>50 μL</td>
<td>-</td>
</tr>
<tr>
<td>Sample supernatant</td>
<td>-</td>
<td>-</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

Above tubes incubated for 5 minutes at 37°C, then the absorbance of the sample was measured against reagent at 505 nm.
Calculation of results

\[
\text{HDL concentration (mg/dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times N \times 2
\]

Where, \( N = \text{Std concentration} \ 40 \text{ mg/dL} \), \( 2 = \text{Dilution factor of the sample} \)

4.7.10 Determination of Triglycerides

Name of the Method: GPO-POD Method

Principle

Lipase hydrolyses serum triglycerides to glycerol and free fatty acids. The liberated glycerol is converted to glycerol-3 phosphate in the presence of ATP and glycerokinase. Glycerol-3-phosphate is oxidized by glycerol-3 phosphate oxidase to yield \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) thus generated reacts with ADPS and 4-aminoantipyrine in presence of peroxidase to form a coloured complex. The intensity of the colour complex formed during the reaction is directed proportional to triglyceride concentration and measured spectrophotometrically at 546 nm (530-570 nm of green filter).

Normal range: 25-200 mg/dl

Principle

\[
\text{Triglyceride} + \text{H}_2\text{O} \overset{\text{Lipoprotein Lipase}}{\rightarrow} \text{Glycerol} + \text{Fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \overset{\text{Glycerol Kinase}}{\rightarrow} \text{Glycerol-3-Phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-Phosphate} + \text{O}_2 \overset{\text{Glycerol phosphate oxidase}}{\rightarrow} \text{Dihydroxy acetone phosphate} + \text{H}_2\text{O}_2
\]

\[
2 \text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{ADPS} \overset{\text{Peroxidase}}{\rightarrow} \text{Colored complex}
\]

(ADPS: N-Ethyl-\text{nsulfopropy}-n anisidine)

Reaction parameters

- Reaction type: end point
- Wavelength: 546 nm (520-570)
- Flow cell temperature: 30°C
- Incubation: 5 min at 37°C
Sample volume : 0.01ml (10mL (10 ul))
Reagent volume : 1.0 mL
Std. conc : 200 mg/dL
Zero setting : Reagent blank

Reagents

Enzymes- It contains-
Lipoprotein lipase- 30 units
Glycerol kinase- 10 units
Glycerol phosphate oxidase- 5 units
Peroxidase- 5 units
Glycerol phosphate 100 ml
Buffer- Phosphate buffer ph 7.2 (50 mmol/l)
p-Cholorophenol reagent- 30mg/dl

Procedure

<table>
<thead>
<tr>
<th>Pipette into tubes</th>
<th>Blanks</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>1 mL</td>
<td>1 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µL</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Incubate for 5 minutes at 37°C. Mix and read the absorbance. The absorbance of test and standard was read against reagent blank at 546 nm (530-570) or with green filter.

Calculation of result

TG concentration (mg/dL) = Absorbance of test / Absorbance of standard × 200

4.7.11 Determination of Lipoprotein fraction LDL and VLDL

Name of the method: Friedwald equation (Friedwald et al., 1972)
Normal range: LDL- 35-130 mg/dl

VLDL- 5-40 mg/dl

Fairly accurate determination of VLDL and LDL-C can be done by the following formulae:

VLDL = Triglyceride / 5

LDL = Total cholesterol – HDL C – (TG /5)

4.7.12 Calculation of the atherogenic indices

The atherogenic indices were calculated as follows:

Cardiac Risk Ratio (CRR) = TC/HDLC (Martirosyan D. M. et al., 2007)

Atherogenic Coefficient (AC) = (TC – HDLC)/HDLC (Brehm A. et al., 2004)

Atherogenic Index of Plasma (AIP) = log (TG/HDLC) (Dobiásová M., 2004)

(Note: for calculation of atherogenic indices we have converted values of TC, HDL-C, and TG into mmol/L)
REFERENCES


Zak, B. (1957) Amer. J. Clin. Path. 27, 583.b