Material and Methods

The present study was carried out at the Department of Biochemistry, Government Medical College and Hospital Miraj and P.V.P.G. Hospital Sangli. The patients with osteoporosis selected for the study, attended orthopedic O.P.D. General Hospital Sangli, Miraj and Ortho Hospital and Post Graduate Institute of Swastiyog Pratishthan, Miraj.

In this study, total numbers of subjects are 120. The distribution of these subjects were as follows,

1) Study group – 60 subjects.
2) Control group – 60 subjects.

All the postmenopausal women included in this study were between the age group of 45 – 60 years.
Selection of patients:

Study group:

It included 60 postmenopausal women in the age group 45 – 60 years and diagnosed as primary osteoporosis by clinicians. The patients had clinical features suggestive of reduced bone mass i.e. backache or generalized weakness or any fracture and radiological evidence of osteoporosis at one or more sites and lower BMD. The study group was given bisphosphonate + calcium + vitamin D therapy.

Dosage:

Alendronate – 70 mg/ week (orally).
CitroMacalvit – once a day (orally).
Calcium citrate - 1200 mg equivalent to elemental calcium 252 mg.
Calcitriol – 0.25 µg.

Patients were instructed to take bisphosphonate on an empty stomach with a glass of plain water and other food, beverages or medication to be avoided for at least 30 minutes.

All the patients were advised to take balanced diet, with adequate calcium and first class proteins.

Inclusion Criteria:

a) Primary type of osteoporotic women.
b) Age group 45-60 years.
c) Cases diagnosed with the aid of bone mineral density by orthopedic surgeons.

Exclusion Criteria:

Patients with secondary type of osteoporosis, liver disease, renal disease, metastatic bone disease, severe ill patients, treatment with estrogen and progesterone were excluded from this study.
Control group:

It included 60 postmenopausal non-osteoporotic women with normal bone density in the age group 45-60 years.

Collection of samples:

In the present study blood and urine samples were collected from control group (non-osteoporotic postmenopausal women) and from osteoporotic postmenopausal women at baseline level. In the follow up study blood and urine samples of osteoporotic women were collected after 3 months of antiresorptive therapy.

Venous blood was collected with the help of disposable syringe and needle and withdrawn in dry centrifuge tube by taking aseptic precautions. Separated nonhemolysed sera were processed for the assay of biochemical parameters. Fasting urine sample was collected in polypropylene tube.

Following investigations were carried out on the samples.

1) Osteocalcin
2) Alkaline phosphatase.
3) Calcium, phosphorus, magnesium.
4) Total Proteins and Albumin.
5) Vitamin C.
6) Tartrate resistant acid phosphatase.
7) Cholesterol.
8) Urinary hydroxyproline.
9) Urinary creatinine.
Bone Mineral Density (BMD):

Bone Mineral Density was measured at the midshaft tibia by Quantitative Ultrasound (QUS) technique (panacea Biotech).\(^{(220)}\)

T-score and Z-score were calculated based on WHO criteria. The WHO T-score thresholds of -2.5 for osteoporosis and -1.0 for osteopenia were used in this study.

**T-Score:** (ratio between patients BMD and that of same gender and race) of > -1 was taken as normal,
Between -1 to -2.5 osteopenic,
< -2.5 as osteoporotic.

<table>
<thead>
<tr>
<th>T-Score</th>
<th>Normal</th>
<th>Osteopenia</th>
<th>Osteoporosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td></td>
<td>-1.5</td>
<td>-2</td>
</tr>
</tbody>
</table>

**Z-Score:** is the number of standard deviations, the measurement is above or below the age matched mean bone mineral density.
Serum calcium

Estimation of serum calcium by O-Cresolphthalein Complexone (o-CPC) method \(^{(221)}\).

**Principle**

In acid medium calcium binds with o-cresolphthalein complexone (o-CPC) to produce a purple colour which absorbs at 530 nm and is proportional to the concentration of calcium.

**Reagents**

1) O-CPC Reagent
   a) O- Cresolphthalein complexone.
   b) 8-hydroxyquinoline.
2) Buffer- Diethanolamine.
3) Calcium standard (10 mg/dl)

**Reagent Preparation**

Buffer reagent (1) and color reagent (2) were mixed in a ratio 1:1 (equal volumes). Working reagent prepared freshly each time when required.

**Procedure**

Test tubes were labeled as Blank (B), Standard (S), and Test (T) and other additions were done as follows.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank ml</th>
<th>Std. ml</th>
<th>Test ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Calcium Standard</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
</tbody>
</table>
The contents of the tubes were mixed well and incubated at room temperature for three minutes. The absorbancies of S and T were read against B on photocolorimeter with green filter (530 nm).

**Calculations:**

\[
\text{Calcium in mg/dl} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard}} \times 10
\]

**Normal Range** – 9.3 to 10.7 mg / dl.
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Serum inorganic phosphorus

Estimation of serum inorganic phosphorus by Fiske & Subbarow Method (222).

Principle

The protein free filtrate of serum is obtained by treating serum with trichloroacetic acid. The inorganic phosphorus present in protein free filtrate reacts with molybdic acid to form hexavalent phosphomolybdic acid. The hexavalent molybdenum of the phosphomolybdic acid is then reduced by means of reducing agent, 1, 2, 4- amino naphthol sulphonic acid to give a blue coloured complex, the absorbance of which is measured colorimetrically at 660 nm. A standard phosphorus solution is treated similarly and the color intensities are compared.

Reagents

1) Trichloroacetic acid (TCA) 10%
2) Ammonium Molybdate I (Higher acidity)
3) Ammonium Molybdate II (Lower acidity)
4) 1,2,4-amino naphthol sulphonic acid (ANSA)
5) Working phosphorus standard: concentration = 0.008 mg/ml.

Procedure

I) Preparation of protein free filtrate.

9 ml of 10% of TCA was taken in a test tube, and added 1ml of serum drop wise. Mixed and allowed the contents to stand for 5 minutes then filtered.
II) Colour development

Test tubes were labeled as Blank (B), Standard (S), and Test (T) and other additions were done as follows.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank ml</th>
<th>Std. ml</th>
<th>Test ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein free filtrate</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>Phosphorus standard</td>
<td>-</td>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>Molybdate I</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Molybdate II</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.6</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>ANSA</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The contents of the tubes were mixed well. The absorbance was read colorimetrically exactly after 4 minutes at 660 nm / red filter.

Calculations

\[
\text{Serum inorganic phosphorus} = \frac{\text{O.D. of T} - \text{O.D. of B}}{\text{O.D. of S} - \text{O.D. of B}} \times \text{conc. of std/ml} \times \text{vol. of std} \times \frac{100}{\text{Vol.of serum}}
\]

\[
= \frac{\text{O.D. of T} - \text{O.D. of B}}{\text{O.D. of S} - \text{O.D. of B}} \times 0.008 \times 5 \times \frac{100}{0.5}
\]

\[
= \frac{\text{O.D. of T} - \text{O.D. of B}}{\text{O.D. of S} - \text{O.D. of B}} \times 8
\]

\[
= \ldots \ldots \; \text{mg / dl}
\]

**Normal Range** – 2.5 – 4.5 mg / dl
Serum magnesium

Estimation of serum magnesium by Calmagite method \(^{(223)}\).

**Principle**

Magnesium forms a red colored complex when treated with calmagite dye in alkaline medium. Interference of calcium and proteins is eliminated by chelating agent and detergent present in the reagent. Intensity of the colour formed is directly proportional to the amount of magnesium present in the sample.

![Diagram of magnesium and calmagite reaction](image)

**Reagents**

1) Buffer reagent (L1)
2) Color regent (L2)
3) Magnesium standard (2.0 mEq / L)

**Procedure**

The test tubes were labeled as Blank (B), Standard (S) and Test (T) and other additions were done as follows.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank ml</th>
<th>Std. ml</th>
<th>Test ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer Reagent</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Colour Reagent</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Magnesium std.</td>
<td>-</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>0.01</td>
</tr>
</tbody>
</table>
The contents of the tubes were mixed well and incubated at R.T. (25°C) for 5 min. The absorbance was read at 525 nm.

**Calculation**

\[
\text{Magnesium in mEq/ L} = \frac{\text{Abs T}}{\text{Abs S}} \times 2
\]

**Normal Range** - 1.5 – 2.5 mEq/L
Serum Osteocalcin

Assay of serum osteocalcin by Enzyme Amplified Sensitivity Immunoassay (EASIA) method\textsuperscript{(224)}.

Proprietary name – Biosource host – EASIA kit.
Cat No       - kap 1381
Manu. By     - Biosource Europe S.A.

Principle
The Biosource hOst – EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtitreplates. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of human osteocalcin. Calibrators and samples react with the capture monoclonal antibody (MAb-1) coated on microtiter well and with a monoclonal antibody (MAb2) labeled with horseradish peroxidase (HRP), after an incubation period allowing the formation of a sandwich: coated MAb1 – MAb2 – HRP, the microtitre plate is washed to remove unbound enzyme labeled antibody. Bound enzyme – labeled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB) is added and incubated. The reaction is stopped with the addition of stop solution and the microtitre plate is then read at the appropriate wavelength. The amount of substrate turnover is determined ELISA reader by measuring the absorbance, which is proportional to the osteocalcin concentration.
**Reagents**

1) Microtiterplate with 96 anti OST (monoclonal antibodies) coated wells.
2) Conjugate HRP labeled anti – OST (monoclonal antibodies) in tris HCL buffer with bovine serum albumin, bovine casein, EDTA, gentamycin and thymol.
3) Zero calibrator in human serum with protease inhibitors and benzamidine.
4) Calibrator N = 1 to 5 human serum with protease inhibitors and benzamidine.
5) Wash solution Tris – HCl
6) Controls – N= 1 or 2 human serum with protease inhibitors, benzamidine and thymol.
7) Chromogen: Chromogenic solution TMB (Tetramethyl benzidine).
8) Stop solution: HCl 2N.

**Reagent preparation.**

1) Calibrators: Reconstituted the zero calibrator with 1.0 ml distilled water and other calibrators with 0.05 ml distilled water
2) Controls: reconstituted the controls with 0.5 ml distilled water.
3) Working wash solution: prepared an adequate volume of working wash solution by adding 199 volumes of distilled water to 1 volume of wash solution.

**Procedure:**

1) The required number of strips were selected for the run.
2) The strips were secured into the holding frame.
3) 25 µl of each calibrator, control and sample were added into the appropriate wells.
4) 100 µl of anti – OST. HRP conjugate was added into all the wells.
5) Incubated for 2 hours at room temp. On a horizontal shakers set at 700 rpm.
6) The liquid from each well was aspirated.
7) The plate was washed 3 times by:
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- Dispensing 0.4 ml of wash solution into each well.
- Aspirating the content of each well.

8) 100 µl of the chromogenic solution was added into each well within 15 minutes following the washing step.

9) The microtiterplate was incubated for 30 minutes at room temp. on a horizontal shaker set at 700 rpm, avoid direct sunlight.

10) 200 µl of stopping solution was added into each well.

11) The absorbencies were read at 405 nm (Reference filter set at 630 nm)

Calculation:

A calibration curve is plotted and OST concentration in samples is determined by interpolation from the calibration curve.

Normal Range – 3 -13 ng / ml.
Standardization of Osteocalcin

Optical Density

concentration of osteocalcin
Alkaline Phosphatase

Assay of alkaline phosphatase by kinetic p-NPP method \(^{(225)}\).

Principle

In alkaline medium serum alkaline phosphatase hydrolyzes pNPP to yellow coloured p- Nitrophenol (pNP). The rate of PNP formation is directly proportional to the ALP activity and is measured in terms of change in absorbance at 405 nm.

\[
\text{Alk PO}_4 \quad \text{p-NPP} + \text{H}_2\text{O} \quad \xrightarrow{\cdot} \quad \text{p- Nitrophenol} + \text{H}_3\text{PO}_4.
\]

Reagents

The reagent for alkaline phosphatase contains:
- P- Nitrophenyl phosphate 17m M.
- Magnesium ions 4 mM.
- Buffer (pH 10.2 ± 0.2). Activator and binder.

Reagent Preparation.

Added 12 ml. distilled water in substrate reagent vial. Reconstituted reagent is stable for 30 days, when stored at 2-8\(^{\circ}\)C.

Procedure: Kinetic assay

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>500 (\mu)l</td>
</tr>
<tr>
<td>Serum</td>
<td>10 (\mu)l</td>
</tr>
</tbody>
</table>

The content of the tube was mixed well. The absorbance was measured at 405 nm by using semiautoanalyzer.

Normal Range – 25 – 90 IU / L
Total Proteins (Estimation of total protein by Biuret method) \(^{226}\).

**Principle:**

Serum proteins are measured by the Biuret reaction in which copper in alkaline medium reacts with peptide linkages of proteins to give violet colour. The intensity of the color is proportional to the protein concentration.

**Reagents:**

1) Biuret Reagent.
   - Cupric sulphate.
   - Sodium and potassium tartrate.
   - Sodium hydroxide.
   - Potassium iodide.
2) Protein Standard – 8.0 gm/ all.

**Procedure:**

The test tubes were labeled as blank (B), standard (S) and Test (T) and other additions were done as follows.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank ml</th>
<th>Standard ml</th>
<th>Test ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biuret Reagent</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The contents of the tubes were mixed well and incubated at \(37^\circ\)C for 5 minutes and the absorbance was read at 530 nm (Green filter) against blank.

**Calculation**

\[
\text{Total Proteins in Gm/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 8
\]

**Normal Range** – 6.5 to 7.5 Gms/dL.
Serum Albumin
Estimation of albumin by BCG method \(^{(227)}\).

**Principle:**
At 4.2 pH, bromocresol green fixes selectively on albumin, producing a blue green colour. The intensity of the colour is proportional to the albumin concentration.

**Reagents**
1) BCG reagents: Bromocresol Green, Succinate Buffer PH 4.2, Brij 35
2) Albumin standard – 4.0 Gms /dL

**Procedure**
The test tubes were labeled as blank (B), standard (S) and Test (T) and other additions were done as follows.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank ml</th>
<th>Std ml</th>
<th>Blank ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG reagent</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The contents of the tubes were mixed well and incubated at RT for 3 mins; the absorbance was read at 620 nm/red filter against blank.

**Calculations**

\[
\text{Serum Albumin in Gm/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 4
\]

**Normal Range** – 4.0 to 5.5 Gms / dl
Vitamin C

Estimation of Vitamin C by photometric method (228).

**Principle**

Ascorbic acid in serum is oxidized by Cu\(^{+2}\) to form dehydroascorbic acid, which reacts with acidic 2, 4 dinitrophenylhydrazine to form a red bis-hydrazine, which is measured at \(A_{520}\).

**Reagents**

1) Metaphosphoric acid solution. 6.0 g / dl
2) Sulphuric acid. 4.5 mol / L
3) Sulphuric acid. 12 mol / L
4) 2,4 Dinitrophenylhydrazine reagent, 2.0 g / dl in sulfuric acid , 4.5 mol/L
5) Thiourea solution, 5.0 g / dl.
6) Copper Sulfate solution, 0.6 g / dl.
7) Dinitrophenylhydrazine thiourea-copper sulfate (DTCS) reagent:
   Combine 5ml. of the thiourea solution, 5ml of the copper sulfate solution and 100ml. of the 2,4- dinitro-phenylhydrazine reagent. Store in a bottle at \(4^0\) C for a maximum of 1 wk.
8) Calibrators.
   a) Ascorbic acid stock calibrator, 50.0 mg / dl.
      50 mg of ascorbic acid was dissolved in metaphosphoric acid (6.0 g/dl) and brought to a final volume of 100 ml with metaphosphoric acid.
   b) Intermediate ascorbic acid calibrator, 5.0 mg/dl.
      10 ml of stock calibrator was added into a 100ml of volumetric flask and diluted with MPA ( 6.0 g/dl )
   c) Working Calibrators.
      In a series of 25 ml volumetric flasks, the following amounts of intermediate calibrator were added: 0.5, 2.0, 4.0, 6.0, 10.0, 15.0 and 20.0 ml. Brought to a final volume of 25 ml with MPA (6.0 g/dl) to yield working calibrators of 0.10, 0.40, 0.80, 1.20, 2.00, 3.00 and 4.00 mg/dl.
Procedure

1) 0.5ml of serum was added to 2.0 ml of freshly prepared metaphosphoric acid in a test tube, and mixed well. Centrifuged for 10 mins at 2500 rpm.
2) 1.2 ml of the clear supernatant was taken into a test tube.
3) 1.2 ml of each concentration of working calibrator was taken into a test tube.
4) 1.2 ml of metaphosphoric acid was taken into test tube used as blanks.
5) 0.4 ml of DTCS reagent was added to all tubes and incubated the tubes in a water bath at 37°C for 3 hrs.
6) The tubes were removed from the water bath and chilled for 10 min in an ice-bath. While mixing slowly added to all tubes 2.0 ml of cold sulfuric acid, 12 mol / l and mixed.

5) The blank was adjusted to read zero at 520 nm, and reading of the calibrators and unknowns was taken. The concentration of each working calibrator versus absorbance values was plotted.

Calculation.

The concentration of the sample is obtained from the calibration curve and is multiplied by 5 (to correct the dilution of the serum by metaphosphoric acid) to get the concentration of ascorbic acid per dl of serum. Normal Range: – 1.2 to 2.0 mg/dl.
Standardization of vitamin C

Optical Density

Concentration of vitamin C
Tartrate resistant acid phosphatase

Assay of tartrate resistant acid phosphatase by King Armstrong method (229).

Principle

When serum is treated with di-sodium phenyl phosphate in acidic medium, the enzymes acid phosphatase acts on phenyl phosphate to liberate phenol. The phenol so formed reacts with folin ciocalteu reagent in alkaline medium to form blue colored complex. The activity of serum acid phosphatase is determined by measuring the light absorption. Tartrate resistant acid phosphatase is assayed by addition of tartrate to the incubation mixture containing serum. The color is measured using colorimeter and concentration of serum tartrate resistant acid phosphatase is determined.

Reagents

1) Substrate (Disodium phenylphosphate)

2.18 gm of disodium phenylphosphate was dissolved in water and made the volume to a liter. Brought quickly to boil, to kill bacteria and cool, and added the chloroform. Kept at 4°C in a brown bottle.

2) Buffer (Sodium citrate buffer)

21 gm. crystalline citric acid was dissolved in water, 188 ml Sodium hydroxide (1 mol/l). And made to 500 ml with water, and Adjusted to pH 4.9.

3) Tartrate solution

15 gm tartaric acid was dissolved in 70 ml water. Added to it 18.5 ml sodium hydroxide. (1 mol/l) to pH 4.9 and make to 100 ml. Store with a few drops of chloroform in the refrigerator.

4) Stock standard (phenol solution 1 g/l)

1 gm of pure crystalline phenol was dissolved in 0.1 N HCL and made up to 1 liter with acid. Kept in a brown bottle at 4°C.
5) Working phenol standard (1 mg / 100 ml) the stock standard diluted 1 ml to 100 ml with distilled water. Preserved with a few drops of chloroform and kept at 4°C in brown bottle.
6) Sodium carbonate – 15%

**Procedure**

Three test tubes were labeled as Test (T), Control (C), Tartrate resistant (Tr) and made the following additions.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>T ml</th>
<th>C ml</th>
<th>Tr Ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Substrate</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
| Mixed and incubated for 5 mins
| Serum             | 0.1  | -    | 0.1   |
| Tartrate          | -    | -    | 50 µl |
| Incubated for exactly 1 hr.
| Folin ciocalteu reagent | 0.9  | 0.9  | 0.9   |
| Serum             | -    | 0.1  | -     |

Centrifuged and the supernatant was taken

5 tubes as T, C, Tr, S, B were labeled and made the following additions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>T ml</th>
<th>C ml</th>
<th>Tr ml</th>
<th>S ml</th>
<th>B Ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D / W</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2 ml</td>
</tr>
<tr>
<td>Working std.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>15% Na₂CO₃</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
All the contents of the tubes were mixed and incubated for 5 mins. The absorbance was read at 620 nm.

**Calculation**-

1 KA unit = 1 mg of phenol liberated by 100 ml of serum in 60 mins. at 37°C.

\[
\text{conc. of serum TRACP} = \frac{\text{Tr-C}}{\text{S-B}} \times \frac{\text{conc. of std.}}{\text{sample dil.}} \times 100
\]

\[
= \frac{\text{Tr-C}}{\text{S-B}} \times 30
\]

\[
= \text{--------- KA units}
\]

**Normal Range** – 1 - 2.7 KA units
Urine Hydroxyproline

Estimation of Urinary hydroxyproline by Bergman and Loxley method (230)

Principle

The peptides present in urine are hydrolyzed by hydrochloric acid, hydroxyproline is oxidized by chloramine T and colour is developed with dimethylaminobenzaldehyde reagent. The technique is applied to urine and to urine which contains known amount of hydroxyproline. For urine blank the oxidizing agent is added after the Ehrlich’s reagent; in this way the chloramine T is inactivated by the acid and any colour development is due to the other substances rather than hydroxyproline.

Reagents
1) Hydrochloric acid 12 mol/l.
2) Lithium hydroxide, saturated solution.
3) Oxidant solution. 4 volumes of (a) and 1 volume of (b) mixed before use:
   a) Buffer solution
      Dissolve 57.0 g sodium acetate (CH₃ COO Na, 3H₂O), 37.5 g trisodium citrate (the dihydrate) and 5.5 g citric acid (the monohydrate) were added in about 500 ml water, add 385 ml isopropanol and made to a liter with water.
   b) Chloramin T, 7.0 g / l
4) Dimethylaminobenzaldehyde solution. For colour development at 60°C 7.6 g was dissolved in 40.8 g perchloric isopropanol. Just before using.
5) Standard of hydroxyproline in isopropanol containing 7.5µmol/l (0.99mg/l).

Procedure

5 ml hydrochloric acid and 5 ml urine were mixed in a poly-propylene centrifuge tube. This tube was heated overnight in an oven at 105°C. To the next day the content of tube was cooled, it was neutralized by lithium hydroxide and few drops of phenolphthalein were added as an indicator. It was
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again made slightly acidic with dilute hydrochloric acid and resultant quantity was brought to 25 ml with distilled water.

The test tubes were labeled as I and II

<table>
<thead>
<tr>
<th>Reagents</th>
<th>I ml</th>
<th>II ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysate</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Internal Standard</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Oxidant solution</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Mixed after 3 to 5 min

<table>
<thead>
<tr>
<th></th>
<th>I ml</th>
<th>II ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylaminobenzaldehyde solution</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Heated at 60°C for 21 min in a water bath, stand 1 h at room temp. Absorbance was read at 562 nm. For urine blank added the oxidant solution after the dimethylaminobenzaldehyde.

**Calculation**

Urinary hydroxyproline (µ mol / 24 h) =

$$\frac{(OD \text{ of unknown-urine blank}) \times \text{conc of std (µmol/l)}}{\frac{(OD \text{ of unknown with added standard} - \text{OD of unknown})}{2} \times \frac{25}{2} \times \frac{24 \text{ h volume (ml)}}{1000}}$$

To convert to mg / 24 h multiply by 0.131. Urinary hydroxyproline is expressed as mg of hydroxyproline/gm of urine creatinine.

**Normal Range** – 70 to 535 µ mol/24 h

(9 to 70 mg/24 h)
Urine Creatinine

Estimation of urine creatinine by Jaffe’s method \(^{(231)}\).

Principle

In an alkaline medium, creatinine reacts with picrate to produce an orange yellow color. The coloration is proportional to the creatinine concentration.

Reagents

1) Picric acid
2) Sodium hydroxide
3) Creatinine standard

Procedure

Diluted urine as 1 to 25 ml in distilled water

Step 1. Protein precipitation:–

Diluted urine: - 1 ml.
Picric Acid: - 3 ml.

Mixed well kept in boiling water bath for one minute. Cooled immediately and separated clear supernatant by centrifuge.
Step 2. Colour developments:-

The test tubes were labeled as blank (B), standard (S) and Test (T) and other additions were done as follows.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank ml</th>
<th>Std ml</th>
<th>Test ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Creatinine standard</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Picric Acid</td>
<td>1.5</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Supernatant from step 1</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>

The contents of the tubes were mixed well. Kept at room temp for 15 mins. The absorbance was read at 530 nm or Green filter against blank.

Calculation

\[
\text{Creatinine in Gms / 24 hrs} = \frac{\text{OD of Test}}{\text{OD of Std}} \times 2 \times \text{Dilution Factor}
\]

**Normal Range** – 1 to 2 Gms / 24 hrs.
Total Cholesterol

Estimation of Cholesterol by Wybenga and Pileggi method \(^{(232)}\).

**Principle**

In hot acidic medium, cholesterol oxidizes ferric ions to a brown colored complex which absorbs at 530 nm and is directly proportional to cholesterol concentration.

**Reagents**

1) Cholesterol Reagent
   - Acetic Ethyl Acetate.
   - Sulphuric Acid.
   - Ferric ion
   - Cholesterol standard in acetic Acid – 200 mgs / dl.

**Procedure**

Three test tubes were labeled as blank (B), standard (S) and Test (T). Made the following additions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank ml</th>
<th>Std ml</th>
<th>Test ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol reagent</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>20 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>20 µl</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
The contents of the tubes were mixed well for 20 secs. Kept in a boiling water bath immediately for exactly 90 seconds. Cooled for 5 minutes. The absorbance was read at 530 nm against blank.

**Calculations.**

\[
\text{Total cholesterol} = \frac{\text{OD of test}}{\text{OD of std}} \times 200
\]

**Normal Range** – 150 to 220 mgs / dl.

**Statistical Analysis:**

All data were expressed as mean ± SD. Statistical analysis was done by using ‘z’ test and **paired‘t’** test.
CONCEN FORM

Date:

The blood is taken by the willingness of the healthy volunteers / patients before
collection of the blood samples.

Name of the Healthy volunteers / patients:

Age :

Sex :

Occupation :

Address :

Name and Signature
PATIENT PROFORMA

DEPARTMENT OF BIOCHEMISTRY

[BIOCHEMICAL STUDIES IN OSTEOPOROSIS OF WOMEN]

Sr.No------ Date: - / / 

Name of patient:-

Age: - Sex:-

OPD Reg. No: ------ IPD Reg.No:--------

Date of Admission: - / / Date of Discharge: - / /

Date of last menses: - / / 

Type of Probable diagnosis:-

Underlying cause:-

Routine investigation:-

Treatment:-
Biochemical analysis:-

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteocalcin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tartrate resistant acid phosphatase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>