Chapter IV

MATERIALS AND METHODS

4.1: Selection of patients and controls :-

The present study was performed in the department of Biochemistry, Shri. Shivaji Science College, Akola. The total number of 100 (65 males and 35 females) patients suffering from nephrolithiasis and 100 (65 males and 35 females) normal healthy individual served as controls, having age group 22-45 years were taken as subjects. Control as well as patient's samples were collected from various hospitals in Marathwada Region. Samples were preserved and analysed. All controls were free from diseases including renal diseases, diabetes, liver diseases, heart diseases, bone diseases, thyroid diseases. Patients of experimental group were also free from all above diseases except nephrolithiasis.

The diagnosis of nephrolithiasis patients were done by the physicians based on imaging test such as ultra sonography and plain abdominal X-ray. Fasting blood samples and twenty four hours urine samples of nephrolithiasis patients and controls were collected and analyzed. The renal stones of nephrolithiasis patients were collected and analyzed. The questionnaire was given to the normal healthy controls and nephrolithiasis patients to elicit information on the following aspects.
i. Age and Sex

ii. Source of drinking water

iii. Daily intake of water (volume).

iv. Dietary habitats

4.2 Selection of Chemicals, Reagents and Equipments :-

4.2.1 Chemicals and Reagents :-

Trichloroacetic acid, Meta phosphoric acid, Sulphuric acid, Potassium bromide, Potassium permanganate, Petroleum ether, Thiourea, Borax, Citric acid, Hydrochloric acid, Potassium hydroxide, Sodium acetate, Acetic acid, Potassium oxalate, Ammonium hydroxide, Disodium hydrogen phosphate, Magnesium sulphate, Sodium nitroprusside, Nitric acid concentrated, Potassium iodide, Mercuric iodide, Sodium hydroxide were of high purity analytical grade chemicals obtained from Merck India Limited, and S. D. Fine chemicals, Mumbai. Culture media used were of Hi-media make.

Sigma diagnostics kits were used for calcium, inorganic phosphorus, chloride, oxalate and magnesium. For the estimation of uric acid and creatinine Teco Diagnostics Kits were used. Agappe diagnostics kits were used for serum total proteins and Albumin. For the estimation of urea Bayer Diagnostics Auto Pack kits was used. Deionized water was used for preparation of reagents. Sodium and potassium standards were used from Biolab Diagnostics.
4.2.2 Equipments :-

Equipments such as photoelectric colorimeter, centrifuge, pH meter, Refrigerator, Incubator, Hot air oven, RA-50 chemistry analyzer, Flame photometer (Mediflame Double Channel Systronic) and auto-pipettes were used.

4.3 Collection of clinical specimens :-

Clinical specimen used in the present study were 24 hour urine, mid stream random urine and serum (Ranail Mukherjee, 1992; Harold Varley, 1969).

A) Urine :-

a. Identification of specimen :-

Containers used for 24 hours urine collection was wide mouth, clean transparent, graduated plastic bottles that was labelled properly before urine collection. First early morning urine specimen was discarded, then time was noted, and the whole urine was passed during the next 24-hour period including early morning urine of the next day was collected.

Appropriate preservatives were added during the sample collection. The following preservative were used.

<table>
<thead>
<tr>
<th>Preservatives</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10N HCl</td>
<td>Calcium, oxalate</td>
</tr>
<tr>
<td>2. 10N H₂SO₄</td>
<td>Citrate.</td>
</tr>
<tr>
<td>3. Thymol crystals</td>
<td>Creatinine, urea, magnesium, phosphorus, sodium, potassium, chloride, uric acid.</td>
</tr>
</tbody>
</table>
After collection of urine specimen volume of urine was noted and used for analysis of various biochemical parameters such as urea, creatinine, uric acid, calcium, phosphorus, oxalate, citrate, magnesium, sodium, potassium and chloride. During the collection of a specimens in female patients, vaginal discharges and menstrual period was avoided.

Midstream random urine samples was collected in sterile container for culture and sensitivity, microscopy and routine urine examination.

**B) Blood:**

Fasting blood samples were collected, the venous blood was drawn using disposable syringes and collected in a plain bulbs and kept for 45 minutes at room temperature. Then serum was separated by centrifugation and subjected for following biochemical parameters such as

- Blood Urea
- Serum creatinine
- Serum uric acid
- Serum calcium
- Serum inorganic phosphorous
- Serum electrolytes - sodium and potassium
- Serum chloride
- Serum albumin
- Serum total proteins
C) Renal stones :-

Renal stones obtained after surgery of the different nephrolithiasis patients were analyzed.

4.4 Analysis of samples :-

4.4.1 Estimation of urea in serum and urine :-

Urea in urine and serum was estimated by urease/glutamate dehydrogenase method. (U.V. kinetic method) (Kaplan L. A., 1984)

Reagents :-

Buffer PH 8.0 50 mmol/l
Urea working reagent 2: Urease > 5000U/L
Glutamate dehydrogenase > 450 U/L
NADH > 0.32 mmol/L
a-ketoglutarate 7.5 mmol/L
ADP 1.20 mmol/L
Standard (Urea) 50 mg/dl (8.32mmol/L)

Reagents were ready to use. All reagents were brought to room temperature before the use.
Procedure: -

1) Test tubes were labelled as Blank (B), Test (T) and standard (S). Urine Test (TU).

2) 1.0 ml of working reagent was taken into each tube

3) 10.0 µl of serum was pipette out in to test tube labelled as "T" and mixed.

4) 10.0 µl diluted urine (1:100) was pipette out into test tube labelled as "TU" and mixed.

5) 10.0 µl urea standard was pipette out into test tube labelled as "S" and mixed.

6) 1.0 ml working reagent was pipette out into test tube labelled as "B".

7) Contents in the tubes were aspirated immediately using wave length 340 nm. Results were displayed in mg/dl.

Calculation: -

\[
\text{Urinary urea in mg/dl} = \text{mg/dl} \times \text{dilution factor}
\]

\[
\frac{24 \text{ hours urinary urea} = \frac{\text{Urinary urea in mg/dl} \times \text{volume of 24hrs urine (mls)}}{100}}{(\text{mg/24 hrs})}
\]

4.4.2 Estimation of creatinine in serum and in urine

Creatinine in serum and in urine was estimated by alkaline picrate (Kinetic method) (Henry J. B., 1974)
Reagents:

- Reagents 1: Picric acid 10mM
- Reagent 2: Sodium hydroxide 240 mM
- Creatinine standard 5mg/dl

(creatinine in hydrochloric acid)

Reagents were ready for use, and brought to room temperature before use.

Procedure:

1) Test tubes were labelled as Blank (B), Test (T), and standard (S). Urine Test (TU).
2) 500 µl of working reagent I and 500 µl of working reagent II were pipette out into each tube and mixed.
3) 50.0 µl of serum was pipette out into test tube labelled as "T" and mixed.
4) 50.0 µl diluted urine (1:100) was pipette out into test tube labelled as "TU" and mixed.
5) 50.0 µl creatinine standard was pipette out into test tube labelled as "S" and mixed.
7) Contents in the tubes were aspirated immediately using wave length 500 nm. Results were displayed in mg/dl.

Calculation:

Urinary creatinine in mg/dl = mg/dl x dilution factor

24 hour to urinary creatinine = urinary creatinine mg/dl \times \frac{\text{vol. of 24 hr urine}}{100} (mg/24hr)
4.4.3 Estimation of uric acid in serum and urine :-

Uric acid in serum and in urine was estimated by enzymatic (Uricase) method (Davidson L. 1974, Henry J. B., 1974)

Reagents :-

All the reagents were brought to room temperature before the use

Reagent 1:-

Reconstituted by adding 12.0ml deionized water to the bottle reagent 1, swirl to dissolve and after 5 minutes used for analysis.

1) Uric acid reagent:-

4 amino antipyrine: 4 mM
3,5 dichloro-2-hydroxybenzene sulfonate: 2mM
Uricase (microbial): >150 U/L
Peroxidase (horse raddish): 10,000 U/L
Buffer pH: 7.5

2) Uric acid standard: 5mg/dl
Procedure: -

1) Test tubes were labelled as Blank (B), Test (T) and standard (S). Urine Test (TU).

2) 1.0 ml of working reagent was pipette out into each tube.

3) 25.0 µl of serum was pipette out in to test tube labelled as "T" and mixed.

4) 25.0 µl diluted urine (1:100) was pipette out into test tube labelled as "TU" and mixed.

5) 25.0 µl uric acid standard was pipette out into test tube labelled as "S" and mixed.

6) All the tubes were incubated at 37°C for 10 minutes.

7) After incubation contents in the tubes were aspirated immidiately using wave length 520 nm. Results were displayed in mg/dl.

Calculation:

Urinary Uric Acid in mg/dl = mg/dl x dilution factor

\[
24 \text{ hours urinary} = \frac{\text{urinary uric acid in mg/dl} \times \text{volume of 24hrs urine (mls)}}{\text{uric acid (mgs/24 hrs)}} \times 100
\]

4.4.4 Estimation of calcium in serum and urine :-

Calcium in serum and urine was estimated arsenazo III method. (Michalylova V. and Likova P., 1971; Adley M., Schumann G. B., 1984)
Reagent:

1) Working Reagent:

Arsenazo III 0.1 mM/L
Buffer pH 6.5 + 0.1

Non-reactive ingredient and stabilizer.

2) Calcium standard: 10 mg/dl

Procedure:

1) pH of urine sample was maintained between 4.0 to 6.0

2) Test tubes were labelled as Blank (B), Test (T) and standard (S). Urine Test (TU).

3) 1.0 ml of working reagent was pipette out into each tube.

4) 10.0 μl of serum was pipette out in to test tube labelled as "T" and mixed.

5) 10.0 μl diluted urine (1:100) was pipette out into test tube labelled as "TU" and mixed.

6) 10.0 μl calcium standard was pipette out into test tube labelled as "S" and mixed.

7) Contents in the tubes were aspirated immediately using wave length 600nm. Results were displayed in mg/dl.
Calculation:

24 hours urinary calcium (mg/dl) = \text{mg/dl} \times \text{Dilution factor}

\[
\text{24 hour Urinary calcium} = \frac{24\text{hrs Urinary calcium in mg/dl} \times \text{24hrs Urine volume (mls)}}{100}
\]

4.4.5 Estimation of inorganic phosphorus in serum and urine

Inorganic phosphorus in serum and urine was estimated UV kinetic method (Daly J. A. and Ertingehausen, G., 1972; Goldenberg H., Fomandoz A., 1966).

Unreduced phosphomolybdate complex.

Reagents: - All reagents brought to room temperature before use.

1) Phosphorus reagent:- Ammonium molybdate 0.40 m Mol/L in sulfuric acid with surfactant.

2) Phosphorus standard:- 5 mgs/dl.

Test tubes were rinsed by glass-distilled water before analysis.

Procedure:

1) Test tubes were labelled as Blank (B), Test (T) and standard (S). Urine Test (TU).

2) 1.0 ml of working reagent was pipette out into each tube.
3) 10.0 μl of serum was pipette out into test tube labelled as "T" and mixed.

4) 10.0 μl diluted urine (1:100) was pipette out into test tube labelled as "TU" and mixed.

5) 10.0 μl phosphorous standard was pipette out into test tube labelled as "S" and mixed.

6) Contents in the tubes were aspirated immediately using wavelength 340nm. Results were displayed in mg/dl.

**Calculations:**

\[
\text{24 hrs. urinary inorganic phosphorus (mg/dl)} = \text{mg/dl} \times \text{dilution factor}
\]

\[
\frac{24 \text{ hrs. urinary inorganic phosphorus (mg/dl)}}{(mg/24\text{hrs.urinary})} = \frac{24 \text{ hrs urinary inorganic phosphorus (mg/dl)} \times \text{volume (ml)}}{100}
\]

4.4 **Estimation of serum and urine electrolytes (sodium and potassium):**

Sodium and potassium in serum and urine were estimated by Flame photometry method (Jahn D. Baur, 1982).

**Reagent :**

I] Ready to use standard

i] Sodium / potassium standard 120 / 2 mEq/L

ii] Sodium / potassium standard 140 / 4 mEq/L

iii] Sodium / potassium standard 160 / 6 mEq/L

All standards were diluted 1:100 and used for analysis
Sample Preparation:-

a) Serum sample was diluted 1:100 by deionised water

b) Urine sample was diluted 1:1000 by deionised water for sodium and potassium analysis.

Procedure:

1) Flame photometer was switched on.

2) Compressor was switched on and output pressure was adjusted to 0.45 kg/cm2.

3) Fuel supply form the fuel source/LPG cylinder was switched on and the flame was ignited immediately with an ignition lighter through the ignition window.

4) Fine flame adjustment was done to get stable flame-having well-defined cones with the help of fuel gas fine adjustment knob.

5) Atomizer capillary tube was dipped in glass-distilled water.

6) Calibration:
   i] \( \text{Na}^+ / k^+ \) 120/2 standard was feed and reading was displayed.
   ii] Then successively Na/k: 140/4 and 160/6 standards were feed and readings were displayed.

7) Diluted serum sample and urine sample 1:100 and 1.1000 respectively were aspirated and readings were reordered in mEq/L.
Calculation :-

24 hrs. urinary sodium and potassium (mEq/24hrs) = meq /L x 24 hrs urinary volume in mls.

2.4.7 Determination of serum and urinary chloride:-

Chloride in serum and urine was estimated by thiocyanate method (Schales O., Schales, S. S. 1964; Schemed R. G. and Lewellen C. J., 1964)

Reagent :-

Working chloride reagent composition :-

Mercuric chloride 0.74 mmol/l
Mercuric nitrate 72.5 umol/l
Mercuric thiocyanate 1.7 mmol/l
Ferric nitrate 22 mmol/l
Sulfuric acid 10 mmol/l
Nitric acid 30 mmol/l
Stabilizer

Chloride reagent was ready to use.

Chloride standard : (carbonate/chloride ) : 100 mEq/L
Procedure:

1) Test tubes were labelled as Blank (B), Test (T) and standard (S). Urine Test (TU).

2) 1.0 ml of working reagent was pipette out into each tube.

3) 10.0 μl of serum was pipette out in to test tube labelled as "T" and mixed.

4) 10.0 μl urine was pipette out into test tube labelled as "TU" and mixed.

5) 10.0 μl chloride standard was pipette out into test tube labelled as "S" and mixed.

6) All tubes were incubated at 37°C for 10 minutes.

7) After incubation contents in the tubes were aspirated immediately using wave length 460nm. Results were displayed in m.Eq/l.

Calculations:

\[ 24 \text{ hrs} - \text{Urinary Chloride} = \frac{\text{Urinary chloride (mEq/L)}}{24 \text{ hrs volume of urine in ml}} \times 24 \text{ hrs} \]

4.4 Estimation of Serum total proteins :-

Serum total proteins was estimated by biuret method

(Gomall, A. J., 1949)

Reagents:

Reagent 1: Potassium sodium- tartarate

Sodium hydroxide

Copper Sulfate
Surfactant.

Protein Standard: 6 gm/dl.

Procedure :-

1) Test tubes were labelled as Blank (B), Test (T) and standard (S).
2) 1.0 ml of working reagent was pipette out into each tube.
3) 10.0 μl of serum was pipette out in to test tube labelled as "T" and mixed.
4) 10.0 μl Protein standard was pipette out into test tube labelled as "S" and mixed.
5) All tubes were incubeted at room temperature for 10 minutes.
6) After incubation contents in the tubes were aspirated immediatly using wave length 630nm. Results were displayed in gm/dl.

4.4.9 Estimation of serum Albumin :-

Serum albumin was estimated by bromocresol Green method

(Doumasa et al., 1971)

Reagents :-

Working Reagent 1 : - Buffer pH= 4.2

Bromocresol green

Preservative

Albumin standard : - 3 gm/dl.
Procedure:-

1) Test tubes were labelled as Blank (B), Test (T) and standard (S).

2) 1.0 ml of working reagent was pipette out into each tube.

3) 10.0 µl of serum was pipette out in to test tube labelled as "T" and mixed.

4) 10.0 µl Albumin standard was pipette out into test tube labelled as "S" and mixed.

5) All tubes were incubated at room temperature for 1 minutes.

6) After incubation contents in the tubes were aspirated immediately using wave length 630 nm. Results were displayed in gm/dl.

4.4.10 Estimation of 24-hour urinary oxalate :-


Reagents: Oxalate standard : 0.50 mMol/L

Oxalate Reagent A :

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMAB</td>
<td>3.2 mMol/L</td>
</tr>
<tr>
<td>MBTH</td>
<td>0.22 mMol/L</td>
</tr>
<tr>
<td>Buffer</td>
<td>pH 3.1 ± 0.1</td>
</tr>
</tbody>
</table>

Non reactive ingredients and stabilizer.
Oxalate reagent B:

Oxalate Reagent (Barley) 3000U/L
Peroxides (Horse radish) 100,00 U/L

Sample diluents:

EDTA 10 m Mol/L
Buffer pH 7.6 ± 0.1.

Sample purifier tubes: Activated Charcoal.

Procedure:

I) Sample Preparation

1. Series of labelled tubes of urine samples were set up.
2. 5 ml of urine taken in to appropriately labelled tube.
3. 5 ml of sample diluents was added into each tube and mixed.
4. pH was checked and if pH was not between 5 & 7.0, then adjusted by using 1N hydrochloric acid or 1N sodium hydroxide.
5. Sample purifier tubes for urine sample was set up.
6. 2 ml of each diluted urine sample was pipett out & taken in a appropriately labelled sample purifier tube and mixed for approximately 5 minutes by intermittent mixing.
7. Tube was centrifuged at 2000 rpm (1500xg).
II) Determination of oxalate concentration in the supernatant.

1. Oxalate reagent was warm at 37°C.

2. Tubes were labelled reagent blank, standard and urine sample.

3. 1 ml of oxalate reagent A was pipette out into each tube.

4. 5.0 ml of supernatants was pipette out into respective tubes, and 5.0 ml-deionized water was added into Reagent Blank and 5.0 ml standard to tube labelled as standard.

5. 0.1 ml of oxalate Reagent B was added into each tube immediately mixed by gentle inversion.

6. All tubes were incubated at 37°C for 5 minutes.

7. Absorbance of Blank, standard, urine sample were taken at 590 nm.

8. Corrected absorbance (ΔA) of standard, samples were determined by subtracting Reagent Blank absorbance from the absorbance readings of standard, and urine Sample.

9. Oxalate concentration in urine sample was calculated using following formula

\[
\text{Oxalate (m mol/L)} = \frac{\text{ΔA Sample}}{\text{ΔA Standard}} \times 0.5 \times 2.
\]

Where 0.5 = Concentration (m mol/L) of oxalate in Standard

2 = Dilution factor.
Urine oxalate (m mol/24 hour) =

\[
\frac{\Delta A_{\text{Sample}}}{\Delta A_{\text{Standard}}} \times 0.5 \times 2 \times \text{volume of urine voided during 24 hours}
\]

Results were calculated in mg/dl. By multiplying concentration in m mol by 90, to obtain oxalate excretion in mg/24hrs.

4.4.11 Estimation of Urinary citrate :-

Urinary citrate was estimated by Pentabromo acetone Method (G. Rajgopal, 1984)

Reagent :-

Deionized water was used for preparation of reagents and analytical procedure.

1. Trichloroacetic acid 10 % : 10 gm of Trichloroacetic acid was weighed & dissolved in Deionized water & volume was made up to 100 ml. With deionized water.

2. Sulphuric acid 9N : 25 ml of conc. $\text{H}_2\text{SO}_4$ AR was diluted to 100ml with water, carefully with cooling.

3. Meta phosphoric acid 40 % : 40g. Meta phosphoric acid was weighed, dissolved in Deionized water, and volume was made up to 100 ml with D/W.
4. Potassium bromide 2 M : 23.8 g. potassium bromide was weighed, dissolved in Deionized water and volume was made up to 100 ml with D/W.

5. Potassium per magnate 6.5% : 6.5 g. of potassium permanganate was weighed dissolved in D/W and volume was made up to 100 ml with D/W (kept in dark bottle).

6. Hydrogen peroxide 6% : 30% \( \text{H}_2\text{O}_2 \) was diluted with water, 1 in 5.

7. Petroleum ether (60 to 80°C).

8. Thiourea borax solution (pH 9.2) : 4 g. thiourea and 2 g. borax was weighed and dissolved in D/W and volume was made up to 100 ml with D/W.

9. Standard citric stock solution : 109.4 mg. of citric acid monohydrate crystal (AR) were taken in a 100 ml standard flask dissolved in 1N H\textsubscript{2}SO\textsubscript{4} volume was made up to 100 ml with the same acid.

10. Standard citric acid working (10mg%). 10 ml of stock citric acid was diluted to 100 ml in a standard flask with D/W (prepared freshly).

**Method :- Preparation of Standard Curve :-**

1) A series of Standards were set up as follows. 25 ml Glass stopper tubes was marked as S1, S2, S3, S4 & blank.

2) 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml working standard was pipett out in to test tubes S1, S2, S3 and S4 respectively.

3) 2.0 ml deionized water was pipette out into test tube labelled as "B".
4) 1.5 ml, 1.0 ml and 0.5 ml deionized water was pipette out into test tube S1, S2 and S3 respectively.

5) 2 ml 10% TCA was pipetted out into each test tube and mixed.

6) 1 ml 9N H₂SO₄ was pipette out into each test tube and mixed.

7) 0.25 ml 40% metaphosphoric acid was pipette out into each test tube and mixed.

8) All the test tubes were kept in ice water for 5 minutes and 0.5 ml of 2 MKBr and 1.5 ml of 6.5% KMnO₄ were added, mixed and left in the ice water bath for 10 minutes.

9) 6% H₂O₂ was added dropwise into each tube for decolorization of KMnO₄ and tubes were returned to a rack.

10) 5 ml petroleum ether was added into each tubes, tubes were stoppered and shaken for 2 minutes for extraction of the penta bromoacetone into petroleum ether. The layers were allowed to separate.

11) 4ml from the petroleum ether layers were transferred quickly into 15 ml glass stopper centrifuge tubes.

12) 6 ml Thiaurea borax solution was added to each tube, stoppered and contents were shaken for 2 minutes. A golden yellow color developed in aqueous layer.

13) The tubes were centrifuged for 5 minutes and top petroleum ether layer from each tubes were aspirated & discarded.

14) The absorbance of the colored aqueous layers were taken using blue violet filter (445 nm), against the reagent blank.
Procedure for determination of urinary citrate :-

For Test :-

1) In 25 ml centrifuge tube the following reagents were taken 3ml. distilled water & 1 ml urine sample and 4 ml of 10% TCA. Content was mixed and centrifuged.

2) From the above content, 4ml was transferred in to 25ml glass stopper tube which is marked as T.

For Blank :-

1) In a 25 ml glass stopper tube 2ml D/W and 2 ml of 10% TCA were taken

2) To the Blank and Test (urine) 1ml of 9N H₂SO₄ was added followed by 0.25ml of 40% meta phosphoric acid, content was mixed after each addition.

3) The tubes were kept in ice water below 10⁰C. taken in a beaker. After 5 minutes, to each tube 0.5ml of 2M KBr and 1.5ml of 6.5% KmNO₄ were added, mixed and left in the ice water bath for 10 minutes.

4) In each tube drop wise 6 % H₂O₂ and 5ml of petroleum ether (60-80⁰C) were added to each tube.

5) The tubes were stoppered and shaken manually about 2 minutes for extraction of PBA in to petroleum ether.
6) The layers were allowed to separate, 4ml from the petroleum ether layer were transferred quickly into 15 ml glass stoppered centrifuge tubes.

7) Thiourea - borax 6ml solution added to each tube, stoppered and contents were shaken for 2 minutes. A Golden Yellow color was developed in the aqueous layer.

8) The tubes were centrifuged at 3000rpm for 5 minutes, the top petroleum ether layer was aspirated and discarded. The absorbance of the colored aqueous layer was taken against reagent blank using blue violet filter 445 nm. The concentration of urinary citrate was determined using standard curve and result was calculated by multiplying 24hrs. urine volume voided.

4.4.12 Estimation of magnesium in 24hrs. Urine

Magnesium in urine was estimated by Mann & Yoe (1956)

(Ralge D. et al., 1986)

Reagents :- Working reagent :-

Magon 0.1m mol/L
Trizma 250 m mol/L
EDTA 0.053 m mol/L
Buffer pH 11.3 ± 0.1
Magnesium standard : 2m Eq/L.

Urine specimen diluted 1:100 with deionized water.
Procedure :-

1) Test tubes were labelled as Blank (B), Test urine (TU) and standard (S).
2) 1.0 ml of working reagent was pipette out into each tube.
3) 10.0 µl of diluted urine (1:100) was pipette out in to test tube labelled as "TU" and mixed.
4) 10.0 µl magnesium standard was pipette out into test tube labelled as "S" and mixed.
5) All tubes were incubated at room temperature for 5 minutes.
6) After incubation contents in the tube were aspirated immediately using wave length 500 nm. Results were displayed in mEq/l.

Calculation :-

Multiplied results (mEq/l) by 1.216 for mg/dl.

Magnesium (mg/dl) = Urinary 24hrs Urinary Magnesium mg/dl x Dilution factor

\[
\text{mg/dl X 24 hrs Urinary volume in mls} \div 100
\]

\[
\text{24hrs Urinary magnesium = } \frac{\text{mg/dl X 24 hrs Urinary volume in mls}}{100}
\]


Preparation: - Stone was crushed to make powder using mortar and pestle.

Powder was divided in to 5 aliquots were place in a separate glass tube.
On first portion following tests were performed.

1. Test for Carbonate:- 3ml. of 5% (vol/vol) HCl was added to the first aliquot of powder. If effervescence was liberates it indicate presence of carbonate.

2. The above content was heated, mixed and divided its contents into 3 approximately equal portions, proceed for following test.

3. Test for ammonia:- To one portion of liquid from step two 10%(wt/vol) KOH was added until the solution tests neutral with litmus paper. The 0.5ml of Nesseler's reagent was added blue color was observed.

4. Test for calcium oxalate: - Another portion of a liquid from step 2 was taken and centrifuged at 2500 x g for 5 minutes and clear supernatant was transferred in to a second tube. 0.2 ml of saturated sodium acetate was added and pH was adjusted to approximately 5 with 10%(vol/vol) acetic acid. (White precipitate indicates presence of calcium oxalate)

5. Test for non oxalate calcium: - In the above contents precipitate was removed centrifugation. In the supernatant potassium oxalate was added to a solution of calculus at pH 5. (Precipitate indicates presence of no oxalate calcium)

6. Test for magnesium and phosphate :- Precipitate formed in a step 5 was centrifuged and supernatant was collected and adjusted to pH 8.0 with ammonium hydroxide.

White precipitate indicates presence of magnesium and phosphate.
7. If solution in step 6 remains clear divided it in to 2 portions.

8. Test for phosphate :- To a portion of solution from step 7, 0.2ml 5% (wt/vol) magnesium sulphate is added.
   White precipitate indicates the presence of phosphate.

9. Test for magnesium :- To the second portion of solution from step 7, 0.2ml 5% (wt/vol) Na2HPO4 was added.
   White precipitate indicates presence of magnesium.

10. Test for Cystine :- To a small amount of powdered stone in a glass tube 2ml. water and 2ml. 5%(wt/vol) NaCl was added. After 5minutes few crystals of sodium introprusside was added.
    Burgundy color indicates presence of Cystine.

11. Test for uric Acid: - To a small amount of powdered stone in a crucible, 1 to 2 drops of conc. HNO₃ was added and evaporated to dryness with slight amount of heat, cooled and 2 drops of H₂SO₄ was added.
    Purple color indicate presence of uric acid.

4.4.14 Urinalysis :- (Free A. H., 1972)

Procedure:-

1. Urine Strip was removed just before use and used for qualitative detection of pH, specific gravity, proteins, blood, etc, in urine.

2. Reagent area of the strip was completely immersed in fresh well mixed urine and removed immediately.
3. The strip was blotted on an absorbent paper to remove excess urine.

4. The reagent area was compared to corresponding color box on the color chart within 1-2 minutes. The results were obtained by comparison with color chart.

4.4.15 Urine microscopy :- (Free A. H., 1972)

1. The urine was mixed and centrifuged for 5 minutes at 1500 to 2500 rpm and supernatant was decanted.

2. The resuspended deposits in the few drops of urine which remains at the bottom of the test tube was placed as a drop on a microscopic slide and the slide was covered with cover slip.

3. The slide was examined under the microscope and observations were noted.

4.4.16 Urine culture and Sensitivity :- (Mackie and McCurtney, 1996; Jhon D Bauer, 1990)

Material:

Clinical Specimen: -

Urine :- Mid stream of first urine passed by the patient was collected in a sterile wide necked leak proof container.
Media :-

Following media were used for the isolation of microorganisms. The media used in this study procured from HI media Mumbai.

1. Mannitol Sah Agar
2. Baird – Parker egg yolk medium
3. Blood Agar
4. Nutrient broth
5. Peptone water
6. Glucose broth

Media for biochemical tests: -

1. Sugar fermentation medium
2. Glucose Phosphate broth (MR –VP medium).
3. Simmon’s citrate agar
4. Christensen’s Urease medium

Stains and Reagents :-

1) Methyl red indicator
2) Barrett’s reagent
3) Kovac’s reagent
4) Oxides reagent
5) 3% Hydrogen peroxide solution (catalos reagent)

6) Andrade's indicator

7) 10% Lead acetate solution

8) Crystal violet solution (stock)

9) Oxalate Solution

10) Safranin (Stock solution)

11) Grams iodine solution

12) 95% ethyl alcohol deodorizer.

Method:

Inoculation :-

Urine sample was inoculated on nutrient agar, mannitol sah agar, brand -parker egg yolk and blood agar for the isolation of staphylococci. One plate of each medium was used for single specimen incubation: all inoculated plates were incubated at 37°C for 18hrs. and then observed for growth chariot erectus.