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ANALYSIS OF BIOMOLECULES PRESENT IN CALCIUM PHOSPHATE (APATITE) & URIC ACID STONES.

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MAY-2008

CERTIFICATE

This is to certify that the work entitled, "**ANALYSIS OF BIOMOLECULES PRESENT IN CALCIUM PHOSPHATE (APATITE) & URIC ACID STONES.**" submitted by **GAURAV AWASTHY** and **RACHIT KAUSHAL** in partial fulfillment for the award of Degree of Bachelor of Technology in Bioinformatics of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Rachit Kaushal.

Gaurav
24/5/08.

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ACKNOWLEDGMENT

We extend our heartfelt gratitude to

Dr. S.K. Singh

MS, MCh (Urology)

Professor

Department of Urology, PGI, Chandigarh.

For providing us the opportunity to work on live samples, without which this work would not have been possible.

We also express our deepest gratitude to

Dr. C. Tandon

Associate Professor

Department of Biotechnology and Bioinformatics

JUIT, Waknaghat.

Without whose guidance, Endeavor and constant motivation this project would not have seen the light of this day.

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SDS – Sodium Dodecyl Sulphate

PAGE – Polyacrylamide Gel Electrophoresis

KDa – Kilo Daltons

PGIMER – Post Graduate Institute of Medical Research

ddH_2O – double distilled water

EDTA – Ethylene Diamine Tetra Acetic Acid

rpm – revolutions per minute

ml – milliliters

OD – Optical Density

nm – Nano meter

gm – Grams

Amm – Ammonium

N – Normality of the solution

mM – millimolar (concentration)

Sol – Solution

µL – micro liters

^{o}C – Degree Celsius

BSA – Bovine Serum Albumin

TEMED – N,N,N',N'-Tetramethylethylenediamine

APS – Ammonium per Sulphate

Conc - Concentration

ABSTRACT

The purpose of the work done was to analyse the various specimens that were procured from various sources and perform qualitative as well as quantitative analysis of both the apatite and uric acid stones. Further the proteins constituting the stone matrix were to be extracted by the various extraction methods and the extracts were to be analyzed for their protein content which was further divided into three groups on the basis of their molecular weights. Also and SDS PAGE analysis of the extracts was to be performed.

For qualitative and quantitative analysis the stone samples were washed and grinded and various Qualitative as well as Quantitative tests were performed. For the analysis of the biomolecules present in the stone matrix two methods of extraction were used: 2% SDS Reducing Buffer and 10% Acetic Acid (James C. Williams Jr, Chad A. Zarse, Molly E. Jackson, Frank A. Witzmann & James A. McAteer, 2006). Proteins were measured using the Lowry's method for estimation of proteins. SDS extracts were also examined using Polyacrylamide Electrophoresis (PAGE).

The qualitative tests on uric acid stone confirmed the presence of phosphate along with uric acid as the main constituent whereas oxalate was found to be absent. The content of Calcium and Phosphate in the apatite stone came out to be 2.156, 2.888, 3.259, 3.577 a against standards of 1mm, 2mm, 6mM & 10mM and 1.952 against 2mM respectively. Out of the two extraction methods that were employed to extract the proteins from the stone matrix SDS method extracted more protein than the Acetic Acid Method.

CHAPTER 1

INTRODUCTION

1.1 – About the disease

Urolithiasis is a disease that has been studied for many years, and the cause of stone formation is not well understood. It is therefore important to fully recognize both the stone's chemical structure and composition. Kidney stones are basically results of the process of bio-mineralization and super-saturation in the kidney, in areas like the nephritic tubules etc.

The crystallization of various chemical substances which are normally excreted out in urine occurs, when their concentrations increase beyond their saturation point. Some of these chemicals are: calcium phosphates, uric acid, purines etc.

1.2 – Components of human urine

The various substances that are normally excreted out in urine are:

Calcium phosphates

Hydroxyapatite

Carbonate-apatite

Brushite

Calcium oxalate

Magnesium ammonium phosphate

Struvite (triple phosphate)

Purines

Uric acid

Xanthine

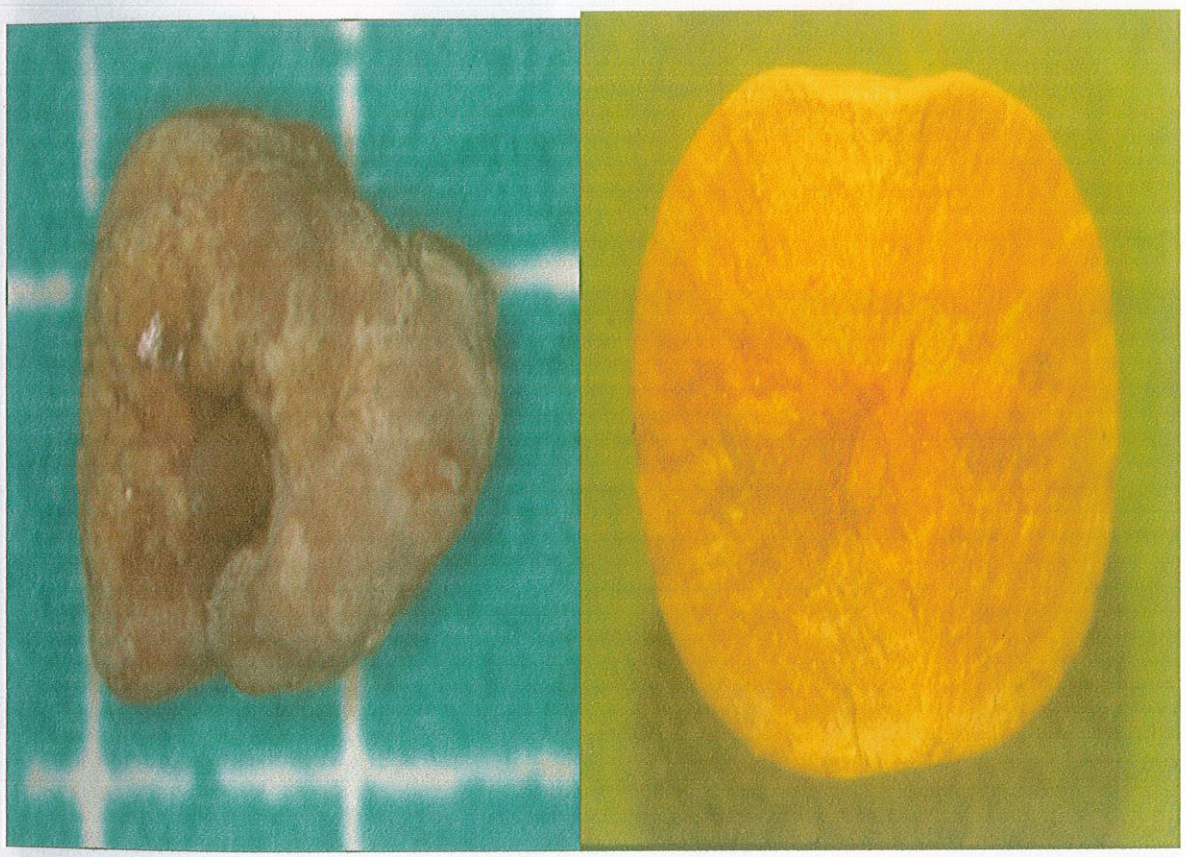
Ammonium acid urate

2, 8-dihydroxyadenine

Cystine

1.3 – Types of stones

Because of varying calculi composition, the stones are divided into six groups: magnesium ammonium phosphate hexahydrate (struvite), calcium phosphate (apatite), mixed phosphates and oxalates, calcium oxalate mono- and dihydrate (whewellite and wedellite), mixed oxalates and uric acid, and uric acid.



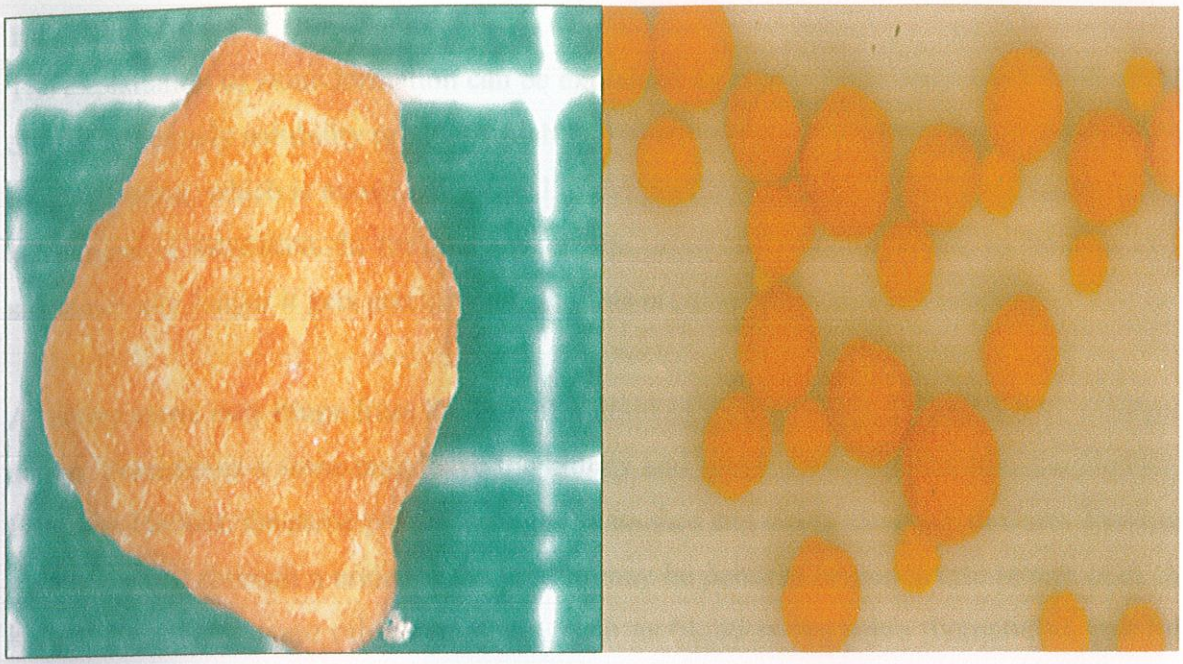


FIG 1 – URIC ACID STONE SAMPLES

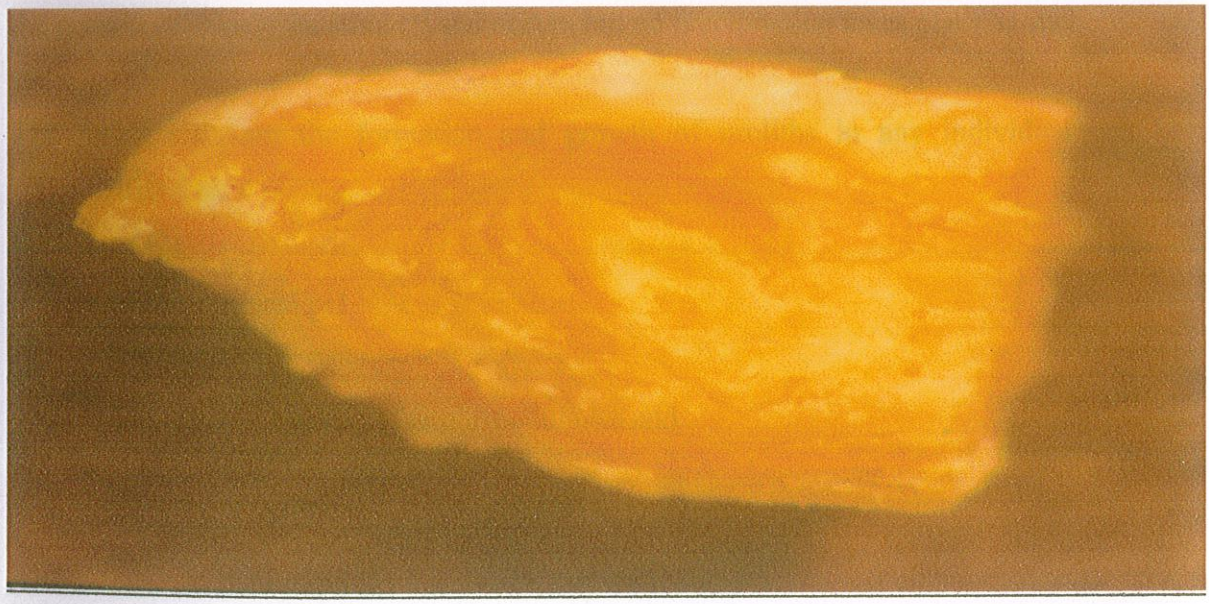


FIG 2 – CALCIUM PHOSPHATE (APATITE) STONE SAMPLES

1.4 – The process of stone formation

The explanation of stone formation can be explained through a simple principle of physical chemistry namely solubility product which is defined as the saturation point of a substance in the solution, which is numerically equal to the product of the molar concentrations of the substances at saturation. In the body when the urine becomes incapable of holding any substance any longer it gets precipitated, and this is called the formation product.

A kidney stone may range from the size of a grain of sand to about 1 in. (2.5 cm) or larger across. Sometimes a stone may leave the kidney and move down a ureter into the bladder. From the bladder, the stone passes through the urethra and out of the body in urine. Passing a kidney stone through a ureter or the urethra may be painless or may cause severe pain. A kidney stone may cause other symptoms, such as blood in the urine (hematuria), painful urination, or urgency to urinate.

Between the solubility product and the formation product lies the metastable state in which the growth of already existing crystal occurs but no precipitation takes place as such.

The factor effecting the formation and growth of uroliths in any individual can be summarized as: -

- Diet
- Physiological pH
- Infection
- Injury to the lining of nephritic tubules and
- Water Intake

Also to note are the symptoms that are usually seen in cases affected with urolithiasis

- Abdominal pain
- Penile Pain
- Painful urination
- Abnormal Urine pH

- Recurrent infection of the urinary Tract.

1.5 – Project Overview

In the present study we performed qualitative analysis of the stone specimens that were procured from various sources. From the results of the qualitative tests we moved on to quantitative analysis of the samples and estimated and established the content of various components in the samples. In the later part of the project we extracted different proteins that are part of the stone matrix with the help of two extraction methods namely 2% SDS reducing Buffer method and the 10% Acetic Acid method. The extract that was obtained was then divided into 3 parts on the basis of the different molecular weight of the proteins comprising of the extract with proteins having molecular weight less that 5 KDa, extract with proteins having molecular weight greater than 5 KDa. And the originally obtained and termed as the crude extract. After the extraction of the proteins on the three extracts Lowry's assay was performed to estimate the protein content in three samples, further SDS – PAGE analysis of the three was also performed.

1.6 - Objectives

1. Qualitative and Quantitative Analysis of the specimen.
2. Extraction of the proteins constituent of the stone matrix.
3. Separation of the proteins on the basis of molecular weight.
4. Estimation of protein content in the extract.
5. SDS – PAGE analysis of the extracts.

CHAPTER -2

MATERIALS AND METHODS

2.1 – MATERIALS

***2.1.1 – Stone Samples*: -**

All the specimens were procured from Department of Urology, PGIMER, Chandigarh and R.G. Stone Clinic, New Delhi.

***2.1.2 – Chemicals*: -**

The chemicals used in the present study were obtained from S.D fine chemicals Limited, Merck Limited and Qualigens fine chemicals Limited.

2.2 – METHODS

***2.2.1 – Preparation of specimen*: -**

The specimens were washed in .15N Sodium Chloride solution in order to make them free of any tissue or blood remains that might have been sticking to the surface.

2.2.2 – Qualitative estimation of uric acid: - (MUREXIDE TEST)

Add 2-3 drops of concentrated Nitric Acid to a small amount of the sample in a small evaporating dish and evaporate to dryness by heating on a water bath or on a small flame. The test is positive if a red or yellow is obtained which after being allowed to cool changes to a purplish red on addition of a drop of dilute Ammonium Hydroxide.

2.2.3 - Qualitative estimation of Oxalate: -

Heat a small amount of powdered stone sample over aluminium foil until it turns into a grey colored ash. Add concentrated Nitric Acid, the presence of effervescence confirms the presence of oxalate.

2.2.4 – Quantitative estimation of Calcium: -

REAGENTS REQUIRED:-

Calcium Reagent:

9.0 Gms of Sodium Chloride dissolved in double distilled water.
280 mg of Napthyl Hydroxanic acid dissolved in 100 ml of solution comprising of 95ml of double distilled water, 5 ml ethanolamine and 2gm tartaric acid.
After mixing 1&2 raise the volume to 1000ml by adding double distilled water.
Filter and store in a brown bottle

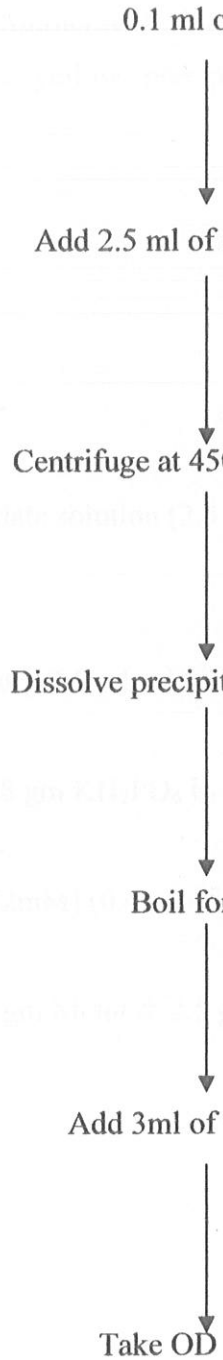
EDTA:

2 gm EDTA dissolved in 100 ml of 0.1 N sodium hydroxide (NaOH)

Color Reagent:

1. 60 gm ferric nitrate dissolved in 500 ml (485 ml double distilled water and 15 ml nitric acid).
2. Dilute it to 1000 ml and store in a brown bottle.

PROTOCOL


0.1 ml of sample
Add 2.5 ml of calcium reagent
Centrifuge at 4500rpm for 15 min
Dissolve precipitate in 1ml EDTA
Boil for 10 min
Add 3ml of color reagent
Take OD at 450 nm.

2.2.5 – Qualitative and Quantitative estimation of Phosphate:-

QUALITATIVE TEST

Dissolve a little of the powdered stone in a few ml of concentrated Nitric Acid and then add an equal volume of Ammonium phosphomolybdate solution. Heat to boiling. If phosphates are present, a yellow precipitate of ammonium phosphomolybdate is obtained.

QUANTITATIVE TEST:-

REAGENTS REQUIRED:-

1. Ammonium Molybdate solution (2.5 gm Amm. Molybdate in 100ml distilled water)
2. 10 N Sulphuric Acid (13.6 ml acid in 36.4 ml ddw)
3. Stock Standard (0.68 gm KH_2PO_4 in 100 ml of ddw)
4. Working Standard [2mM] (0.4 ml of stock in to 10 ml of ddw)
5. Metol Reagent (0.5 gm Metol & 2.5 gm $NaHSO_3$ in 100 ml of ddw)

PROTOCOL

Take 0.2 ml of sample. Add 1.2 ml of Amm. Molybdate sol. to it.

Mix the solution and stand for 10 min.

Add 6.8 ml of ddw

Add 0.5 ml metol reagent with stirring.

Stand for 20-30 minutes. Take O.D at 660 nm

2.2.6 - Extraction of matrix proteins by 2% SDS reducing buffer method:-

For the SDS method, 100 mg stone powder was mixed with 400 µL SDS reducing buffer (0.06 M TRIS HCl, pH 6.8, 10% glycerol, 5% beta-mercaptoethanol, and 2% SDS) and heated (100°C, 30 min). Samples were centrifuged (700g, 15 min, 4°C) and the supernatant was collected. This process was repeated for the pellet two more times. After the third centrifugation, all three supernatants were pooled and dialyzed.

2.2.7 – Extraction of matrix proteins by 10% Acetic Acid method:-

For the acetic acid method, 100 mg stone powder was vortexed with 500 µL 10% acetic acid and incubated for 30 min. Samples were centrifuged (2,000g, 20 min, 4°C) and the supernatant was retained and dialyzed.

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2.2.8 – Separation of the extracts on the basis of molecular weight:-

In Order to see whether the proteins that constitute the stone matrix are majorly large molecular weight proteins or small molecular weight proteins, AMICON tubes for separating proteins greater than 5 Kda and less than 5 Kda were used.

2.2.9 – Estimation of protein content using Lowry's Method:-

This can be achieved by many procedures such as Biuret test, Lowry method etc out of which we have employed Lowry's method for extraction of proteins.

PRINCIPLE

The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most proteins

estimation techniques use Bovine Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10 µg/ml and is probably the most widely used protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to10.5 is essential.

PROCEDURE

1. Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube.

2. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ ml.

3. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2 ml of alkaline copper sulphate reagent (analytical reagent).

4. Mix the solutions well. This solution is incubated at room temperature for 10 mins.

5. Then add 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min.

6. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 660nm.

2.2.10 – SDS PAGE Analysis:-

PRINCIPLE

The purpose of SDS-PAGE is to separate proteins according to their size, and no other physical feature. Since we are trying to separate many different protein molecules of different shapes and sizes, we first want to it denatured so that the proteins no longer have any secondary, tertiary or quaternary structure. We use SDS to denature all proteins to the same linear shape. SDS (sodium dodecyl sulfate) is a detergent (soap) that can dissolve hydrophobic molecules but also has a negative charge (sulfATE) attached to it. So a protein that started out in its original configuration will be converted into just its primary structure in a straight chain. The end result has two important features: 1) all proteins retain only their primary structure and 2) all proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field.

Now if the proteins are denatured and put into an electric field, they will all move towards the positive pole at the same rate, with no separation by size. So we need to put the proteins into an environment that will allow different sized proteins to move at different rates. The environment of choice is polyacrylamide, which is a polymer of acrylamide monomers. When this polymer is formed, it turns into a gel and we will use electricity to pull the proteins through the gel so the entire process is called polyacrylamide gel electrophoresis (PAGE). A polyacrylamide gel is not solid but is made of a laberynth of tunnels through a meshwork of fibers. Now If all the proteins enter the gel at the same time and have the same force pulling them towards the other end and hence the ones with greater molecular weight tend to move slowly that the ones that are light weight. The bands are then calibrated with the help of a known molecular marker in order to calibrate the bands after the staining process by comassie brilliant blue or silver staining.

REAGENTS & CHEMICALS USED:

1. Upper gel buffer
2. Lower gel buffer
3. 30% stock acrylamide
4. TEMED
5. Ammonium persulphate (APS)
6. ddH_2O
7. Glass plate, combs & spacers, assembly (for electric field)
8. Running buffer & sample buffer
9. Destainer

PROCEDURE

1. The glass plates were first taped with the help of brown tape together along with the spacers.
2. The taped plates are often sealed with the help of a plug gel in order to prevent any leakage of lower gel from the bottom of the plate
3. Then the lower gel or the resolving gel (12.5%) is prepared by mixing the various constituents.
4. This solution was then poured into the glass plate upto a certain level & then a small amount of water was put on top so as to prevent oxidation of the gel with air.
5. After that the upper gel or the stacking gel (4.4%) was prepared by mixing the various constituents in the following amounts:

a. Acrylamide – 1.2 ml
b. ddH_2O – 4.4 ml
c. TEMED – 25 µl
d. APS – 25 µl
e. Upper gel buffer (UGB) – 1.9 ml

6. The comb is then placed & after the gel gets polymerized, the comb was removed & the gel was placed in the assembly.

7. The assembly was filled with running buffer & the samples were then loaded into the wells, with the help of loading tips after mixing them with 10µL of sample buffer & heating for 2 minutes.

8. The gel was run till the samples reached the end of the gel.

9. The tape is then carefully removed so as not to damage the plates.

10. The gel is then stained in Comassie Brilliant blue dye for 45 minutes; it was then destained with the help of destainer.

11. The gel was then kept on gel rocker and destained overnight.

12. The destainer was replaced in the morning and then replaced every hour for 24 hours.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 – Outcome of Qualitative test of Uric Acid:-

The qualitative estimation of uric acid indicated the presence of Uric Acid in the given specimen.

3.2 – Outcome of Qualitative Test of Oxalate:-

The uric acid stone sample was also checked for the presence of oxalate and the same indicated its presence too in the given sample.

3.3 – Outcome of Quantitative test for Calcium:-

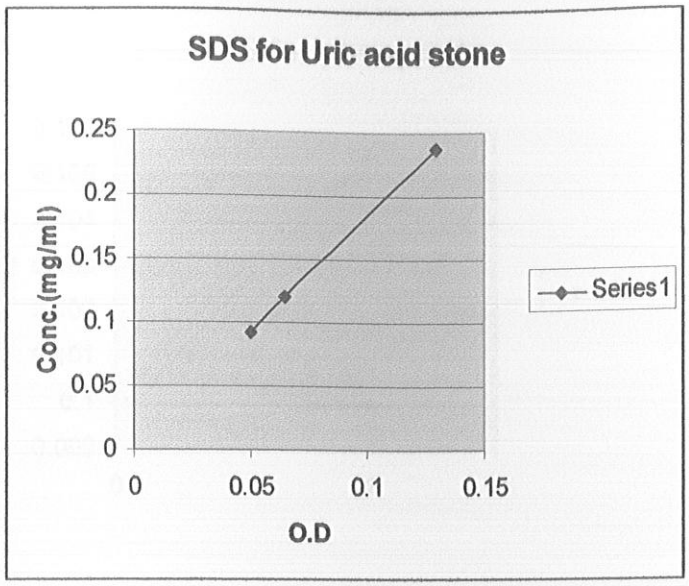
The Apatite stone sample tested for the concentration of calcium revealed that against a standard of 2 mM the concentration was 1.952 mg/ml

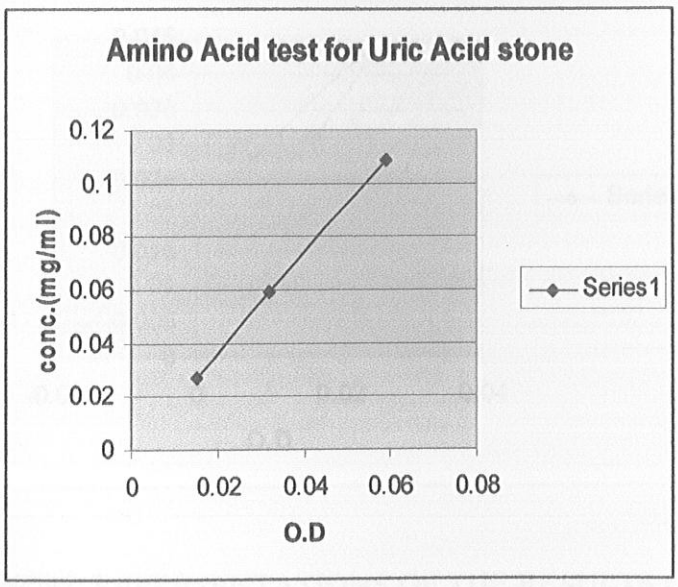
3.4 – Outcome of Quantitative test of Phosphate:-

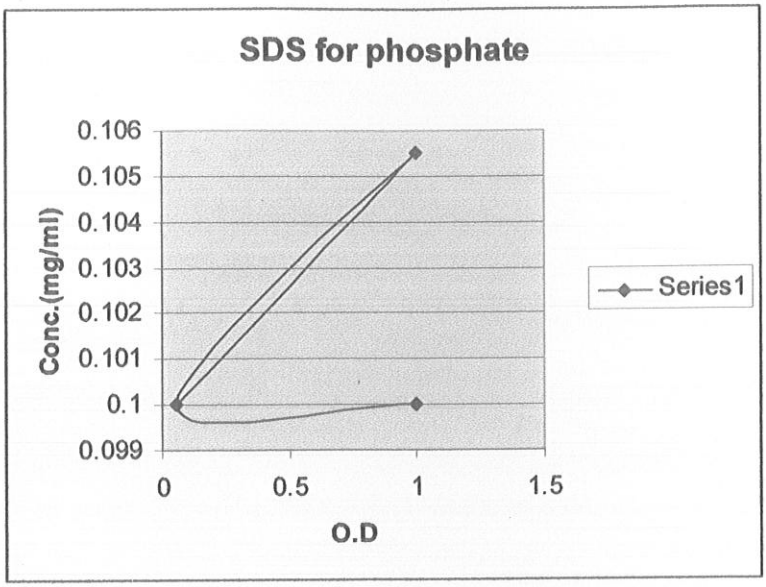
The Apatite Stone sample was also analyzed for the content of phosphate in it. The four standards that were employed (1Mm, 2Mm, 6Mm, 10Mm), gave the concentrations of phosphates as 2.156 mg/ml, 2.888 mg/ml, 3.259 mg/ml, 3.577 mg/ml respectively.

3.5– Outcome of Concentration of proteins in the extracts obtained by SDS and Acetic Acid Methods:-

The following are graphical representations of the concentrations of the extracts obtained from the two extraction methods employed to extract matrix proteins.


SDS for Uric acid stone
Conc.(mg/ml)
0.25
0.2
0.15
0.1
0.05
0
0
0.05
0.1
0.15
O.D
Series1


Amino Acid test for Uric Acid stone
conc.(mg/ml)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.02
0.04
0.06
0.08
O.D
Series1



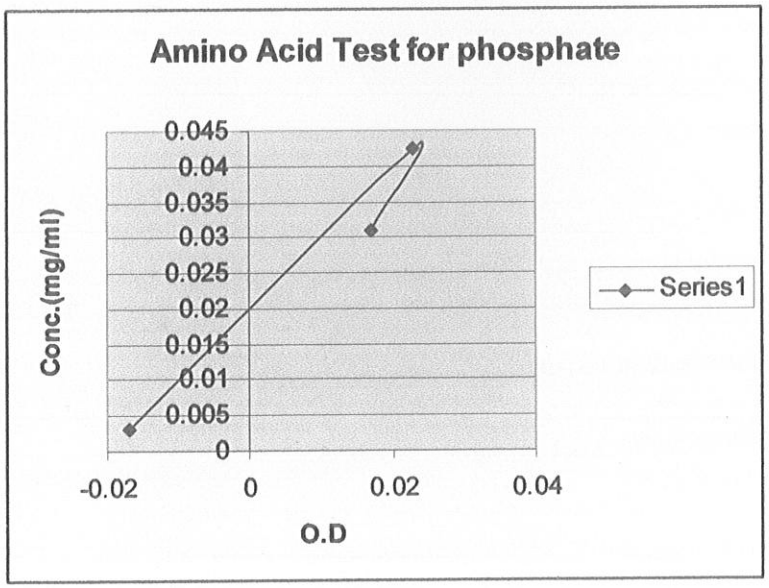


FIG – 3 GRAPHICAL REPRESENTATIONS OF THE RESULTS OF PROTEIN ESTIMATION BY LOWRY'S METHOD AFTER EXTRACTION OF MATRIX PROTEINS.

DISCUSSION

The stone samples obtained from different sources showed similar chemical composition when the different qualitative and quantitative tests were performed, though it was seen that uric acid uroliths can have deposits of phosphates along with uric acid. Also SDS buffer method was more sensitive than the acetic acid method. (James C. Williams Jr, Chad A. Zarse, Molly E. Jackson, Frank A. Witzmann & James A. McAteer, 2006),

The optical density values for apatite obtained by Lowry's colorimetric method (Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. , 1951) were ambiguous as the crude extract was found to have less concentration of protein than the individual extracts containing proteins less than and greater than 5 KDa. This error can be attributed to the fact that the there should not be much time difference when we finish the Lowry's estimation and proceed to measure Optical Density.

Silver staining of the gel in the SDS-PAGE analysis was done to enhance the bands on the gel which were not very clearly visible after staining with Commassie Brilliant Blue dye. This allows a more enhanced image of the gel which can then be analyzed on gel dock.

CONCLUSION

Urolithiasis, as described earlier is a disease which is quiet prevalent in our country and has not yet been fully understood as to what factors play a crucial role in its incidence. In order to achieve this competency it is reasonable to assume that proteins constituting the matrix of these uroliths play a crucial role in its formation. The present work was aimed at extracting these proteins and performing their SDS PAGE analysis so as to support the hypothesis to a greater extent and at the same time to gain an insight into the chemical structure of the specimen as well. Though at this stage it is difficult to conclude as to whether the matrix proteins are actually responsible in the formation of these uroliths or not, it can be clearly stated that

1. Uric acid stones can be made up of more than just uric acid alone, though it being the major constituent, such as phosphate etc.
2. Out of the Extraction methods that were employed for extraction of matrix proteins, SDS reducing buffer is more efficient and extracted more protein that the Acetic Acid method.

Further studies related to checking the specific activity of the biomolecules present in the stone matrix shall be done so as to see whether they influence the formation of these stones and to what extent.

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