CHAPTER - III

MATERIALS
AND
METHODS
3. MATERIALS AND METHODS

The prevalence of skeletal fluorosis in the Chittoor district of Andhra Pradesh and the assessment of fluoride, bone mineral, hormonal and nutritional levels in males and females residing in fluorotic and non-fluorotic areas have been taken as the object of the present study.

3.1 Geographical details:

Andhra Pradesh is situated in India between 12°41' and 22°N latitude and 77° and 84°40'E longitude with a geographical area of 2,75,000 sq. km (Directorate of Economics and Statistics, Hyderabad A.P.). It comprises of 23 districts and a total population of 7,57,27,000 (Director, Census operations, Hyderabad, A.P., 2001). Chittoor District in the Andhra Pradesh is situated between 12°37' to 14°8' N latitude and 78°33' to 79°55' E longitude, with a geographical area of 15,152 sq. km, comprising of 66 mandals with a total population of 37,45,875. The present study is carried out from Tirupati in the Chittoor District which is situated between Lat.13.40° N, Long. 77.2° E.

3.2 Survey:

A survey was conducted to collect drinking water samples in the urban and rural areas in and around Tirupati region and to check the fluoride levels using an ion-sensitive electrode. The preliminary data pertaining to water fluoride levels were obtained from the Water works department, Tirupati, Andhra Pradesh.

3.2.1 Selection of the area:

Peddatippasamudram and KV Bommaraju Puram are the two mandals afflicted with fluorosis in the Chittoor district of Andhra Pradesh, where the fluoride contents in drinking water range from >1.0 to 2.0 ppm as per the records available with the rural water works department, Tirupati, Chittoor district of Andhra Pradesh.

Based on this realities, drinking water samples were collected at a random from 10 villages in each of the above two Mandals. These water samples were analyzed for fluoride levels using ORION fluoride ion-electrode. With the
Map 3: Study areas of selected mandals and villages in Chittoor district
preliminary studies of water fluoride levels and distance from the place for carrying out experiments in Tirupati, four villages were further selected. They are Sandramakulapalli, Peddatippasamudram (alias PTM), Kandluru and Adaram, wherein water fluoride contents range from >1.0 to 2.18 ppm. Further, two control areas in the neighbouring Mandals were selected which are non-fluorosis areas. They are: Sathyavedu with water fluoride levels ranging between 0.03 – 0.74 ppm (in the Satyavedu Mandal) and Rajanagaram with water fluoride levels ranging between 0.21 – 0.45 ppm (in Pichatur mandal). The location of the selected mandals and villages are shown in Map - 3.

The total population surveyed in these four fluorosis afflicted villages was 3,480 of which 1,827 were males and 1,653 were females. Details of water fluoride levels in these villages in proportion to the population and their location to the equator are shown in Table - 6.

The total population surveyed in the non-fluorosis villages was 14,941 of which 7,398 were males and 7,543 were females. Details of water fluoride levels in these villages in proportion to the population and their location to the equator are shown in Table - 7.

3.2.2 Technique adopted for observing influence of fluorosis:

The prevalence of dental and skeletal fluorosis were studied among a total population of 3,480 individuals in the fluorosis afflicted areas in the age group ranging from 1 month – 90 years old. The World Health Organization criteria (1997) were used to grade the symptoms and signs of dental and skeletal fluorosis. The prevalence of dental and skeletal fluorosis (grades) was computed according to the WHO criteria.

3.3 Selection of subjects:

This is a cross-sectional study, as the disease and exposure status were measured simultaneously in a given population. This type of data is used to assess the prevalence of acute or chronic conditions in population. Like other cross-sectional analysis, it pertains to how variables affect each other at the same time.
Table 6: Statement showing the water fluoride levels in fluorosis afflicted areas in proportion to the population and exact location of its place to the equator.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Villages</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Total population</th>
<th>Males</th>
<th>Females</th>
<th>Water fluoride levels (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sandramakulapalli</td>
<td>13.40°N</td>
<td>78.14°E</td>
<td>578</td>
<td>313</td>
<td>265</td>
<td>1.4-1.76</td>
</tr>
<tr>
<td>2</td>
<td>Peddatippasamudram</td>
<td>13.43°N</td>
<td>78.13°E</td>
<td>821</td>
<td>441</td>
<td>380</td>
<td>1.88-1.97</td>
</tr>
<tr>
<td>3</td>
<td>Adaram</td>
<td>13.37°N</td>
<td>79.47°E</td>
<td>1122</td>
<td>567</td>
<td>555</td>
<td>1.38-1.7</td>
</tr>
<tr>
<td>4</td>
<td>Kandluru</td>
<td>13.36°N</td>
<td>78.47°E</td>
<td>959</td>
<td>506</td>
<td>453</td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>3480</td>
<td>1827</td>
<td>1653</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Statement showing the water fluoride levels in areas not afflicted with fluorosis in proportion to the population and exact location of its place to the equator.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Villages</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Total population</th>
<th>Males</th>
<th>Females</th>
<th>Water fluoride levels (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Satyavedu</td>
<td>13.26°N</td>
<td>79.57°E</td>
<td>14,172</td>
<td>7035</td>
<td>7137</td>
<td>0.03 - 0.74</td>
</tr>
<tr>
<td>2</td>
<td>Rajanagaram</td>
<td>13.27°N</td>
<td>79.33°E</td>
<td>769</td>
<td>363</td>
<td>406</td>
<td>0.21 - 0.45</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>14,941</td>
<td>7398</td>
<td>7543</td>
<td></td>
</tr>
</tbody>
</table>
Three hundred and twenty five adults (10 per cent of population) with an age range of >15 to ≤70 years of age of both sexes (Males - 158 and Females – 167) were selected randomly. Two hundred and twenty eight non-fluorotic subjects (Males - 97 and Females – 131) who matched well in demographic features were selected randomly from the neighboring non-fluorotic areas.

Three hundred and twenty five fluorotic subjects were divided based on the drinking water fluoride levels. Two hundred and forty two subjects residing in Sandramakulapalli, Peddatimmasamudram and Adaram where drinking water fluoride levels range from 1.3 to 1.88 ppm were categorized into “Fluorotic group I”, 83 fluorotic adults residing in Kandluru where drinking water fluoride level was 2.18 ppm were categorized into “Fluorotic group II”.

Two hundred and twenty five non-fluorotic subjects residing in Satyavedu and Rajanagaram where drinking water fluoride levels range from 0.03 to 0.74 were categorized into non-fluorotic group.

3.4 Demographic features:

A door-to-door survey was carried out using an interview method in order to collect information on the demographic features of the fluorotic groups I and II and NFLG. Details regarding family type and size, income, caste and education were collected.

3.5 Collection of samples:

Blood and urine samples were collected from all the selected 325 fluorotic and 228 non-fluorotic subjects.

3.5.1 Blood Samples:

All the selected 325 fluorotic and 228 non-fluorotic subjects were asked to remain fasting on the day of collection of blood sample. Ten milliliters of blood samples were collected from the most accessible peripheral vein between 08.00 to 09.00 hours in the fasting state without applying tourniquet for the estimation of
Transportation to the study villages for collection of blood and urine samples

Collection of blood samples from the study population
Storage and transport of blood samples in cool packs, immediately after collection
serum fluoride, calcium, phosphorus, creatinine, protein, albumin, alkaline phosphatase, tartrate resistant acid phosphatase and on ice for parathyroid hormone and 25-hydroxy vitamin D. They were transported under cool packs. Sera were separated in refrigerated centrifuge at 2000 rpm for 10 min at 4°C and stored at –20°C for further analysis.

3.5.2 Urine samples:

All the selected 325 fluorotic subjects and 228 non-fluorotic subjects were requested to collect first urine samples of the day (mid-stream) in collection bottles provided to them. After collecting these samples 1 mL toluene, a preservative was added to each bottle and kept in refrigerator until further analysis of urinary fluoride, calcium, phosphorus, creatinine and free hydroxyproline.

3.5.3 Drinking water samples:

The samples from all the sources of water supply for the families of fluorotics and non-fluorotics used for drinking and cooking purpose were collected in polyethylene bottles (since fluoride may be adsorbed by glass, collections were done in plastic containers). All the collection bottles prior to use were washed with 50 per cent nitric acid and rinsed thoroughly with deionised water.

3.5.4 Geochemical analysis of water samples:

Drinking water samples collected were analyzed chemically for pH, total hardness, alkalinity as carbonate, calcium, chloride, bicarbonate, potassium and sodium following standard American Public Health Association procedures (1985).

3.5.5 Food Samples:

Samples of most commonly consumed food such as rice, ragi, groundnuts and tomato and others which were cultivated in the local areas were collected from fluorotic and non-fluorotic areas. These samples were sun dried, powdered, and ashed by the standard method (Oser, 1979) and then used for fluoride estimation. The details of the ashing procedure are given below.
Procedure for ashing:

Five grams of sample by weight was taken. The samples of grains were dried, powdered, and placed them in a clean silica crucible. The sample was ignited in the silica crucible gently over a small flame until thoroughly charred. The silica crucible was placed in a muffle furnace and heated at 555°C to dull redness for 2-3 hours. Switched off muffle furnace and allowed to cool overnight. Added 5 mL of conc. HCl to the ash formed and dissolved. The contents of the silica crucible were transferred carefully into 50 mL volumetric flask, using a funnel, rinsed the dish for 5 times with distilled water. The volume was made up with distilled water.

3.6 Determination of fluoride content:

Fluoride content of drinking water samples and different food samples grown and consumed were estimated by fluoride ion electrode method (ORION 740). The details of the procedure are given below.

Estimation of fluoride content in samples:

The fluoride content in samples was estimated using a fluoride ion-sensitive electrode.

Apparatus:

A complete electrode-measuring device consists of ORION combination of F⁻ electrodes, which are connected to an ORION electrode Model 740. Each electrode potential is transmitted through the electrode switch to an ORION digital pH/ mV meter. This mV meter is in turn connected to a recorder.

Reagents:

The required standard solutions were prepared from an ORION 100 ppm aqueous fluoride ion stock solution. ORION TISAB III (Ammonium acetate, 384 gm /L; HCl (11.6 M) 213.5 mL; CDTA (1.2 cyclohexylenedinitrilotetra acetic acid) 19.8 g/L; cresol red 0.07 g/L; the pH was adjusted to 5.0-5.2 using HCl or NH₄ OH as per the conditions.
Procedure:

Standards: Different fluoride concentrations (ppm) $10^{-2}$, $10^{-1}$, $10^1$, $10^2$ were prepared with $\text{F}^-$ stock solution. Different standards were diluted with double distilled water. Nine mL of standard + 1 mL of TISAB were added in flat-bottomed polystyrene tubes and mixed well. The electrodes were cleaned by double distilled water and dried. Electrodes were dipped into standard tubes and mixed well. Using standards, the slope and measuring range of the electrodes and linearity of the calibrations were checked in the range from 0.01 ppm to 100 ppm. The ideal standard slope was taken as 57.0 mV for the estimation of samples.

Similar procedure was followed for the estimation of samples, by adding the sample in the place of standards and read the ppm.

3.7 Assessment of Fluoride Status for Fluorotics and Non-Fluorotics:

3.7.1 Estimation of serum and urine fluoride contents:

Fluoride content was estimated by fluoride ion selective electrode method.

3.7.2 Estimation of fluoride intake:

The fluoride intake of the fluorotic and non-fluorotic subjects was calculated from the estimated fluoride content of different foods and drinking water samples collected along with the quantity of food intake.

3.7.3 Estimation of dietary and nutritional status:

The most popular method for obtaining information about dietary intake is 24 hr recall method for 7 days. In this recall method, oral questionnaire and a set of standard cups were used. Before undertaking dietary assessment a conversion table for raw and cooked food weights and measures was prepared, which was used in estimating the intake of cooked foods consumed by the subjects and their conversion into raw food weights.

Another independent observer conducted a re-check of the diet survey at a random. The dietary intake of calcium, phosphorous and phytates were documented by recalling the diet consumed in the previous 5 to 7 days. From the raw weights, the
calcium and phosphorous intakes were calculated using the published food composition table detailing the nutritive value of Indian foods (Gopalan et al., 1998).

The amount of food consumed was expressed in terms of cups. Food items like idly, roti etc. were expressed in size and numbers. The cooked food consumed was converted into raw weights. Based on this, the raw food intake per day for 3 to 5 days and the mean intake was calculated and compared with the recommended daily allowances (ICMR, 1981).

3.7.4 Energy and nutrient intakes:

Based on the mean intake of foods per day, energy, protein, fat, iron, calcium, vitamin A, vitamin B₁ and vitamin C intake was calculated individually using the nutritive value of Indian foods (Gopalan et al., 2000). These intakes were compared with recommended dietary allowances (Gopalan et al., 2000).

3.8 Anthropometric measurements:

Nutrititional anthropometry is concerned with measurements of the physical dimensions and gross composition of the human body. These body measurements can give valuable information concerning certain types of malnutrition in which body size and gross body composition are affected (Jelliffe, 1966).

Anthropometric measurements used for assessing nutritional status were height, weight and BMI, waist and hip circumferences in adults. The procedures followed were as per those given by Jelliffe (1966).

3.8.1 Height:

Standing height was measured to the nearest 1 cm with the subject wearing no foot wear, standing erect on a horizontal surface with heels together, the shoulders relaxed and arms by the sides. The subjects were made to look straight. Standing on the left side of the subject, the height was measured and recorded in centimeters.
3.8.2 Weight:

Weight has been observed to be a sensitive indicator of growth failure and current nutritional status of the individual. Krups spring balance was used for this purpose. This weighing machine was placed on an even floor and the subjects after instructing to wear light clothing were made to stand at the center of the foot rest plate, with head erect. The weight was recorded in kilograms without any parallax error.

BMI was calculated using the formula: \( \text{BMI} = \frac{\text{Weight (kg)}}{\text{height (m}^2)} \). The adults were classified into different nutritional grades based on BMI.

**Anthropometric classification for adults is as follows:**

<table>
<thead>
<tr>
<th>BMI Class</th>
<th>Grades</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 18.5</td>
<td>Under weight</td>
</tr>
<tr>
<td>18.0 – 24.9</td>
<td>Normal</td>
</tr>
<tr>
<td>25.0 – 29.9</td>
<td>Over weight</td>
</tr>
<tr>
<td>30.0 – 34.9</td>
<td>Obese, Class I</td>
</tr>
<tr>
<td>35.0 – 39.9</td>
<td>Obese, Class II</td>
</tr>
<tr>
<td>≥ 40</td>
<td>Obese, Class III</td>
</tr>
</tbody>
</table>

BMI = Body mass index (kg/m\(^2\)).

(Simone Lemieux et al., 2004)

3.8.3 Waist:

Measurement was recorded midway between the inferior margin of the last rib and the crest of the ilium in a horizontal plane. It was recorded in centimeters.

3.8.4 Hip:

The hip measurement was recorded in centimeters by fitting the tape round the widest part of the trochanters.
Waist and hip ratio was calculated.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference</td>
<td>Males: ≥ 85 cm</td>
</tr>
<tr>
<td></td>
<td>Females: ≥ 80 cm</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>Males: ≥ 89 cm</td>
</tr>
<tr>
<td></td>
<td>Females: ≥ 81 cm</td>
</tr>
</tbody>
</table>

(Chamukuttan Snehalatha et al., 2003)

3.9 Skin-fold thickness measurements and techniques:

A simpler procedure for estimating body fat is the skin fold thickness, particularly in adults. A pinch of skin is precisely measured by Harpenden skin fold caliper at several standardized points on the body to determine the subcutaneous fat layer thickness. These measurements are converted to an estimated body fat percentage by an equation. Skin fold thickness provides the best field information about the level of fatness of an individual, as persons suffering from chronic energy deficiency would have minimal quantities of fat in the body and, therefore, very small amounts of subcutaneous fat is seen. But, a very large amount of subcutaneous fat is seen in obese persons, since the fat is stored (Durnin and Womersely, 1974).

In the present study four such measurements at biceps, triceps, sub scapular and supra iliac were taken. These four sites were found to be the best established sites, as proposed by Durnin and Rahman (1967) and developed later by Durnin and Womersely (1974). The skin fold measurements were measured with Harpenden skin fold caliper with an accuracy of ± 0.5 mm.

3.9.1 Biceps skin fold:

The measurement was taken over the center of the biceps of the muscle of the left upper arm. The arm of the subject was in a relaxed state and loosely hung. The skin fold was lifted about a centimeters below the midpoint along the long axis of the muscles. The caliper, in a horizontal position was allowed to compress the skin fold about the point where the thumb and finger grasps the skin folds. Biceps skin fold is measured as the thickness of a vertical fold in the front of the upper left arm, directly
above the center of the cubical fossa at the same level as the triceps skin fold (Weiner and Lourie, 1969).

3.9.2 Triceps skin fold:

As the fat deposition in the upper arm is not uniform in thickness, the site selected was the left mid upper arm between the tip of acromial process of the scapula and the olecranon process of the ulna. The measurement was made with the elbow slightly fixed and the site on the triceps was marked. The thickness of the fat fold was measured with the hand hanging freely at the side. The fat fold thickness was noted to the nearest 0.5 mm. Triceps skin fold was measured in millimeters at the midpoint of the back of the upper left arm (Weiner and Lourie, 1969).

3.9.3 Sub scapular skin fold:

The skin fold was taken on the diagonal line coming from the vertebral border to between 1 and 2 cm from the inferior angle of the scapulae. (A diagonal fold about 1 to 2 cm below the point of the shoulder blade and 1-2 cm toward the arm). The caliper was used about 1 cm laterally downwards from this point. Sub scapular skin fold was measured just below, with the shoulder and left arm relaxed. The skin fold was angled 45° from horizontal, in the same direction as in the inner border of the scapula in medially upward and laterally downward according to Jette (1981) and Lohman et al., (1988). It was recorded in mm.

3.9.4 Supra iliac skin fold:

The skin fold was lifted just above the crest of the ilium. The fold was lifted to follow the natural diagonal line at this point. Supra iliac skin fold measured in the mid auxiliary line immediately superior to the iliac crest. The skin fold was picked up obliquely just posterior to the mid auxiliary line and parallel to the cleavage lines of the skin according to Lohman et al., (1988). It was recorded in mm.

Sum of skin fold thickness (SSFT) mm = Skin fold thickness of Biceps + Triceps + Sub scapular + Supra iliac skin folds
3.9.5 Body Density:

Behnke (1969) pioneered the measurement of body density as an index of obesity. The body density can be calculated with the help of age and sex matched regression equation (Durnin and Womersley, 1974) using SSFT.

\[
\text{Body density (Kg/m}^3\text{): } c - (m \times \log \text{of sum of SFTs})
\]

where the values of 'c' and 'm' were taken from the tables of linear regression equation for the estimations of body density are presented below.

Linear regression equation for the estimation of body density \( \times 10^3 \) (kg/m\(^3\)) from the logarithm of the sum of skin fold thickness: 
\[
\text{Density} = c - m \times \log \text{skin fold thickness}
\]

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c</td>
<td>M</td>
</tr>
<tr>
<td>17-19</td>
<td>1.1620</td>
<td>0.0630</td>
</tr>
<tr>
<td>20-29</td>
<td>1.1631</td>
<td>0.0632</td>
</tr>
<tr>
<td>30-39</td>
<td>1.1422</td>
<td>0.0544</td>
</tr>
<tr>
<td>40-49</td>
<td>1.1620</td>
<td>0.0700</td>
</tr>
<tr>
<td>50+</td>
<td>1.1715</td>
<td>0.0779</td>
</tr>
</tbody>
</table>

3.9.6 Body fat:

Body fat was calculated as body fat percent and later computed to body fat in kilograms. These two are calculated as follows:

**Calculation of Body fat:**

The amount of fat present in the body was calculated from the fat percent. The formula used to calculate body fat in kilograms is

\[
\text{Body fat (Kg)} = \frac{\text{Body weight (Kg)} \times \text{Fat per cent}}{100}
\]

**Calculation of Body fat percent:**

Although the densitometric method has been used as the most accurate method of determining percent body fat, the formulae that translate body density to percent body fat assume a constant value for the density of lean tissue for all individuals.
Calculations for the per cent body fat were based on equation given by Siri (1956).

\[
\text{Fat percent} = \left(\frac{4.95}{\text{Body density} - 4.5}\right) \times 100
\]

3.9.7 Lean body mass (LBM):

The lean body mass is composed approximately 72 per cent water, 20 per cent protein, 1 per cent carbohydrate and 7 per cent minerals. The variability is less when compared to body fat. Neutral fat does not bind water or electrolytes; consequently, the measurement of total body water or total body potassium offers a means for estimating non-fat component of the body. With the body weight of the subject and the body fat worked out earlier, the Durnin and Rahman formula is used for the calculation of LBM.

\[
\text{LBM (Kg): Body weight (Kg) – Body fat (Kg)}
\]

3.10 Assessment of bone mineral parameters for fluorotics and non-fluorotics:

3.10.1 Estimation of serum calcium:

Serum calcium was estimated by the method of Clark and Collip (1925).

Reagents:

1. Ammonium oxalate solution, 4 per cent
2. Dilute ammonia solution: 2 mL of liquor ammonia was dissolved in 98 mL of distilled water.
3. 1 N H₂SO₄
4. 0.01 N Potassium permanganate solution
5. 0.01 N Oxalic acid: 0.67g of sodium oxalate is dissolved in distilled water, 5.0 mL concentrated H₂SO₄ is added and solution made up to 1 lit.

Procedure:

Two mL of sample was taken into a 15 mL centrifuge tube. Added 2 mL of distilled water and 1 mL of 4 per cent ammonium oxalate solution and mixed thoroughly by holding the tube at the mouth and giving it a circular motion by tapping the lower end. The tube is allowed to stand for 30 minutes. Mixed the contents again and centrifuged for 10 min at 1500 RPM. Poured off the
supernatant liquid carefully and washed the precipitate with dilute ammonia solution by blowing with a pipette directly on to the precipitate in the tube. Placed the tube in hot water bath for 1 to 2 minutes and then titrated (using micro burette) with 0.01 N KMnO4 till definite pink colour persists for at least a minute.

Serum calcium was expressed as mg/dL.

3.10.2 Estimation of serum inorganic phosphorus:

Serum phosphorus was estimated by the method of Fiske and Subbarow (1925).

Reagents:

1. Trichloroacetic acid, 10 per cent (w/v): Dissolved 10 grams in water and make up to 100 mL.

2. Sulphuric acid, 10 N: Added 450 mL of concentrated sulphuric acid slowly while cooling to 1,300 mL of distilled water.

3. Molybdate I: 2.5 per cent ammonium molybdate in 5 N sulphuric acid.
   Dissolved 25 grams of the salt in about 200 mL of water and transferred to a litre flask containing 500 mL of 10 N sulphuric acid. Diluted it to 1 lit with water.

4. Molybdate II: 2.5 per cent ammonium molybdate in 3 N sulphuric acid. Made as molybdate I except that 300 mL of the acid is used instead of 500 mL.

5. Sodium bisulphate, 15 per cent solution: Dissolved 15 g of sodium bisulphate in 100 mL of water.

6. Sodium sulphite, 20 per cent: Dissolved 20 g of sodium sulphite in 100 mL of water.

7. 0.25 per cent 1,2,4-aminonaphthosulphonic acid: Added 0.5 g of dry powder to 195 mL of 15 per cent sodium bisulphate and 5 mL of the 20 per cent sodium sulphite. Stoppard and shaked well until it was dissolved.

8. Standard phosphate solution: Dissolved 0.351 g of potassium dihydrogen phosphate in water in a litre flask. Added 10 mL of 10 N sulphuric acid and made to the mark with water. 5 mL contain 0.4 mg phosphorus.

9. Dilute standard for use: Diluted the stock standard 1 to 10. One mL contains 0.008 mg phosphorus.
Procedure:

Four mL of 10 per cent trichloroacetic acid was taken into a suitable test tube. Added 1 mL of serum and mixed well. Added 1.0 mL of molybdate II reagent and mixed. Then added 0.4 mL of the aminonaphthosulphonic acid and made to 10 mL with distilled water. 5 mL of the standard phosphate solution was taken into a suitable test tube. Added 1 mL of molybdate I reagent and mixed. Then added 0.4 mL of the aminonaphthosulphonic acid and made to 10 mL with distilled water. After standing 5 minutes read the absorbance, using a red filter at 680 nm.

Serum phosphorus was expressed as mg/dL.

3.10.3 Estimation of serum alkaline phosphatase:

Serum alkaline phosphatase was estimated by the method of King and Armstrong, (1934).

Reagents:

1. Disodium phenyl phosphate, 0.01 M: Dissolved 1.09g in water and made to 500 mL. Brought quickly to the boil. Cooled and added a little chloroform and kept in the refrigerator.

2. Sodium carbonate-sodium bicarbonate buffer, 0.1 M: Dissolved 3.18g of anhydrous sodium carbonate and 1.68g of sodium bicarbonate in water and made up to 500 mL.

3. Buffer substrate for use: Prepared by mixing equal volumes of solutions 1 and 2. This has a pH of 10.

4. Standard phenol stock solution: One hundred mg of phenol per 100 mL of solution. Dissolved 1 gram of pure crystalline phenol in 0.1N HCl and made to 1 L with the acid.

5. Dilute phenol standard for use: Diluted the stock standard 1 in 10 to obtain a standard solution containing 10 mg phenol per 100 mL of solution.

6. Sodium hydroxide 0.5N

7. Sodium bicarbonate 0.5M

8. 4-amino-antipyrine, 0.6 percent in water
9. Potassium ferricyanide, 2.4 grams per 100 mL in water

Procedure:

Two mL of buffer substrate was added into each of two test tubes and placed in a water bath at 37°C for a few minutes. To one tube (the test) added 0.1 mL of serum and incubated the both the tubes for exactly 15 minutes. Removed the tubes from the bath and added 0.8 mL of 0.5 N sodium hydroxide and 1.2 mL of 0.5 M sodium bicarbonate to both tubes and then 0.1 mL of serum to the second tube (the blank). To both tubes added 1 mL of amino-antipyrine reagent and 1.0 mL of potassium ferricyanide. For the standard 1.1 mL of buffer was taken and 1.0 mL of phenol standard containing 0.01 mg of phenol and for the standard black, 1.1 mL of buffer and 1.0 mL of water. To both then added sodium hydroxide, bicarbonate, amino-antipyrine and ferricyanide as above. Read the absorbance at 520 nm.

Serum alkaline phosphatase was expressed as IU/L.

3.10.4 Estimation of serum creatinine:

Serum creatinine was estimated by the method of Brod and Sirota (1948).

Reagents:

1. 5 gm of sodium tungstate (Na₂WO₄.2H₂O) was dissolved in 100 ml of distilled water.
2. 2/3 N sulphuric acid
3. Picric acid: 9.16 gm of picric acid was dissolved in 1 lit of distilled water.
4. 0.75 N Sodium hydroxide
5. Creatinine stock standard: 100 mg of creatinine was dissolved in 10 mL of 0.1 N hydrochloric acid.
6. Creatinine working standard: 5 mL of stock was made up to 100 mL with distilled water.

Procedure:

Two mL of serum was dissolved in 2 mL of distilled water and precipitated the proteins by adding 2 mL of 5 per cent sodium tungstate and 2
mL of 2/3 N sulphuric acid, and stand it for 10 min and filtered. 3 mL of filtrate was pipetted and to that 1 mL of picric acid and 1 mL of 0.75 N sodium hydroxide were added. This was incubated for 15 min and colour was read at 540 nm. A blank was maintained with distilled water. A series of test tubes containing hierarchy of concentrations of creatinine were also maintained for standard curve.

Serum creatinine was expressed as mg/dL.

3.10.5 Estimation of serum tartrate resistant acid phosphatase:
Serum tartrate resistant acid phosphatase was estimated by the method of Otto et al., (1946).

Reagents:
1. Acid buffer (0.09 mol/L): 18.91g of citric acid monohydrate were mixed in 500 mL of double distilled water placed in a 1-litre volumetric flask. Then, 180 mL of 1N sodium hydroxide (NaOH) and 100 mL of 0.01N hydrochloric acid (HCl) were added. The pH was checked and adjusted to 4.85 with the acid or alkali and then diluted to 1000 mL with double distilled water.

2. Tartrate-Citrate Buffer (pH 4.85) 0.09 mole citrate and 0.04 mole tartrate/L: 1.5g of L(+) tartaric acid was dissolved in 250 mL of citrate buffer.

3. Stock substrate of PNPP: (4mg/mL or 15.2 mmol/mL or 15.2 μmol/L): 0.4g of p-nitrophenyl disodium phosphate was dissolved in 100 mL of double distilled water.

4. Working buffered substrate:
   a. Working citrate substrate: Citrate buffer and stock substrate were mixed in equal volumes.
   b. Working tartrate-citrate substrate: Tartrate-citrate buffer and stock substrate solutions were mixed in equal volumes.

5. Standard solution of p-nitrophenol:
   a. Stock standard (1mmol/L): 139.1mg of high purity PNP was dissolved in double distilled water to make 1000 mL of solution in a 1-litre Volumetric flask. This solution was kept in dark for stability purpose.
b. PNP working standard (0.04 mmol/L): 1.0 mL of the stock standard was pipetted into a 25 mL volumetric flask and diluted the volume with 0.05 N NaOH solution. It was mixed thoroughly. This was prepared freshly for the test.

6. Sodium hydroxide solutions:
   a. 1N NaOH: 40g sodium hydroxide was dissolved in about 800 mL of double distilled water placed in a 1-litre volumetric flask and diluted the solution to 1000 mL volume with double distilled water.
   b. Other strengths (0.2N, 0.1N and 0.05N): 1N sodium hydroxide was diluted 1:5, 1:10 and 1:20 for getting 0.2N, 0.1N and 0.05N sodium hydroxide solutions.

Procedure:

Two sets of tubes were taken. The first set of tubes was labeled as citrate substrate tubes and the second set of tubes was labeled as tartrate-citrate tubes. 1.0 mL of working citrate substrate was added to citrate substrate blank and sample tubes. One mL of working tartrate-citrate substrate was added to tartrate citrate tube. All the tubes were placed in a water bath (37°C) for five minutes in order to equilibrate. Test serum of 0.2 mL was added to sample tubes. The contents were mixed by swirling the tubes rapidly and replace in the water bath. All the tubes were incubated exactly for 30 minutes at 37°C. At the end of 30 minutes, 4.0 mL of 0.1 N NaOH was added to all tubes to stop the enzyme reaction. Then all the tubes were removed from the water bath, and 0.2 mL of double distilled water was added to the citrate-substrate blank tube and tartrate-citrate blank tube. The contents of all tubes were mixed and absorbances of all solutions were read at 410 nm against double distilled water as an instrument blank.

Serum tartrate resistant acid phosphatase was expressed as U/L.
3.10.6 Estimation of urinary calcium:

Urinary calcium was estimated by the method of Clark and Collip, 1925.

Reagents:

1. Bromophenol blue indicator: 0.04g of Bromophenol blue was dissolved in
   0.6 mL of 0.1M sodium hydroxide and make up to 100 mL.
2. 0.5 N NaOH
3. Saturated ammonium oxalate solution
4. Dilute ammonia solution: 2.0 mL of liquor ammonia was dissolved in 98 mL
   of distilled water.
5. 1 N H₂SO₄
6. 0.01 N Potassium permanganate solution
7. 0.01 N Oxalic acid: 0.67g of sodium oxalate is dissolved in distilled water,
   5.0 mL concentrated H₂SO₄ is added and solution made up to 1 lit.

Procedure:

One mL of urine sample was taken in conical centrifuge tube. Treated it
with two drops of bromophenol blue indicator and 1.0 mL of saturated
ammonium oxalate solution. Added 0.5 N NaOH, drop wise until the solution
turned to blue. The contents were mixed thoroughly by holding the tube at the
mouth and giving it a circular motion by tapping the lower end. The tube was
allowed to stand for 30 minutes. Mixed the contents again and centrifuged for
10 min at 2000 RPM. Poured off the supernatant liquid carefully and washed the
precipitate with dilute ammonia solution by blowing with a pipette directly on to
the precipitate in the tube. The supernatant was poured off carefully. To the
pellet added 2.0 mL of 1 N H₂SO₄. Placed the tube in hot water bath for 1 min
and then titrated (using micro burette) with 0.01N KMnO₄ till definite pink
colour persists for at least a minute.

Fractional excretion of calcium was expressed as per cent.
3.10.7 Estimation of urinary inorganic phosphorus:

Urinary phosphorus was estimated by the method of Fiske and Subbarow (1925).

Reagents:

1. Trichloroacetic acid, 10 per cent (w/v): Dissolved 10 grams in water and make up to 100 mL.
2. Sulphuric acid, 10 N: Added 450 mL of concentrated sulphuric acid slowly while cooling to 1,300 mL of distilled water.
3. Molybdate I: 2.5 per cent ammonium molybdate in 5 N sulphuric acid. Dissolved 25 grams of the salt in about 200 mL of water and transferred to a litre flask containing 500 mL of 10 N sulphuric acid. Diluted it to 1 lit with water.
4. Molybdate II: 2.5 per cent ammonium molybdate in 3 N sulphuric acid. Made as molybdate I except that 300 mL of the acid is used instead of 500 mL.
5. Sodium bisulphate, 15 per cent solution: Dissolved 15 g of sodium bisulphate in 100 mL of water.
6. Sodium sulphite, 20 per cent: Dissolved 20 g of sodium sulphite in 100 mL of water.
7. 0.25 per cent 1,2,4-aminonaphthosulphonic acid: Added 0.5 g of dry powder to 195 mL of 15 per cent sodium bisulphate and 5 mL of the 20 per cent sodium sulphite. Stoppard and shaked well until it was dissolved.
8. Standard phosphate solution: Dissolved 0.351 g of potassium dihydrogen phosphate in water in a litre flask. Added 10 mL of 10 N sulphuric acid and made to the mark with water. 5 mL contain 0.4 mg phosphorus.
9. Dilute standard for use: Diluted the stock standard 1 to 10. One mL contains 0.008 mg phosphorus.

Procedure:

The urine sample was diluted to 1 in 5. One mL of diluted urine was taken in a 10 mL test tube. To this added 7 mL of distilled water, followed by 1
mL of molybdate reagent I and 0.4 mL of 0.25 per cent Aminonaphthosulphonic acid. The contents were mixed gently after each addition. 5 mL of the standard phosphate solution was taken into a suitable test tube. Added 1 mL of molybdate I reagent and mixed. Then added 0.4 mL of the aminonaphthosulphonic acid and made to 10 mL with distilled water. After standing 5 minutes read the absorbance, using a red filter at 680 nm.

Fractional excretion of phosphorus was expressed as per cent.

3.10.8 Estimation of urinary Creatinine:

Urinary creatinine was estimated by the method of Bonsnes and Taussky (1945).

**Reagents:**

1. 0.04M Picric acid: 9.16g of picric acid was dissolved in one litre of distilled water.
2. 0.75 N Sodium hydroxide solution: 3 grams of sodium hydroxide was dissolved in 100 mL of distilled water.
3. Standard stock solution (50 µg/mL): 5 mg of pure dry creatinine was dissolved in 100 mL of 0.1N hydrochloric acid.

**Procedure:**

One mL of urine was diluted 100 times, from that 1.0 mL of diluted urine was taken. To that 1.0 mL of 0.04 M picric acid and 1.0 mL of 0.75 N NaOH and 1.0 mL of picric acid were added. Simultaneously a series of test tubes containing a series of concentrations of creatinine were also maintained in the same way. All the tubes were incubated at room temperature for 15 min. Then color developed was read at 540 nm. From the graph, the concentration of sample was determined and the values were calculated.
3.10.9 Estimation of urinary free hydroxyproline:

Urinary free hydroxyproline was estimated by the method of Robert and Milan, (1949).

Reagents:

1. 0.01M aqueous solution of Copper (II) sulphate: 2.497 grams of Copper (II) sulphate was dissolved in double distilled water and made up to 1 litre.

2. Hydrogen peroxide, 6 per cent: 6.0 mL of aqueous solution of hydrogen peroxide was diluted to 100 mL.

3. 6N solution of Sulphuric acid: 60.0 mL of concentrated sulphuric acid was diluted to 360 mL.

4. Ehrlich's aldehyde reagent, 5 per cent: 5.0 grams of p-dimethylaminobenzaldehyde in 100 mL n-propanol.

5. 2.5M aqueous solution of Sodium hydroxide: 25.0 grams of sodium hydroxide was dissolved in 150 mL of distilled water; dilute the solution to 250 mL distilled water.

6. Stock Hydroxyproline (1 mg/mL): 100 mg of Hydroxyproline was dissolved in 100 mL double distilled water.

7. Working Hydroxyproline (0.1 mg/mL): 10.0 mg of stock Hydroxyproline was diluted to 100 mL with distilled water.

Procedure:

To the two test tubes (sample and control) containing 1.0 mL of filtered urine sample, 1.0 mL of copper (II) sulphate, 1.0 mL of sodium hydroxide, and 1.0 mL of hydrogen peroxide solution were added. Contents were stirred with a glass rod for 5 minutes. Then the test tubes were placed in a thermostat set at 70°C for 5 minutes with shaking at intervals until no more gas bubbles are observed to evolve. The test tubes were cooled in a beaker of ice or snow water. 4.0 mL of sulphuric acid and 2.0 mL of Ehrlich's aldehyde reagent were poured into the cooled sample test tubes and boiled in a boiling water bath for 80
seconds and the control test tube were kept in a beaker of ice water for the same period of time.

The absorbance for sample and control solutions against distilled water was measured at 550 nm. The absorbance difference (obtained by subtracting the control absorbance from sample absorbance) was used for determination of hydroxyproline concentration in the sample solution by analytical curve.

Analytical curve was plotted, by taking nine 1.0 mL volumes of hydroxyproline solutions at concentrations of 1, 2, 3, 4, 5, 10, 15, 20, and 25 µg/mL, into test tubes (standard solutions). Further the standard solutions were treated in a manner similar to that described above for the sample solution. The absorbance for standard solutions was measured against blank solution at 550 nm in a photometer.

3.11 Assessment of protein status for fluorotics and non-fluorotics:

3.11.1 Estimation of serum total protein and albumin:

Serum total protein and albumin was estimated by the method of Reinhold (1953).

Reagents:

1. Sulphate – sulphite solution, 27.8 percent. Weighed out 208 grams of sodium sulphate (anhydrous) and 70 grams of sodium sulphate (anhydrous) and dissolve with stirring in about 900 mL of water. To which 2 mL of concentrated sulphuric acid has been added, in a 2 litre beaker. Transfer to a litre volumetric flask and make to the mark with water. The pH should be above 7.0. Keep above 25°C. the 37°C incubator is convenient in a stoppered bottle.

2. Stock Biuret reagent: Dissolve 45 gm of Rochelle salt in about 400 mL of 0.2 N sodium hydroxide and add 15 gm of copper sulphate, stirring continuously until solution is complete. Add 5 gm of potassium iodide and make up to a litre with 0.2 N sodium hydroxide.

3. Biuret solution for use: Dilute 200 mL of the stock reagent to a litre with 0.2 N sodium hydroxide which contains 5 gm of potassium iodide per litre.
4. Tartrate-Iodide solution: Dissolve 9 gm of Rochelle salt in 0.2 N sodium hydroxide containing 5 gm of potassium iodide per litre.

5. Ether, AR


Procedure:

a) **Total Protein**: Six mL of the sulphate-sulphite solution was taken into a 90x15mm centrifuge tube and on it layer 0.4 mL of serum. Inverted the tube to mix the contents. Two mL of the mixture was taken and added to 5 mL of the biuret reagent in a tube.

b) **Albumin**: Added about 3 mL of eather to the rest of the serum sulphate-sulphite mixture, put the stopper and mixed the contents well. The tubes were centrifuged for 5 min. After centrifuging, tilted the tubes and inserted a pipette into the clear solution below the globulin layer. Pipetted out 2 mL of this and added 5 mL of the biuret reagent in a test tube.

c) **Serum blank**: Added 2 mL of the serum sulphate sulphite mixture to 5 mL of the tartrate-iodide solution and mixed.

d) **Biuret blank**: Added 2 mL of the sulphate-sulphite mixture to 5 mL of the biuret reagent.

e) **Standard**: Taken 0.4 mL of the standard serum into 6 mL of sulphate- sulphite solution as above and transferred 2 mL of the mixture into 5 mL of the biuret reagent in a test tube.

f) **Standard serum blank**: Prepared this as described for the test serum (c).

Mixed the contents in the tubes well and then placed the tubes in a water bath at 37° C for 10 minutes. Allowed the contents to cool for five minutes at room temperature, then read the the absorbance in absorptiometer at 555 nm.

Serum protein, albumin and globulin were expressed as g/dL.
3.12. Assessment of hormonal status for fluorotics and non-fluorotics:

3.12.1 Estimation of 25(OH)D:

25(OH)D assessment was done using 25(OH)D $^{125}$I radioimmunoassay kit, supplied by Diasorin, Stillwater, Minnesota, U.S.A, (Catalogue No: 68100E A).

Extraction Procedure:

1. The disposable glass tubes of 12 × 75 mm were labeled for each calibrator, control and patient sample.
2. Added 500 µl of acetonitrile to each tube.
3. Placed pipette tip containing 50 µl of calibrator, control or patient sample below the surface of the acetonitrile and slowly added into the acetonitrile.
4. Vortexed the tubes for 10 seconds.
5. The contents in the tubes were centrifuged using 1200 × g for 10 minutes at 20 to 25°C.
6. The duplicate 25 µl aliquots were pipetted out from the supernatant into separate appropriate labeled 12 × 75 mm tubes.
7. Assay supernatants according to the assay procedure.

Assay Procedure:

1. All reagents and samples were allowed to equilibrate to room temperature.
2. Disposable glass tubes of 12 × 75 mm were labeled in duplicate according to the scheme of the Assay.
3. Added the reagents as follows:
   a. Total count tubes
      50 µl of $^{125}$I 25(OH)D
      1.0 mL of NSB/Addition buffer
   b. Nonspecific binding tubes (NSB)
      25 µl of 0 calibrator (extracted)
      50 µl of $^{125}$I 25(OH)D
      1.0 mL of NSB/Addition buffer
   c. Calibrators, controls and unknown samples
25 µl of calibrator, control, or unknown sample (extracted)
50 µl of $^{125}$I 25(OH)D
1.0 mL of 25(OH)D antiserum

4. Vortexed the tubes gently without foaming and incubated for 90 (±10) minutes at 20-25°C.

\[ g = \left(1.118 \times 10^8\right) \left(\text{radius in cm}\right) \left(\text{rpm}\right)^2 \]

5. Added 500µl DAG precipitating complex (Donkey anti goat [DAG] precipitating complex mixed thoroughly before and during use) to all tubes except the total count tubes.

6. Mixed tubes well and incubated for 25 - 25 minutes at 20-25°C.

7. Added 500 µl of NSB/Addition buffer to all tubes except the total count tubes. Vortexed gently to mix tubes well. Care was taken when performing this step to avoid splashing due to high liquid volume in tube.

8. Centrifuged all tubes for 20 minutes at 20-25°C at 1800 x g, except the total counts.

9. Decanted the supernatants, except the total count tubes into an appropriate waste container. The inverted rack was placed onto absorbent paper for 2-3 minutes. Blotted the tubes gently to ensure all liquid is removed.

10. In a gamma scintillation counter, counted each tube for a minimum of 1 minute.

Serum 25(OH)D was expressed as ng/mL.

3.12.2 Estimation of N-tact Parathyroid hormone (N-tact PTH):

PTH intact assessment was done using N-tact PTH immunoradiometric assay kit, supplied by Diasorin, Stillwater, Minnesota U.S.A (Catalogue No: 26100).

Procedure:

1. The lyophilized reagents were reconstituted and allowed frozen specimens to thaw completely. Kept reagents and thawed specimens on ice while setting up assay.
2. Labeled 12 x 75 mm borosilicate glass tubes in duplicate according to the Scheme of the Assay.

3. The reagents were added to the tubes as follows:
   a. Total count tubes - 100 µl $^{125I}$ N -tact PTH SP Antibody (red)
   b. Calibrator 0 - 200 µl calibrator 0, 100µl $^{125I}$ N -tact PTH SP Antibody (red)
   c. Calibrators (1-5) - 200 µl calibrator, 100 µl $^{125I}$ N -tact PTH SP Antibody (red)
   d. Controls and unknown samples - 200 µl sample, 100 µl $^{125I}$ N -tact PTH SP Antibody (red)

4. Vortexed all tubes.
\[ \text{rpm} = (1118 \times 10^8) \left( \frac{\text{radius in cm}}{\text{rpm}} \right)^2 \]

5. Dispensed one bead into each tube with Teflon-coated forceps.

6. The tubes were covered with parafilm.

7. Incubated the tubes for 22 (± 2) hours at 20-25°C.

8. Aspirated the reaction mixture from each tube.

9. Washed the beads vigorously by dispensing 1 mL of wash solution into each tube with sufficient force to raise the bead from the bottom of the test tube.
   Aspirated wash solution. Repeated wash procedure 3 times.

10. Measured the radioactivity present in each tube using a gamma counter.

    Serum PTH was expressed as pg/mL.

3.13 Statistical analysis:

   The following analyses were done on the data using SPSS package (version 11.5). Computation of Means and SEM for the following parameters of fluorotics and non-fluorotics and between the two sex groups:

   Fluoride status, Anthropometric measurements, Body composition, Food intake, Bone mineral status, and Hormonal status.
The difference in mean values of different parameters among the fluorotic group I, fluorotic group II and non-fluorotic group has been compared with the help of one way ANOVA followed by a multiple comparison test.

Pearson's correlation was used to compute the correlations between the following parameters:

a) Fluoride intake and serum fluoride  
b) Fluoride intake and urinary fluoride  
c) Fluoride intake and anthropometric measurements  
d) Fluoride intake and body composition  
e) Fluoride intake and bone mineral markers  
f) Fluoride intake and protein status parameters  
g) Fluoride intake and hormonal parameters  

Discriminant analysis was used to identifying the fluorotic status of individual subjects based on different parameters and classifying a new individual into one of the two groups namely fluorotic and non-fluorotic.