CHAPTER 3  
METHODS AND MATERIALS

Any new research study planned is initiated as a trial and error based approach. This approach provides a margin to rectify the gaps which arise during the trials. Thus this study aimed in conducting research trials on efficacy of double fortified salt (DFS) supplementation in improving iodine and iron profile amongst pregnant women and school children. The study design was an experimental-control (intervention) based longitudinal approach.

3.1 OVERVIEW OF THE STUDY PHASES

The study was comprised of three phases:

3.1.1 Phase I: Impact Assessment of Double Fortified Salt Supplementation amongst Pregnant women

**Study population:** Pregnant women (n=256) (during first trimester, ≤12 weeks) with singleton pregnancy and without known thyroid dysfunction. Further subsampled to n=150 due to various reasons for dropouts (abortion, miscarriages, missing follow-ups, unwillingness to participate and non-reachability). N=121 could be followed up till the end of gestation.

**Study area:** An antenatal clinic of Jamnabai Hospital, urban Vadodara. It is a semi-government hospital including second highest number of deliveries per month in the state.

**Sampling technique:** Random sampling during screening. Further, stratified sampling was done to distribute subjects into experimental (n=75) and control (n=75) groups.

**Sample size:** Using OpenEpi calculator: Open Source Epidemiologic Statistics for Public Health, Version 2.3.1., calculated sample size for screening was N=246 required at 95% CI, assuming 80% prevalence of anemia during pregnancy.

Experimental design of phase I has been depicted in Figure 3.1, indicating an overview of the work conducted.
Figure 3.1: EXPERIMENTAL DESIGN – PHASE I

Phase I (A): Baseline Data

Screening Pregnant women from an antenatal clinic in urban Vadodara (N=256) (1st trimester)

Basic Indicators: Obstetric History
- Anthropometry: Height, Weight, BMI
- Iodine Status: Urinary iodine, thyroid hormones
- Iron Status: Haemoglobin Estimation
- Other parameters: obstetric history

Phase I (B): Intervention

Subsampled subjects (N=150) (2nd trimester)

Experimental Group (n=75)
- DFS supplementation
- Nutrition Consultation: Dietary guidelines, Best practices for DFS usage
- Awareness on iodine and iron nutrition
- 24 hr. Dietary recall and FFQ
- Assessment of UIE, Thyroid hormones and Hb

Control Group (n=75)
- Ensure Iodized Salt Usage (by H.H. Sample testing)
- Nutrition Consultation: Dietary guidelines, Best practices for Iodized Salt usage
- Awareness on iodine and iron nutrition
- 24 hr. Dietary recall and FFQ
- Assessment of UIE, Thyroid hormones and Hb

Phase I(C): Impact Assessment of Intervention

Lost to follow-up = 5, IUFD = 1, Migrated = 2 (Total = 8)

n = 67

Lost to follow-up = 15, IUFD = 1, Not willing to participate = 5 (Total = 21)
n = 54

Post Data Collection (3rd trimester):
- Anthropometry Parameters: Weight, BMI
- Iodine Status: Urinary iodine, thyroid hormones
- Iron status: Haemoglobin
- Other parameters: KAP, 24 Dietary recall
3.1.2 Phase II: Impact Assessment of Double Fortified Salt Supplementation amongst School children

Study population: School aged children (6-15 yrs.) studying in 1\textsuperscript{st} to 6\textsuperscript{th} standard were selected. N=1184 subjects made the baseline sample size. However, n=947 subjects could complete the study due to various reasons.

Study area: This phase was carried out in rural area- Waghodia of Vadodara district.

Sampling technique: Rural area was divided into 4 demographic regions based on population and number of schools available. This area comprises of 172 primary government schools. Four schools on a same belt were selected randomly. Out of these four schools, two schools were chosen as control group, where there was better availability of iodized salt than rest two schools (experimental group).

Sample size: The sample size formula for determining the sample size is as follows:

\[ N = \frac{16p (100-p)}{w^2} \]

where, \( p \) = estimated prevalence based on earlier study or pilot trial

\( w \) = width of CI eg. if the interval is 95% the width will be 10 (±5)

Now for this phase, considering the prevalence of anemia to be 60% the sample size was estimated as, \( N = 16 \times 60 \times (100-60)/10^2 \)

\[ = 384 \]

Thus, we screened 1184 children and 947 completed the study.
Experimental design of phase II has been depicted in Figure 3.2, indicating an overview of the work conducted.

**Figure 3.2: EXPERIMENTAL DESIGN- PHASE II**

**Phase II (A): Baseline Data**

Waghodia Block Village Schools (n=172)

N=4 schools were selected. All the school children (6-15 yrs.) made baseline sample population (n=1184)

**Baseline Parameters:**

1. **Anthropometry** – Height, Weight
2. **Biochemical Parameters** – Urinary Iodine Excretion and Hb
   - Thyroid hormones (TSH, FT4, TT4, Tg)
3. **IQ and Cognitive Tests** - Draw-a-man, Clerical and Memory

**Experimental Group** (N=2 schools)

- Deworming (N=1)
  - n=256
  - Supplementation, BCC, NHE
  - Other parameters: SES, KAP, 24hr. dietary recall, FFQ
  - Lost to follow-up =11, Not willing to participate=6 (Total =17)

**Control Group** (N=2 schools)

- Non deworming (N=1)
  - n=221
  - BCC, NHE
  - Other parameters: SES, KAP, 24hr. dietary recall, FFQ
  - Lost to follow-up =64, Not willing to participate =51, migrated = 3, not reachable =11 (Total =139)

**Phase II (B) Intervention**

- Deworming (N=1)
  - n=353
  - BCC, NHE
  - Other parameters: SES, KAP, 24hr. dietary recall, FFQ
  - Lost to follow-up =31, Not willing to participate =27, death = 1, not reachable =3 (Total =62)

- Non deworming (N=1)
  - n=353
  - Supplementation, BCC, NHE
  - Other parameters: SES, KAP, 24hr. dietary recall, FFQ
  - Lost to follow-up =11, Not willing to participate=6 (Total =17)
• **Anthropometry Parameters** - Height, Weight
• **Biochemical parameters** - Urinary Iodine excretion and Hb
  - Thyroid hormone analysis
• **IQ and Cognition Tests** - Draw-a-mar, Clerical and Memory
• **Other parameters** - KAP of the mothers, 24 hr. dietary recall and FFQ of children

3.1.3 Phase III: Upgrading salt iodization at local level and feasibility for Double Fortified Salt Production at local level

This phase for DFS production at local level was a first step towards future research- as an approach and a feasible method to combat 2 micronutrient deficiencies.

**Study population and area:** Based on the availability of local salt producers within and around **Anand, Kheda, Nadiyad and Baroda districts**; and also due to higher prevalence of still birth in the region, these local salt producers were selected as study participants. Timely monitoring and improved salt iodization were the short term goals to attain quality production.

**Sampling technique:** Purposive sampling

The long run target of DFS production was carried out by motivating the producers for further entrepreneurship. The producers were provided with information on availing technology transfer from National Institute of Nutrition-NIN Hyderabad. The producers were made to meet the officials who were responsible for the needful support. The crux of business, trade etc was explained to them. Their role as a societal contribution was explained and was well received by the producers.

It is a known fact that, any new venture takes time to establish its roots. This venture follows the same pattern. Here, the setup is in slow progress due to financial limitations of the producers. Laisoning is being carried out to provide further directions; work is in progress towards achievement of the same.
Experimental design of phase III has been depicted in **Figure 3.3**, indicating an overview of the work conducted.

**Figure 3.3: EXPERIMENTAL DESIGN- PHASE III**

- Mapping and upgradation of salt iodization amongst small and medium scale salt producers (n=34) from Anand, Kheda, Nadiyad, Bharuch

- Purposive selection of small and medium scale salt of Bharuch for Double Fortified Salt production initiation (n=3)

- Advocacy measures towards production requirements, technical and ethical need for Double Fortified Salt Production

- Consultations with NIN for Double Fortified Salt Premix

- Correspondence to avail consents and scheduling training programmes for production and technology transfer to the producers

**STUDY DURATION**

**Phase I:** Study duration - October 2009 to November 2010  
Report writing - December 2010 to March 2011

**Phase II:** Study Duration- March 2010 to June 2011  
Report writing- July 2011 to December 2011

**Phase III:** Iodization upgradation and monitoring - January 2008 to May 2009  
DFS production discussion and correspondence- May 2009 to May 2011  
Report writing- January 2012 to February 2012
ETHICAL ISSUES

Approval for the study was obtained from the ethical committee of the home institution ethical board in compliance with the guidelines issued by Indian council of Medical research (No. F. C. Sc FN ME70).

Phase I: Written permission from the hospital authorities and concerned doctors was availed before the commencement of the study. All the pregnant women were explained the purpose of the study and signed written consent were collected (in local language).

Phase II: Permissions from all the schools and District education officer were availed to carry out the work. All the children from 1st to 6th standard were enrolled for the study. Written consent from the parents of the children (in local language) and oral consent from the children was also availed.

Phase III: Oral consent from the salt producers was availed after explaining the purpose of the study.

3.2 INDICATORS FOR DATA COLLECTION

Study indicators used for all the three phases have been mentioned in Table 3.1 along with the required sample size and methods references details.
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Indicators</th>
<th>Tools</th>
<th>Phases</th>
<th>Samples Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Socio-Economic Status</td>
<td>Structured Questionnaire</td>
<td>Phase I</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phase II</td>
<td>212</td>
</tr>
<tr>
<td>2</td>
<td>Anthropometry</td>
<td>Standard methods and tools</td>
<td>Phase I</td>
<td>256(Baseline)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Phase II</td>
<td>121(Final)</td>
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<td>3</td>
<td>Hemoglobin Estimation</td>
<td>Cynmet-hemoglobin method</td>
<td>Phase I</td>
<td>256 (Baseline)</td>
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<td></td>
<td></td>
<td></td>
<td>Phase II</td>
<td>121 (Final)</td>
</tr>
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<td>4</td>
<td>Urinary iodine excretion</td>
<td>Sandell-kolthoff reaction (Modified microplate technique)</td>
<td>Phase I</td>
<td>256 (Baseline)</td>
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<td></td>
<td>Phase II</td>
<td>121 (Final)</td>
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<tr>
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<td>DFS content estimation</td>
<td>BIS standards</td>
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<td>6</td>
<td>Thyroid hormones-TSH, FT₄, TT₄, Tg</td>
<td>RIA technique</td>
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<td>256(Baseline)</td>
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<td></td>
<td></td>
<td>Phase II</td>
<td>121 (Final)</td>
</tr>
<tr>
<td>7</td>
<td>Cord blood Analysis- TSH, FT₄, TT₄, Tg</td>
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<td>Phase I</td>
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<td>121 (Baseline and Final)</td>
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<td></td>
<td></td>
<td>Phase II</td>
<td>212(Baseline and Final)</td>
</tr>
<tr>
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<td>Knowledge, attitude and practices</td>
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<td>121 (Baseline and Final)</td>
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<td></td>
<td></td>
<td>Phase II</td>
<td>212(Baseline and Final)</td>
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<td>Household salt samples</td>
<td>Spot testing kit</td>
<td>Phase I</td>
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<td></td>
<td></td>
<td></td>
<td>Phase I</td>
<td>302</td>
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<td>Salt samples from salt units</td>
<td>Titrimetric method</td>
<td>Phase III</td>
<td>250</td>
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<td>12</td>
<td>IQ and Cognition Tests- Draw-a-man, Visual Memory test, Clerical Test</td>
<td>Standardized methods</td>
<td>Phase II</td>
<td>823-864 (Baseline)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>700 (Final)</td>
</tr>
</tbody>
</table>
3.3 METHODS OF DATA COLLECTION

3.3.1 NUTRITIONAL STATUS ASSESSMENT

3.3.1.1 Weight

It was a key anthropometric measurement of body mass. Weight deficiency is a best indicator for the detection of protein energy malnutrition (Gibson 1989).

Procedure: Subjects were asked to stand bare foot on the Standardized bathroom scales. They were used for weighing with the least count of 0.5 kg. The subjects were asked to stand straight on the scale without touching anything and look straight ahead. Then the weights were recorded. Weight for age was calculated using CDC standards in the case of school children.

3.3.1.2 Height

Height is a linear measurement of body. Deficit in the height is associated with chronic insufficient food intake and frequent infections.

Procedure: Height was measured by a wall mounted fiber glass tape with the least count of 0.5 cm. The tape was mounted accurately on the wall perpendicular to the floor and the subjects were asked to stand against it. The head, shoulders and heels touched the wall and the subjects were asked to look straight. The flat surface of the floor and the wall was taken into consideration for even measurements. A thin scale was kept straight on the head, perpendicular to the wall, so as to slightly press the hair. This was the feasible method in the rural field level and a government hospital setting. Height for age was calculated using CDC standards in the case of school children.

3.3.1.3 Body mass index (BMI)

BMI has been recommended by WHO (1995) as an indicator of choice for measuring undernutrition. It is calculated as follows:

_________________
Z score was used for defining underweight, stunted and wasted based on CDC growth standards (2005).

>-2SD – marginally malnourished.

-2SD to -3SD – moderately malnourished

<-3SD – severely malnourished

WHO growth standards (2007) have now been recommended as the reference standard for children. However, weight-for-age norms for children above 10 years have not been available. Hence, to use all the three Z scores using same standards would give better homogeneity and avoid complications in concluding the results on the anthropometry. Hence, CDC growth standards 2005 have been used for comparing anthropometric indices.

3.3.2 BIOCHEMICAL PARAMETERS

3.3.2.1 Determination of Urinary Iodine Excretion

Urinary iodine excretion is a good marker of the recent dietary intake of iodine and therefore used as an index as an index for evaluating the degree of iodine deficiency.

Principle

Iodine in urine occurs as the iodine ion (I\(^{-}\)). Most of the popular methods for urinary iodine concentration determination are based on Sandell-Kolthoff reaction. Iodide is measured by its catalytic action on the reduction of the ceric ion (Ce\(^{4+}\)) to the cerous ion (Ce\(^{3+}\)) coupled to the oxidation of arsenous to arsenic, As\(^{3+}\) to As\(^{5+}\).

\[
2\text{Ce}^{4+} + 2I^{-} \rightarrow 2\text{Ce}^{3+} + I_2
\]

\[
I_2 + \text{As}^{3+} \rightarrow \text{As}^{5+} + 2I^{-}
\]

The Ceric ion (Ce\(^{4+}\)) has a yellow colour, while the Cerous (Ce\(^{3+}\)) is colourless. The course of reaction can be followed by the disappearance of yellow colour as the Ceric ion is reduced and can be measured colorimetrically. With other reactants held stable, the speed of this colour disappearance is directly proportional to the amount of catalyzing
iodide. Because of its specificity and high sensitivity, this reaction has been the basis for almost all chemical methods for the detection of iodine in urine.

However, many studies have indicated that there are interfering substances such as nitrite, thiocynate or ferrous ion in the urine that might interfere by reducing or oxidizing the Ceric or arsenic reactants and thus needs to be removed initially. Different methods are used such as dry ashing, dialysis or digestion with strong acid. At present the safest course is to include digestion step prior to colorimetric determination for urinary iodine.

Chloric acid digestion is the most commonly used method. Although it provides an accurate measurement, the method also has following disadvantages:

- Production of toxic wastes (>5ml/test) from arsenic trioxide in Sandell-Kolthoff reaction.
- Leakage of gas during sample digestion, requiring a special fumehood
- Difficulty in locating Chloric acid from chemical vendors because of its instability

On the other hand, an alternative method that uses ammonium persulphate digestion has been reported recently as a non-hazardous, non-explosive and easy to use method. The persulphate digestion makes possible a comparatively non-hazardous (no chlorine gas) measurement. However, this method is still not completely suitable for testing because it is time consuming and produces a non-negligible amount of toxic waste. To further minimize the amount of toxic wastes as well as simplify and speed up the procedure simple microplate method using ammonium persulphate digestion is used. (Ohashi, et al.2000)

Reagents

1. Standards: Measure 168.5 mg of KIO₃ and put in 100ml volumetric flask. Make up the volume to 100 ml by deionized water. Mix it well. This is the stock solution 1. Take out 100 μL of this stick solution 1 in 2nd 100 ml volumetric flask and make up the volume to 100 ml by deionized water. This is stock solution 2.
Table 3.2: Preparation of working standards

<table>
<thead>
<tr>
<th>Standard (µg/L)</th>
<th>Stock Solution 2 (µL)</th>
<th>Deionized water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100</td>
<td>9.9</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>9.8</td>
</tr>
<tr>
<td>100</td>
<td>1000</td>
<td>9</td>
</tr>
<tr>
<td>150</td>
<td>1500</td>
<td>8.5</td>
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<td>200</td>
<td>2000</td>
<td>8</td>
</tr>
<tr>
<td>300</td>
<td>3000</td>
<td>7</td>
</tr>
<tr>
<td>400</td>
<td>4000</td>
<td>6</td>
</tr>
</tbody>
</table>

2. *Ammounium persulphate solution*: Measure 5 mg of ammonium persulphate and add in 15 ml of deionized water. Mix it properly. It should be prepared fresh.

3. *Arsenous acid solution (500 ml)*: Arsenic trioxide (5 g) was dissolved in 100 ml NaOH solution (3.5 w/v). Keep it in ice bath and add 16 ml of conc. H₂SO₄ slowly. After cooling add 12.5 g NaCl and dilute upto 500 ml with water. Filter and store in amber colored bottle.

4. *Ceric ammonium sulphate solution (250 ml)*: Weigh 3 gm of ceric ammonium sulphate and add 250 ml of 3.5 N H₂SO₄. Store the solution in amber color bottle.

**Procedure:**

- 50 µl of each calibrators (with known concentration of 0, 10, 20, 100, 150, 200, 300 and 400 µg/L) and urine samples are pipetted into the wells of a polypropylene plate (PP).

- Into the sample wells 100µl of 3% ammonium per sulphate is added. The plate is then set into a cassette.

- The cassette is tightly closed and kept for 60 minutes in an oven adjusted at 110° C.

- After digestion, the bottom of the cassette is cooled to room temperature with tap water to avoid condensation of vapour on the top of wells and to stop the digestion.
• The cassette is opened and 50 µl aliquots of the resulting digestes are transferred to the corresponding wells of a polystyrene 96 wells microtiter plate.

• Arsenious acid solution (100µl) is added to the wells and mixed; 50µl of ceric ammonium sulphate solution is then added quickly (within 1 minute), using a multichannel pipette. The reaction mixture is allowed to sit for 30 mins.

• The absorbance is measured at 405 nm with an ELIZA reader.

Table 3.3: Epidemiological criteria for assessing iodine nutrition based on the median or range in urinary iodine concentrations of pregnant women and children *(WIIO/UNICEF/ ICCIDD 2007)*

<table>
<thead>
<tr>
<th>Population Group</th>
<th>Median Urinary iodine excretion (µg/L)</th>
<th>Iodine concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant Women</td>
<td>&lt;150</td>
<td>Insufficient</td>
</tr>
<tr>
<td></td>
<td>150-249</td>
<td>Adequate</td>
</tr>
<tr>
<td></td>
<td>250-499</td>
<td>Above requirements</td>
</tr>
<tr>
<td></td>
<td>≥ 500</td>
<td>Excessive b</td>
</tr>
<tr>
<td>Children</td>
<td>&lt;20</td>
<td>Severely deficient</td>
</tr>
<tr>
<td></td>
<td>20-49</td>
<td>Moderately deficient</td>
</tr>
<tr>
<td></td>
<td>50-99</td>
<td>Mildly deficient</td>
</tr>
<tr>
<td></td>
<td>≥ 100</td>
<td>Normal</td>
</tr>
</tbody>
</table>

* For lactating women and children < 2 years of age median urinary iodine concentration of 100 µg/L can be used to define adequate iodine intake, but no other categories of iodine intake are defined. Although lactating women have the same requirements as pregnant women, the median urinary iodine is lower because iodine is excreted in breast milk. b the term “excessive” means in excess of the amount required to prevent and control iodine deficiency.

3.3.2.2 Determination of Haemoglobin Concentration

Estimation of Haemoglobin was carried out by,

(A) Sahli’s haemoglobinometer (Phase I)

(B) Finger prick blood Spot  Cyanmethemoglobin (Phase II)

(A) Sahli’s haemoglobinometer
Blood collection was done on the spot using Sahli’s method. Blood was collected using disposable lancets.
**Principle - Haemeoglobin (Hb)** is converted to acid haemeatin by addition of 0.1 N Hydrochloric acid and the resulting brown colour is compared with standard brown glass reference blocks.

**Procedure**

- 0.1 N HCL is placed in the graduated tube to the mark 10.
- Blood is drawn upto the 20 mark in the Sahli’s pipette, added to the acid in the tube and the pipette rinsed thoroughly.
- The mixture is allowed to stand for 10 min. for complete conversion to acid haemeatin. (Maximum colour develops in 1 hr.).
- The solution is diluted with a few drops of distilled water at a time until the colour matches with the standard glass reference block.
- The height of the solution corresponds to the Hb content.

**(B) Finger prick blood Spot- Cyanmethemoglobin**

Haemogloin was assessed using finger prick blood sample by cyanmethaemoglobin method using filter paper technique.

**Procedure:**

- Label the Whatman No.1 filter paper strip appropriately (with identification particulars) before collection of blood sample.
- Clean the middle finger of left hand with spirit and cotton and allow it to dry. Squeeze the finger slightly and hold it firmly with left hand.
- Using a lancet, puncture the skin at right angles to the tip of the finger. Discard the lancet after folding it. Wipe off the first drop of the blood with tissue paper and press the finger gently so as to form a drop of blood. Collect the blood into clean hemoglobin pipette.
- Using a clean and dry hemoglobin pipette draw the blood slowly up to a little above the 20μl mark on the pipette. Care should be taken to avoid any air bubbles in the blood column. Clean the tip of the pipette using a tissue paper. Adjust the volume to 0.02 ml (up to the mark on the pipette) by touching the tip of pipette with a wet tissue paper.
• Transfer the blood from the pipette by slowly blowing it out on to the coded filter paper, in the form of a circular spot of about 1 cm diameter, by keeping the pipette perpendicular with its tip touching the filter paper and moving in a circle. Take care not to splash the blood, by blowing very slowly. No trace of blood should remain, either in the pipette or on its tip.

• As soon as the sample blood collection is over, wipe the finger with dry cotton. Press the site of puncture with spirit swab. Avoid direct contact with blood.

• Discard the sample in case the blood sticks to the inner surface of the pipette. Pipette the blood once again with a fresh pipette.

• Fold the filter paper diagonally and place it in a breadbox and allow it to dry in shade. Protect the sample from sunlight, flies and dust. Pack the dried samples in polythene cover.

**Haemoglobin estimation**

Principle: When blood is mixed with drabkin’s reagent containing potassium cyanide and potassium ferricyanide, haemoglobin reacts with ferricyanide to form methemoglobin which is converted to stable Cynmethemoglobin by the cyanide. The intensity of the color is proportional to haemoglobin concentration and it is compared with a known cynmethemoglobin standard at 540 nm (green filter).

**Requirement**

1. Drabkin’s reagent: It contains in 1000 ml of distilled water.
   a. Potassium ferricyanide: 400 mg
   b. Potassium dihydrogen phosphate: 280 mg
   c. Potassium cyanide: 100 mg
   d. Ninidet: 1 ml

2. Cynmethemoglobin standard: It is commercially available. This standard is directly pipetted in a cuvette and optical density is measured at 540 nm. The reading obtained, corresponds to ______ g/dl haemoglobin.

3. Hb-pipette (20 μl calibrated)
4. Test tubes (15 X 125 mm)
5. Spectrophotometer (Spectronic 20D)

Procedure

- Exactly 5 ml of the diluted Drabkin’s reagent was pipetted out in test tubes.
- The portion of filter paper with blood spot was cut carefully using scissors and the same was transferred into a pre-coded test tube having 5 ml of Drabkin’s solution.
- These test tubes with soaked blood spot on filter paper, was kept for sufficient length of time (overnight/6-8 hrs) in Drabkin’s reagent for complete extraction of the blood. On complete extraction, the filter paper appeared white.
- On complete extraction, content was mixed thoroughly before reading.
- Absorbance of test was read at 540 nm by setting blank to 100% T
- Absorbance of standard and samples were read by pipetting directly in a cuvette.

Calculation Formula

\[
\text{Hb in g/dl} = \frac{\text{OD of sample} \times \text{Concentration of Hb standard} \times \text{Dilution factor (251)}}{\text{OD of standard} \times 100}
\]

Where concentration of Hb standard is 60mg/dl, and OD stands for optical density.

Table 3.4: Hemoglobin cutoffs used to define anemia in people living at sea level

<table>
<thead>
<tr>
<th>Age or Sex group</th>
<th>Hemoglobin below (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children 6 months to 59 months</td>
<td>11.0</td>
</tr>
<tr>
<td>Children 5 – 11 years</td>
<td>11.5</td>
</tr>
<tr>
<td>Children 12- 14 years</td>
<td>12.0</td>
</tr>
<tr>
<td>Nonpregnant women (above 15 years of age)</td>
<td>12.0</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>11.0</td>
</tr>
<tr>
<td>Men (above 15 years of age)</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Source: (WHO/UNICEF/UNU 2001)
3.3.2.3 DETERMINATION OF THYROID ANALYTES

Collection of blood and separating serum
Venous blood was collected using a plain Vacutainer (i.e. not containing any anticoagulant) for all subjects. Blood collection was carried out by a technician. These vacutainers were centrifuged at 3000 RPM for 5 minutes. Serum is separated as supernatant. Carefully serum is transferred to other screw capped tube and deep freeze at (-20°C) till it is used. From these samples Tg, T4, FT4 and TSH were analyzed.

(A) Measurement of serum TSH

Method of analysis.

TSH was measured from serum by Immuno-radiometric assay (IRMA) using kit. The kit was IRMAK-9, procured from The Board of Radiation and Isotope Technology (BRIT) Government of India, Navi Mumbai.

Principle

In immuno-radiometric assay (IRMA), two antibodies generated against different epitopes of the same antigen are used. One antibody is bound to a solid-phase, usually a tube, while the other antibody is labelled with \(^{125}\)I. Thus, when an antigen is present, it simultaneously binds both antibodies in a “bridge” fashion (i.e. it gets sandwiched between two antibodies. This entire complex remains bound to the tube. The radioactivity in the bound fraction may then be quantified using a Gamma Counter. The radioactivity measured, is directly proportional to the concentration of antigen. A standard curve is constructed from which unknown concentration of human thyroid stimulating hormone can be extrapolated.

Reagents

Reagents were provided with kit.

1. hTSH monoclonal antibody tubes: 100
2. \(^{125}\)I-Anti-hTSH: 1 vial 10 ml
3. hTSH standards: 8 vials (10 ml each)(concentration- 0, 0.15, 0.5, 1.5, 5.0, 15, 50 µIU/ml)
4. Wash diluent: 1 vial (50 ml)
5. Control: 2 vials (10 ml each)

**Reagent preparation**

1. hTSH standards: ready to use
2. $^{125}$I-Anti-hTSH: ready to use
3. Wash diluent: Dilute with 950 ml double distilled water

**Protocol**

**Table 3.5: Defined dose for standard graph (TSH estimation)**

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Tube number</th>
<th>Defined dose (μIU/ml)</th>
<th>Percentage bound (%B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$S_A$</td>
<td>0.00</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>$S_B$</td>
<td>0.15</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>$S_C$</td>
<td>0.50</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>$S_D$</td>
<td>1.50</td>
<td>2.8</td>
</tr>
<tr>
<td>5</td>
<td>$S_E$</td>
<td>5.00</td>
<td>8.2</td>
</tr>
<tr>
<td>6</td>
<td>$S_F$</td>
<td>15.00</td>
<td>23.4</td>
</tr>
<tr>
<td>7</td>
<td>$S_G$</td>
<td>50.00</td>
<td>68.3</td>
</tr>
<tr>
<td>8</td>
<td>$S_H$</td>
<td>100.00</td>
<td>100.0</td>
</tr>
</tbody>
</table>

**Figure 3.4: Standard graph for TSH**
### Table 3.6: Steps in measurement of TSH

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Tube number</th>
<th>Standard/control/ Sample (µl)</th>
<th>Tracer $^{125}$I-Anti-hTSH (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Standard A</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>2.</td>
<td>Standard B</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>3.</td>
<td>Standard C</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>4.</td>
<td>Standard D</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>5.</td>
<td>Standard E</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>6.</td>
<td>Standard F</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>7.</td>
<td>Standard G</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>8.</td>
<td>Standard H</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>9.</td>
<td>Control 1</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>10.</td>
<td>Control 2</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>11.</td>
<td>Control 3</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>12.</td>
<td>Test sample</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Incubate for 60 min. at Room Temp. with gentle shaking. Add 2ml diluted wash solution to each tube. Mix. Decant the solution. Repeat wash step once more. Count the tubes in Gamma Counter set for $^{125}$I for 2 min.

---

**(B) Measurement of serum FT$_4$ and TT$_4$**

**Method of analysis**

$T_4$ from serum was measured by radioimmunoassay.

**Principle**

This assay is based on the competition between unlabeled $T_4$ and fixed quantity of $^{125}$I-labeled $T_4$ (tracer) for limited number of binding sites on $T_4$ specific antibody. These $T_4$ antibodies are coupled to magnetic particles. Allowing to react a fixed amount of tracer and antibodies (bound to magnetic particles) with different amount of unlabeled antigen,
the amount of tracer bound by antibody will be proportional to the concentration of unlabeled antigen.

On providing a magnetic field, (using magnetic racks) all antigen-antibody immune complexes settle at the bottom of the tube. In presence of magnetic field, the reaction mixture is discarded and tubes are dried. Then, radioactivity is measured in Gamma Counter.

The concentration of antigen is inversely proportional to the radioactivity measured in test tubes. By plotting binding values against known amount of T₄, a standard curve is constructed, from which the unknown concentration of T₄ in patient’s sample can be determined.

Reagents

Reagents were supplied with kit.

- Standard T₄: 5 vials 10 ml each (concentration- 0, 2.5, 5, 10, 20 µg/dL)
- Tracer: 1 vial of 10 ml
- Antibody-magnetic particles: 1 vial of 20 ml
- Control: 2 vials (10 ml each)

Reagent preparation
All the reagents were ready to use. Hence, they were directly used without reconstitution.

Protocol

**Table 3.7: Defined dose for standard graph (FT₄ & TT₄)**

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Tube number</th>
<th>Defined dose (µg/dL)</th>
<th>Percentage bound (%B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sₐ</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>S₉</td>
<td>2.5</td>
<td>67.5</td>
</tr>
<tr>
<td>3</td>
<td>S₀</td>
<td>5.0</td>
<td>49.8</td>
</tr>
<tr>
<td>4</td>
<td>S₉</td>
<td>10.0</td>
<td>33.6</td>
</tr>
<tr>
<td>5</td>
<td>Sₐ</td>
<td>20.0</td>
<td>20.9</td>
</tr>
</tbody>
</table>
Figure 3.5: Standard graph for T₄

Table 3.8: Steps in measurement of FT₄

<table>
<thead>
<tr>
<th>Tube Reagents</th>
<th>Std/Control/Sample (µl)</th>
<th>Tracer (µl)</th>
<th>Ligand (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD A</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>STD B</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>STD C</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>STD D</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>STD E</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>QC1 (kit)</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>QC1 (in-house)</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>QC2 (in-house)</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>Samples</td>
<td>25</td>
<td>400</td>
<td>100</td>
</tr>
</tbody>
</table>

Vortex, incubate for 60 min. at 18-25°C, with shaking. Aspirate carefully the contents. Count.
Table 3.9: Steps in measurement of TT4

<table>
<thead>
<tr>
<th>Tube Reagents</th>
<th>Std/Control/Sample (µl)</th>
<th>Tracer (125I-T4) (µl)</th>
<th>T4 – Ab magnetic particles (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD A</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>STD B</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>STD C</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>STD D</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>STD E</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>P1</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>P2</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>P3</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>Samples</td>
<td>10</td>
<td>400</td>
<td>500</td>
</tr>
</tbody>
</table>

Vortex. Incubate for 90 min. Water bath. Place the tubes for 20 mins on magnetic rack. Decant, wipe the rim of the tubes with tissue paper and count.

(C) Measurement of serum Tg

Thyroglobulin from serum was measured by radioimmunoassay.

Principle

In radioimmunoassay (RIA), a fixed concentration of labeled tracer (antigen) is incubated with a constant amount of antiseraum such that the number of antigen binding sites on the antibody is limiting. If unlabeled antigen is added to this system, there is a competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody and thus the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody bound from free tracer and counting the bound fraction in Gamma Counter. The radioactivity measured is inversely proportional to concentration of unlabeled antigen. A calibration or standard curve is set up with increasing amounts of known antigen and from this curve the amount of antigen in the unknown samples can be calculated.

Reagents:

Reagents were provided with kit.
1. **Standard Tg**: 8 vials containing 10 ml each (concentration 0, 12.5, 25, 50, 100, 200, 400, 800 ng/ml)

2. **Tg free serum**: 1 vial (10 ml)

3. **r-antiTg-antibodies**: 1 vial (10 ml)

4. **Labeled Tg**: 1 vial (10 ml)

5. **Control**: 2 vials (10 ml each)

6. **DAB-magnetic particles**: 1 vial (10 ml)

**Reagent Preparation**: (Carried out by BARC Laboratory, RIA Unit, Mumbai)

**Protocol for serum thyroglobulin**

**Table 3.10: Defined dose for standard graph (Tg)**

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Tube number</th>
<th>Defined dose (ng/ml)</th>
<th>Percentage bound (%B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B₀</td>
<td>0.61</td>
<td>18.76</td>
</tr>
<tr>
<td>2</td>
<td>S₁</td>
<td>12.5</td>
<td>16.04</td>
</tr>
<tr>
<td>3</td>
<td>S₂</td>
<td>25.0</td>
<td>12.98</td>
</tr>
<tr>
<td>4</td>
<td>S₃</td>
<td>50.0</td>
<td>10.24</td>
</tr>
<tr>
<td>5</td>
<td>S₄</td>
<td>100.0</td>
<td>7.38</td>
</tr>
<tr>
<td>6</td>
<td>S₅</td>
<td>200.0</td>
<td>5.59</td>
</tr>
<tr>
<td>7</td>
<td>S₆</td>
<td>400.0</td>
<td>4.22</td>
</tr>
<tr>
<td>8</td>
<td>S₇</td>
<td>800.0</td>
<td>3.33</td>
</tr>
</tbody>
</table>
Figure 3.6: Standard graph for serum thyroglobulin

Table 3.11: Steps for measurement of Serum Tg

<table>
<thead>
<tr>
<th>Tube</th>
<th>Buffer (µl)</th>
<th>Free serum (µl)</th>
<th>125I (tracer) (µl)</th>
<th>Standard/Sample/control (µl)</th>
<th>Antiserum (µl)</th>
<th>DAB-magnetic particles (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₀</td>
<td>---</td>
<td>---</td>
<td>100</td>
<td>---</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S₁</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S₂</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S₃</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S₄</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S₅</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S₆</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S₇</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>---</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>samples</td>
<td>100</td>
<td>---</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Incubate at 4°C for 3 overnights.

Keep for 2 hours on shaker. Add 0.4 ml assay buffer and leave it for 20 minutes on magnetic racks, decant and count.
3.3.3 QUALITY ESTIMATIONS

3.3.3.1 Determination of iodine from iodized salt

(A) Titrimetric Method

Description of reaction: The iodine content of salt iodated samples is measured using an iodometric titration, as described by Dc. Macyer, Lowenstein and Thilly (1979). The reaction mechanism can be considered in two steps:

Reaction 1: Liberation of free iodine from salt.

- Addition of H$_2$SO$_4$ liberates free iodine from the iodate in the salt sample.
- Excess KI is added to help solubilize the free iodine, which is quite insoluble in pure water under normal conditions.

Reaction 2: Titration of free iodine with Sodium thiosulfate.

- Free iodine is consumed by sodium thiosulfate in the titration step. The amount of thiosulfate used is proportional to the amount of free iodine liberated from the salt.
- Starch is added as an external (indirect) indicator of this reaction. It reacts with free iodine to produce a blue colour. When added towards the end of the titration (that is, when only a trace amount of free iodine is left) the loss of blue colour or endpoint, which occurs with further titration, indicates that all remaining free iodine has been consumed by thiosulfate.

- Reaction steps for Iodometric Titration of iodate:

1. IO$_3^{-}$ + 5I$^{-}$ + 6H$^+$ $\rightarrow$ 3I$_2$ + 3H$_2$O.

2. 2Na$_2$S$_2$O$_3$ + I$_2$ $\rightarrow$ 2NaI + 2Na$_2$S$_4$O$_6$.

(Sodium Thiosulfate) (Iodine) (Sodium Iodide) (Sodium Tetrathionate)
Reagent Preparation:

- **Water requirements for Reagent Preparation:**

  Water required for this method should be boiled distilled Water, which requires provision of a distillation unit as a simpler alternative.

- **0.05 N Sodium thiosulfate (Na₂S₂O₃):** Dissolve 1.24 gm Na₂S₂O₃·5H₂O in 1000 ml water. Store in a cool dark place. This volume is sufficient for 100-200 samples, depending on the iodine content of samples. The solution is stable at least for 1 month, if stored properly.

- **2N Sulfuric Acid (H₂SO₄):** slowly add 6ml concentrated H₂SO₄ to 90 ml distilled water. Make up the volume to 100 ml with distilled water.

- **10% Potassium iodide (KI):** Dissolve 100 gm KI in 1000 ml water. Store in a cool dark place. This volume is sufficient for 200 samples. Properly stored solution is stable for 6 months.

- **Starch indicator solution:** Make 100ml of a saturated solution of NaCl. By adding NaCl to approximately 80 ml distilled water in a beaker, with stirring and/or heating until no further solid will dissolve. This solution is stable for at least one year. Weigh 1 gm soluble starch into a 100ml beaker; add 10ml water and heat to dissolve. Add saturated NaCl solution to the hot starch solution to make up to 100 ml. Store in a cool dark place. This volume is sufficient for 50 samples.

Procedural Steps:

- Weigh 10g of the salt sample into a 250 ml Erlenmeyer flask with a stopper.
- Add approximately 30 ml water, swirl to dissolve salt sample.
- Add distilled water to make the volume upto 50 ml.
- Add 1 ml 2N H₂SO₄.
- Add 5 ml 10% KI. The solution should turn yellow if iodine is present.
- Stopper the flask and put in the cupboard or drawer (dark) for 10 minutes.
- Rinse and fill burette with 0.005 M Na₂S₂O₃ and adjust level to zero.
• Remove flask from drawer, and add some Na₂S₂O₃ from the titration burette until the solution turns pale yellow.

• Add approximately 2ml of starch indicator solution (the solution should turn dark purple) and continue titrating until the solution becomes pink, and finally colourless.

• Record the level of thiosulfate in the burette and convert to parts per million (ppm) using the conversion table in (Annexure V).

**Precautions:**

• The reaction mixture should be kept in the dark before titration because a side reaction can occur when the solution is exposed to light that causes iodide ions to be oxidized to iodine.

• Inaccurate results may occur if starch solution is used while still warm.

• If starch indicator is added too early, a strong iodine starch complex is formed which reacts slowly, and gives falsely elevated results.

• The reaction should be performed at mild room temperature (>30°C) since the iodine is volatile, and the indicator solution loses sensitivity when exposed to high temperature.

**(B) Spot Test Kit**

It is used for semi quantitative test of iodine in salt. This kit consists of two ampules of test solutions and one ampule of recheck solution.

• **The test solution** contains starch solution in acidic medium, a drop of which when added to iodized salt, it gives blue colour. The colour developed is compared with the reference colour chart from assessment of the ppm (parts per million) of iodine.

• **The recheck solution** contains 50% Phosphoric acid. It is used when salt contain excess of alkaline materials, impurities or additives which interfere with the reaction of iodates with starch. When a drop of test solution added to iodized salt doesn’t give blues colour, a drop of recheck solution is added to the salt.
**Principle:** The reaction mechanism for iodate spot test is that iodate from salt, in the presence of free hydrogen ion, oxidizes added iodine to give free iodine; this then turns starch to give a blue colour:

\[
\text{IO}_3^- + 5\Gamma^- + 6H^+ \rightarrow 3\text{I}_2 + 3\text{H}_2\text{O}.
\]

\[
\text{I}_2 + \text{Starch} \rightarrow \text{Blue colour.}
\]

**Procedure:**

- One drop of test solution is added to a spoonful of salt sample. The change in colour of salt sample ranges from white to light blue to dark violet depending on the iodine content of salt, i.e. Nil, 7 ppm, 15 ppm, 30 ppm. This is compared to the standard colour chart provided with the kit.

- If on addition of test solution, no change in colour of salt is observed, the recheck solution is added. This is done to make the salt medium acidic, (in case the salt has alkaline constituents), and then the test solution is added once again. The intensity of blue colour is directly proportional to the iodine content of the salt.

**Precautions:**

- Contamination may occur if measuring spoon and plate are not washed.

- The kit should show an expected shelf life, usually 12-18 months. Thus kits, which have outlasted their shelf life, should not be used.

- In addition to the date of expiration of the kit as a whole the test solution also has a limited shelf life, once the dropper bottle has been opened and used.

3.3.3.2 Determination of Iodine and iron content from DFS was carried out by a standardized method established by (Ranganathan et al 2007). The estimations were carried out by one of the QC laboratory at DFS production unit, Gujarat.

**3.4 QUESTIONNAIRES**

**3.4.1 STRUCTURED QUESTIONNAIRES**

Background information was obtained from the study subjects on socio-economic status availing details on names of the subjects, names of spouse (Phase I)/ parents (Phase II) of
the subjects, age, sex, education, occupation and other relevant information. For pregnancy obstetric history and previous adverse experiences were also reported (Annexure VI and VII).

3.4.2 SEMI STRUCTURED QUESTIONNAIRES

Background and post interventional effect of NHE on KAP was assessed using this tool. The information on iodine nutrition, iron nutrition, dietary, cooking and behavioral practices related to different aspects were collected, which were addressed during the education sessions and covered based on their baseline levels of information (Annexure II to VI).

3.5 DIETARY INTAKE ASSESSMENTS

Food and nutrient intake is an important component of nutritional status assessment. The 24 hour dietary recall and food frequency methods were used to record the dietary intake of the subjects. Both baseline and end intervention data regarding dietary intake was collected using these tools.

3.5.1 DIETARY RECALL- 24 HOURS

This method is based on the process of recall of food consumption over a specified period of time (24 hour), prior to the survey. The ingredients recalled by the respondents are measured using standard cups and spoons. From the cooked amount, the raw ingredients as well as their nutritive values are calculated using recipes of the conventional cooked foods. In certain cases on the day of survey, if the subject had not consumed sufficient diet than their routine life due to some constraint, then they were motivated to recall the previous days diet/ their regular dietary practice. Thus, it helped to avoid the chances of under estimation of dietary intake.

Procedure: The food intake of subjects was noted for the previous day through individual interviews. Amount of cooked food eaten in each meal was recorded using standard measures. Food consumed by the subjects outside home was also recorded. The nutrient content of food consumed was calculated using the food composition tables (NIN 2009). (Annexure VII).
3.5.2 FOOD FREQUENCY PATTERN

Food frequency method is usually used to assess the habitual food intake of the subjects and their families qualitatively. The subjects were asked to respond to frequencies of consumption of each food from a list of foods, rich in iron, vitamin C, iodine, protein rich and green leafy vegetable (Annexure VIII). The frequencies were listed from daily to never into 9 different categories. It is an accepted method to estimate usual dietary intake (Thompson and Byers 1994).

3.6 IQ AND COGNITIVE FUNCTION TESTS

Iodine and iron deficiencies adversely affect the ability of the school children to learn. The IQ and cognitive functions of the school children were assessed using selected tests from the Gujarati version of Wechsler Intelligence Scale for children (WISC) (Bhatt 1973 and Phatak P 2002), which has been pretested on different groups of population and were modified for the present study groups. The WISC is a battery of tests for 6-17 years olds, which assesses intellectual abilities. The various tests used were Draw-a-man, Visual memory test and Clerical test.

3.6.1 DRAW-A-MAN TEST

This test is used to assess the intelligent quotient of the children and their brain development. Hence, this test was selected considering the effect of iodine deficiency on IQ of the children.

Procedure: A child is asked to draw a picture of a full human being in the space on a blank sheet of paper and score were analyzed further using specific scales mentioned in the manual “Draw a Man Test for Indian Children” (Pramila Pathak, 2002). The use of human figure to measure maturity (intelligence) is based upon the development of concept of human body. The scale includes scoring of major body parts and their crude proportions for 25 different scoring points.

Scoring: The scoring was carried out for the quality of each body part drawings, matched with the age wise standard drawings provided in the manual. The scores ranged from 1-5 for each body part. Then the total scores for all the 25 parts made the final score (leads to
mental age calculation using table 8 in the manual), which in turn made the final IQ score using a formula as below:

\[ \text{IQ} = \frac{\text{Mental Age}}{\text{Chronological age}} \times 100 \]

3.6.2 VISUAL MEMORY TEST

Visual memory test is used to assess the short term memory of the school children.

**Procedure:** Twenty commonly used items (pictures), which they observed in their daily life which were a part of their routine activity was selected. These pictures were presented in a coloured picture form on a visible chart form a 10 feet distance. The children were allowed to observe the objects for one minute, and then the chart was removed from the display. The children were asked to recall and list down the items which they had observed within 2 minutes. A single score point was rewarded for each item listed correctly.

**Scoring:** The score was calculated as the ratio of number of items correctly identified against the total number of items, i.e. 20. The highest score was one.

3.6.3 CLERICAL TEST

Clerical test helps to assess the ability of the children to concentrate and discriminate

**Procedure:** The children were given a typed sheet of Gujarati alphabets pre-tested in a different school who belonged to the same group (Kuruvilla. A. Nair S and Patel A 2005; Nair S and Dutta S 2009). They were required to encircle a particular letter assigned by the investigator in Gujarati within 5 minutes. The children were instructed not to encircle any other letters at all. The investigator motivated the children to encircle as many as they could in 5 minutes.

**Scoring:** Score points were rewarded by providing the maximum points for the maximum letters encircled. All the particular letters in sheet was repeated 32 times in the given section. The children had to find and circle them. The final score was calculated as the ratio of the number of letters encircled by the children against the total number of letter repeated in the section i.e. 32. The highest score was one.
3.7 DATA ANALYSIS

All the data were processed, entered and analyzed in the Statistical Package for Social Sciences for windows version 15.00 (SPSS 15.0) and growth indices were analyzed in Epi-Info, Version 3.5.3 (2011), Microsoft Excel 2007, Windows XP and WHO Anthroplus (Neonatal anthropometric indices- phase I).

Simple descriptive analysis of the data was carried out. Statistical analysis was performed using Chi-square ($\chi^2$) when appropriate for categorical data.

Normality of the data was assessed by the Kolmogorov-Smirnov test. Where indicated, the data was normalized using log transformation to facilitate the use of normal-theory analytic methods.

Nonparametric (Mann-Whitney U test, Kruskal-Wallis test and Wilcoxon test) or parametric (Student’s t-test, paired t test and ANOVA) statistical tests, depending on the normality of the data, were used to detect within-group and between group differences. Further post-hoc bonferroni analysis was done. To determine associations between analytes Pearson’s correlation or Spearman’s rank correlation were calculated. 95% CI was calculated to reveal $\pm 2\text{SD}$ from the mean values or percent prevalence. A two-tailed p values $<0.05$ was considered statistically significant.

3.8 Conduction of research work

This section contains pictures captured in the field and laboratory during the work was being conducted.