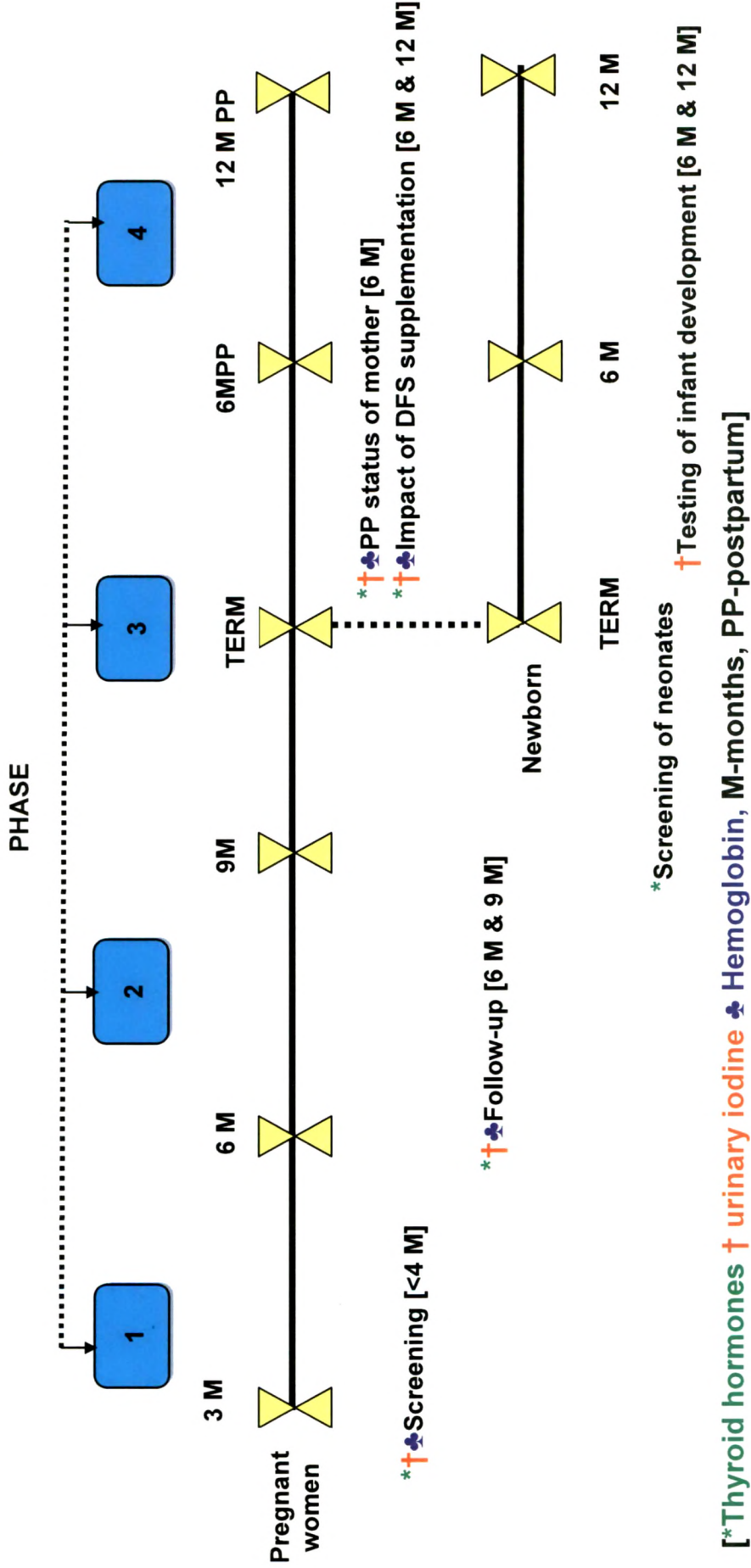# MATERIALS
AND
METHODS

MATERIALS AND METHODS

Present study is carried out in Vadodara district of Gujarat. Iodine deficiency disorder (IDD) is a public health problem in Gujarat and Vadodara district was considered as a new pocket of IDD (Brahmbhatt et al, 2001). Iodine Deficiency was identified as a public health problem in Porbandar district in 2011 (Kotecha et al, 2011). Many studies have been conducted in Different states of Gujarat to study the prevalence of iodine deficiency and thyroid function in school aged children. However, no data is available on prevalence of iodine deficiency and thyroid dysfunction in pregnant women and infants of Vadodara.

The study is divided into four phases:

Phase I	
[Jan. to Mar. 2010]	Screening of pregnant women for thyroid dysfunction during early pregnancy
Phase II:	
[Apr. to Sept 2010]	Follow-up of pregnant women, Nutrition Health Education (NHE)
Phase III:	
[Sept. Oct. Nov. 2010]	Screening of neonates
Phase IV:	
[Jan. Feb. Mar. 2011]	Postpartum status of mother, Testing of Infant Development
[Apr. May. June 2011]	Impact of double fortified salt (DFS) supplementation on maternal iodine and iron status

STUDY TIMELINE

PHASE

Pregnant women: 3 M — 6 M — 9 M — TERM — 6MPP — 12 M PP

Newborn: TERM — 6 M — 12 M

*†♣Screening [<4 M]

*†♣Follow-up [6 M & 9 M]

†♣PP status of mother [6 M]
†♣Impact of DFS supplementation [6 M & 12 M]

*Screening of neonates

†Testing of infant development [6 M & 12 M]

[*Thyroid hormones † urinary iodine ♣ Hemoglobin, M-months, PP-postpartum]
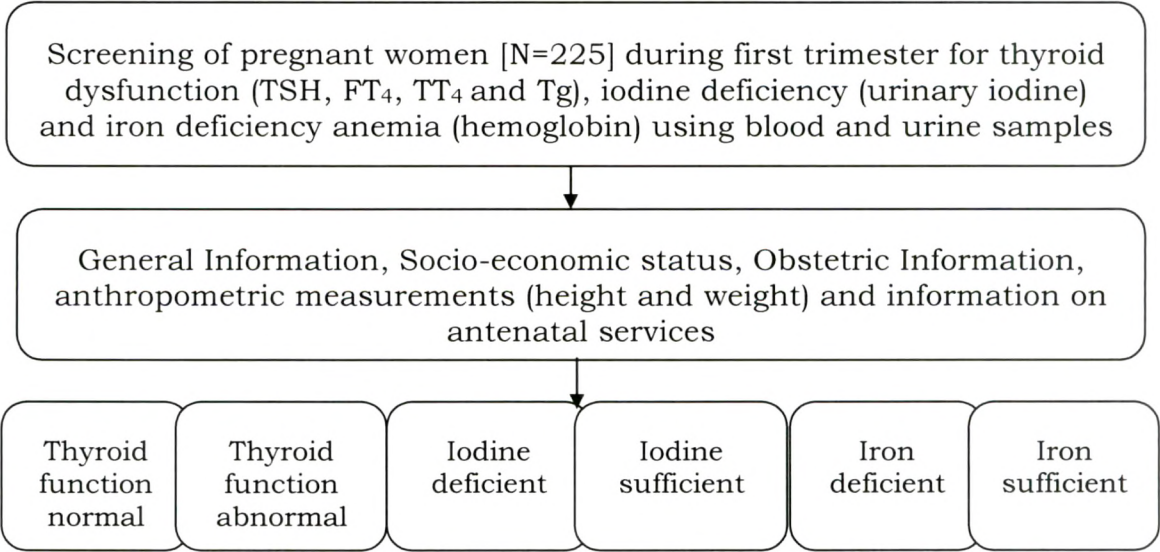
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Phase I: Screening of pregnant women during early pregnancy

In December 2009, a survey was carried out in all (low budget) government/general hospitals of Vadodara for identifying a hospital which is having highest delivery rate and where most of the low income group (LIG) pregnant women go for delivery. Hence the study was focused using a single hospital enrolment of pregnant women, who represent the general population of pregnant women of urban Vadodara.

Between January to March 2010, pregnant women who checked in for antenatal assessment in Jamnabai General Hospital (JGH) were enrolled for the study. JGH is Vadodara's most popular hospital among LIG people. It has a delivery rate of 250-300/month, which is highest among all hospitals in Vadodara. People from different parts of Vadodara and nearby villages, come to JGH for availing antenatal services, immunization and delivery.
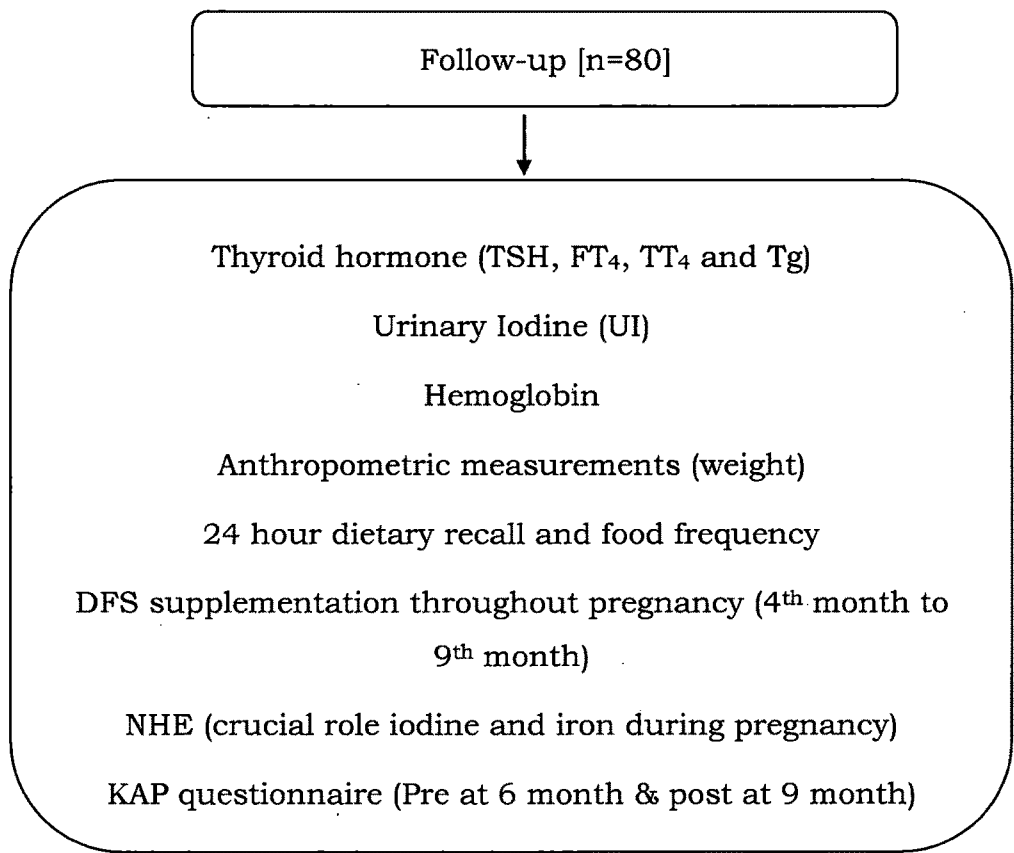
A total of 225 pregnant women during first trimester were screened for thyroid dysfunction, iodine deficiency and iron deficiency anemia. All pregnant women were given a consent form in local language (Gujarati) and the purpose of the study was explained to them. After obtaining consent from them, general information, socio-economic status, obstetric history, anthropometric measurements, etc. were recorded (Appendix- i).

Screening of pregnant women [N=225] during first trimester for thyroid dysfunction (TSH, FT_4, TT_4 and Tg), iodine deficiency (urinary iodine) and iron deficiency anemia (hemoglobin) using blood and urine samples

↓

General Information, Socio-economic status, Obstetric Information, anthropometric measurements (height and weight) and information on antenatal services

| Thyroid function normal | Thyroid function abnormal | Iodine deficient | Iodine sufficient | Iron deficient | Iron sufficient |

Phase II: Follow-up of pregnant women and Nutrition Health Education (NHE)
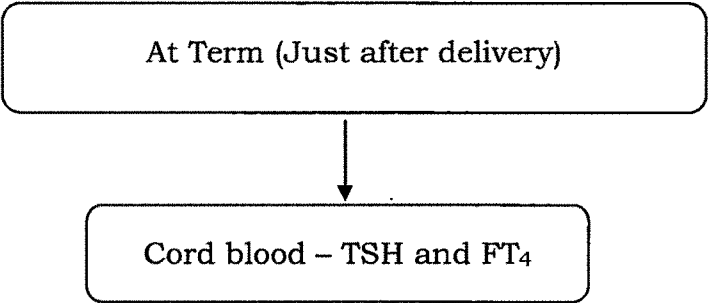
In the second phase, follow-up of pregnant women during second trimester and third trimester was carried out on a sub sample (n=100). Due to various reasons (change in address, change in contact number or not interested in further participating in the study) all 100 pregnant women were not continued in the follow-up. All three trimesters data was collected from 80 pregnant women.

During this phase NHE was provided to all the pregnant women irrespective of their screening result. Again blood and urine samples were collected for thyroid hormone status, iodine deficiency and iron deficiency anemia. Additional information was also availed like-dietary information, anthropometric measurements and testing their knowledge regarding importance of iodine and iron during early pregnancy. Each component mentioned in Mamta card was explained to them (TT injection, BP check-up, regular weight check-up, danger signs etc.).

Follow-up [n=80]

Thyroid hormone (TSH, FT₄, TT₄ and Tg)

Urinary Iodine (UI)

Hemoglobin

Anthropometric measurements (weight)

24 hour dietary recall and food frequency

DFS supplementation throughout pregnancy (4th month to 9th month)

NHE (crucial role iodine and iron during pregnancy)

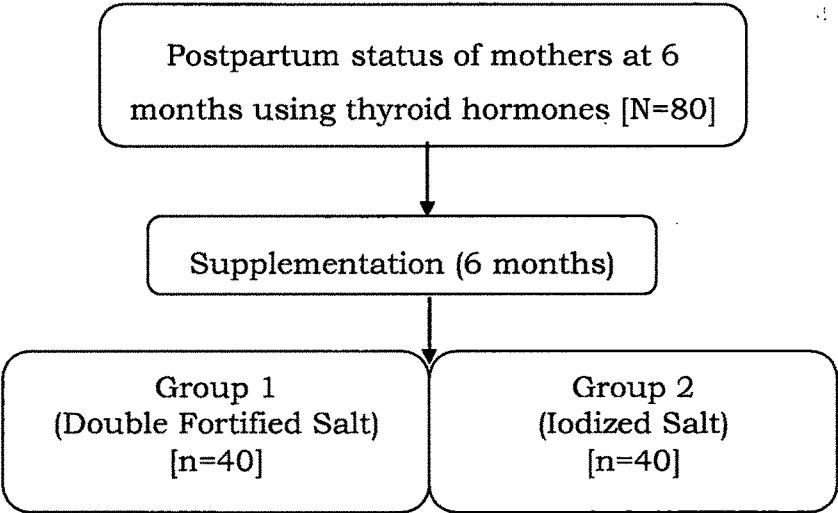KAP questionnaire (Pre at 6 month & post at 9 month)

Phase III: SCREENING of neonates

Screening of neonates was carried out using cord blood samples. During this phase delivery care information of mothers was also recorded. NHE was given to them using Manta Card regarding- care of newborn and care of herself, importance of colostrum and exclusive breastfeeding. Anthropometric measurements (birth weight, length and head circumference) of the neonates were also recorded.

```
┌─────────────────────────────────────────┐
│      At Term (Just after delivery)       │
└─────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────┐
│        Cord blood – TSH and FT4          │
└─────────────────────────────────────────┘
```

Phase IV: Postpartum status of mother

After 6 months postpartum, mothers were again called up with the baby for checking their thyroid status. Blood samples were collected from mothers for testing thyroid hormones. Weight of the mothers was also recorded. NHE was provided to mothers using Mamta card regarding initiation of complementary feeding along with breastfeeding. Next immunization details for their child were explained to mothers.
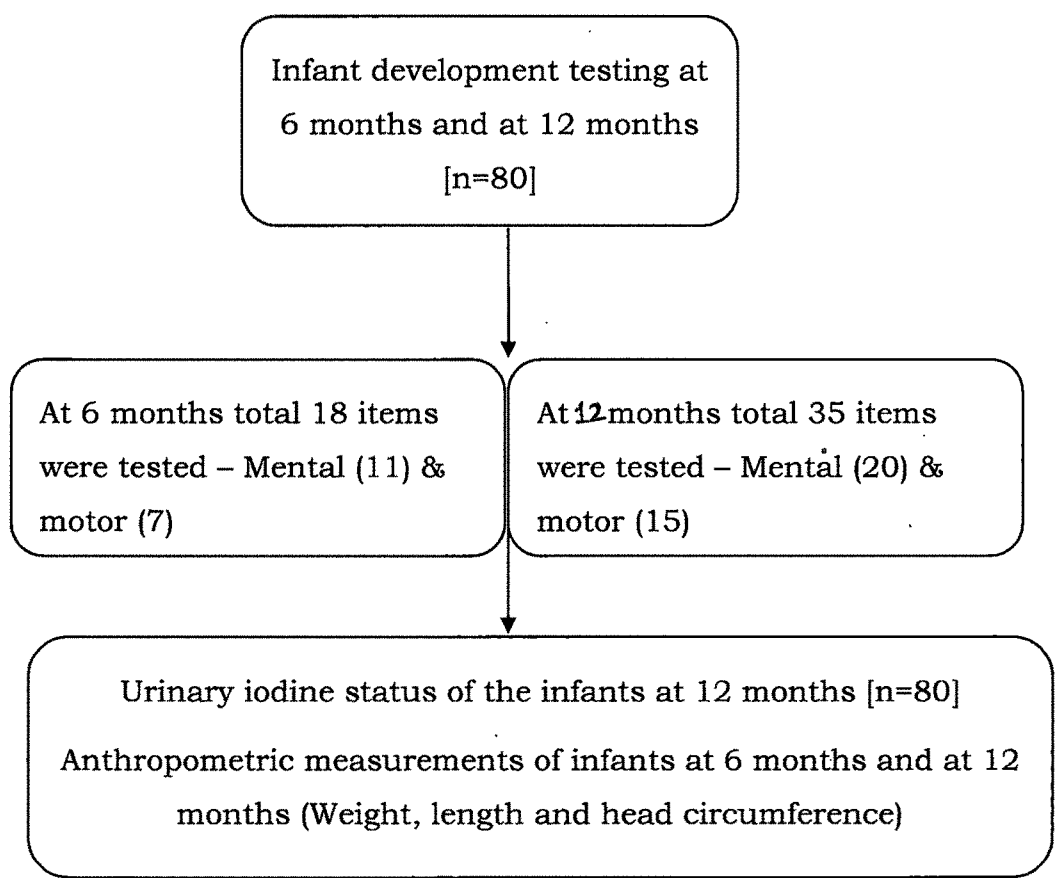
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┌─────────────────────────────────────────┐
│   Postpartum status of mothers at 6      │
│   months using thyroid hormones [N=80]   │
└─────────────────────────────────────────┘
                     │
                     ▼
        ┌─────────────────────────┐
        │  Supplementation (6 months) │
        └─────────────────────────┘
                     │
                     ▼
┌──────────────────────┬──────────────────────┐
│      Group 1         │      Group 2         │
│ (Double Fortified Salt) │   (Iodized Salt)   │
│      [n=40]          │      [n=40]          │
└──────────────────────┴──────────────────────┘
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Impact of double fortified salt (DFS) supplementation on maternal iodine and iron status

Mothers were provided DFS for 6 months along with control group. Urine and blood samples were collected from mothers at 6 months (pre data) and at 12 months (post data) for urinary iodine and hemoglobin.

Testing of Infant Development

Infant development was tested twice, first at 6 months and second at 12 months using BDSTI (Baroda Norms). Anthropometric measurements of the infants were also recorded at 6 months and at 12 months along with immunization details. Urine samples were collected from infants at 12 months for checking their iodine status. Additional information was also recorded regarding health of the child.

```
┌─────────────────────────────────┐
│  Infant development testing at  │
│  6 months and at 12 months      │
│  [n=80]                         │
└─────────────────────────────────┘
                │
                ▼
┌──────────────────────┐ ┌──────────────────────┐
│ At 6 months total 18 │ │ At 12 months total 35│
│ items were tested –  │ │ items were tested –  │
│ Mental (11) & motor  │ │ Mental (20) & motor  │
│ (7)                  │ │ (15)                 │
└──────────────────────┘ └──────────────────────┘
                │
                ▼
┌─────────────────────────────────────────────────┐
│ Urinary iodine status of the infants at 12       │
│ months [n=80]                                    │
│ Anthropometric measurements of infants at 6      │
│ months and at 12 months (Weight, length and      │
│ head circumference)                              │
└─────────────────────────────────────────────────┘
```

Methods Used

3.1 Collection of urine samples and storage

3.2 Collection of blood samples and storage

3.3 Anthropometric Measurements

3.4 Dietary Assessment Methods

3.5 Nutrition Health Education (NHE) and Knowledge Attitude and Practices (KAP)

3.6 Double Fortified Salt supplementation

3.7 Determination of Urinary Iodine

3.8 Thyroid Hormone Analysis

   Determination of Thyroid Stimulating Hormone (TSH)

   Determination of Free Thyroxine (FT4)

   Determination of Thyroglobulin (Tg)

   Determination of Total Thyroxine (TT4)

3.9 Determination of Hemoglobin (Hb) Concentration

   Method: Sahli (Acid Hematin) method

   Method: Cyanmethemoglobin method

3.10 Infant Development Testing

Ethical approval

Statistical analysis

3.1 Collection of Urine samples and storage

Urine samples were collected in sterile 50 ml bottles (Tarson). After collection of urine samples in sterile bottles, they were transferred to ependof tubes of 1.5 ml (Tarson) rest of the sample was stored in the bottle with addition of toluene till analysis. A total of 625 bottles were utilized for the study.

3.2 Collection of blood samples and storage

Hemoglobin: Blood samples were collected in EDTA coated tubes (BD) of 4ml capacity. Hemoglobin was estimated on the same day. A total of 160 tubes were used.

Thyroid hormones: Blood samples were collected in plain tubes (BD) of 4 ml capacity and for cord blood 7 ml tubes were used. Sample was allowed to clot, after 15 minutes samples were centrifuged and serum was separated. Serum was transferred in small vial of 1.5 ml (Tarson) capacity and stored at -18⁰C in deep freezer. A total 515 of plain tubes were used.

3.3 ANTHROPOMETRIC MEASUREMENTS

Weight

Mother: Weight of pregnant women was measured in kg (to the nearest 100 gm) using a simple weighing scale (standardized).

Baby: Weight of the baby was measured in kg (to the nearest 10 gm) using electronic infant weighing scale. Infants were weighed with minimum clothing and excessive infant movements were avoided during measurement.

Height/Length

Mother: Height of pregnant women was measured in cm (to the nearest 0.1 cm) using a tape mounted on wall. While measuring height following things were taken care off-

- Subject was bare footed
- Was standing with heels together, arms to the side, legs straight, shoulders relaxed
- Position the head in the Frankfort horizontal plane ("look straight ahead")

- Heels, buttocks, scapulae (shoulder blades), and back of the head should be against the wall.
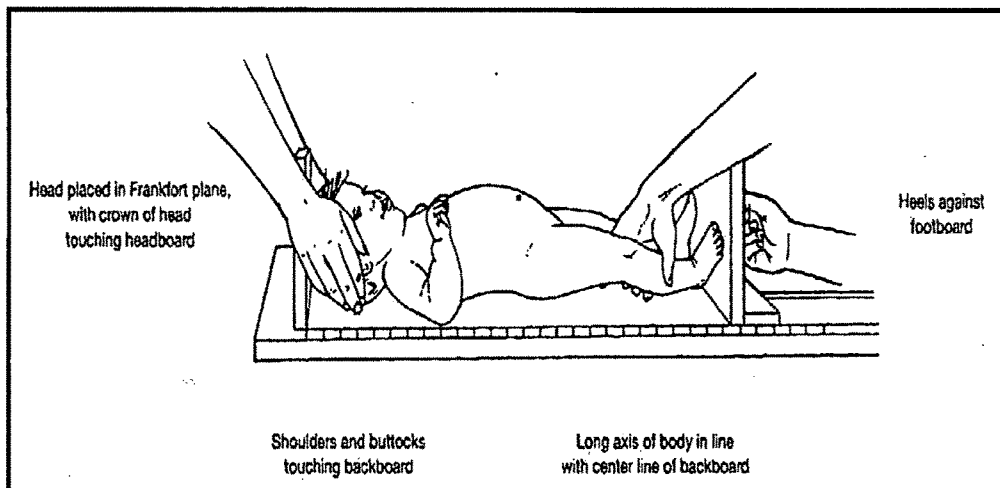

Figure 3.1: Measurement of length of infant

Baby: Length of the baby was measured in cm (to the nearest 0.1 cm) using an infantometer. Two persons were required for taking the measurement. One person holds the child's head against the backboard with the crown securely against the headboard and with Frankfort plane perpendicular to the backboard. This person also keeps the long axis of the child's body aligned with the center line of the backboard, the child's shoulders and buttocks securely touching the backboard, and the shoulders and hips at right angles to the long axis of the body. The other person keeps the child's legs straight and against the backboard, slides the footboard against the bottom of the feet (without shoes or socks) with the toes pointing upward, and reads the measurement. The footboard should be pressed firmly enough to compress the soft tissues of the soles but without diminishing the vertebral column length.

Head Circumference

Head circumference of the baby was measured (to the nearest 0.1 cm) using a flexible tape. It was used as a measure of brain development.

Body Mass Index (BMI)

BMI was used as an indicator of nutrition status.

$$BMI = Weight in kg / Height in (m)^2$$

Table 3.1: BMI reference for adults

Classification	BMI(kg/m²)	
	Principal cut-off	Additional cut-off
Underweight	**<18.50**	**<18.50**
Severe thinness	<16.00	<16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal range	**18.50 - 24.99**	**18.50 - 22.99**
		23.00 - 24.99
Overweight	**≥25.00**	**≥25.00**
Pre-obese	25.00 - 29.99	25.00 - 27.49
		27.50 - 29.99
Obese	**≥30.00**	**≥30.00**
Obese class I	30.00 - 34.99	30.00 - 32.49
		32.50 - 34.99
Obese class II	35.00 - 39.99	35.00 - 37.49
		37.50 - 39.99
Obese class III	≥40.00	≥40.00

Source: WHO, 2004

Child growth standards for infants

Weight, length and head circumference were used to assess the growth of infant and size of their brain. Four anthropometric index were used which are- weight-for-age, length-for-age, weight-for-length and head circumference-for-age.

1. **Weight-for-age** reflects body weight relative to age and is influenced by recent changes in health or nutritional status. It is not used to classify infants, children and adolescents as under or

overweight. However, it is important in early infancy for monitoring weight and helping explain changes in weight-for-length.

2. **Length-for-age** describes linear growth relative to age. Length-for-age is used to define shortness or tallness.

3. **Weight-for-length** reflects body weight relative to length and requires no knowledge of age. It is an indicator to classify infants and young children as overweight and underweight.

4. **Head circumference-for-age** is critical during infancy. Head circumference measurements reflect brain size. Very small and very large are both indicators of health or developmental risk.

Each of these indices was expressed in standard deviation units (Z-scores) from the median of the reference population. These were calculated using WHO anthro plus software (version 3.1 2011).

Table 3.2: Cut-offs used for classifying nutritional status of infants based on WHO reference standards (2006)

Classification	Cut-off points
Weight-for-age	
Underweight	<-2SD
Normal	-2SD to +2SD
Overweight	>+2SD
Length-for-age	
Under length/short	<-2SD
Normal	-2SD to +2SD
Above average/tall	>+2SD
Weight-for-length	
Thin	<-2SD
Normal	-2SD to +2SD
Overweight	>+2SD
Head circumference-for-age	
Very small	<-2SD
Normal	-2SD to +2SD
Very large	>+2SD

3.4 DIETARY ASSESSMENT METHODS

24-hour dietary recall

For the 24-hour dietary recall, the respondents were asked to remember and report all the foods and beverages consumed in the preceding 24 hours or in the preceding day (Appendix-iv).

Food Frequency

For food frequency respondents were asked to report their usual frequency of consumption of each food, mainly foods rich in iron and vitamin C (Appendix-v).

3.5 NUTRITION HEALTH EDUCATION (NHE) AND KNOWLEDGE ATTITUDE AND PRACTICES (KAP)

NHE was provided as intervention to pregnant women using posters. Key messages included in the poster were

- Importance of iodine during early brain development.
- Role of iron in reducing risk of maternal and child mortality
- How to recognize iodized salt
- Healthy cooking and storage practices to minimize iodine loss from iodized salt

The idea was to provide knowledge regarding the importance of iodine and iron nutrition during early pregnancy and to change their incorrect practices of cooking and storing iodized salt.

For measuring the outcome of NHE, a KAP questionnaire was used (before and after NHE intervention).

3.6 DOUBLE FORTIFIED SALT SUPPLEMANTATION

Iodine (30 ppm) and iron (1 ppm) fortified salt was given to pregnant women throughout pregnancy and 6 months postpartum. This was procured from ANKUR Chem. Foods Ltd., Gandhidham. A total of 720 kg of DFS was used. However, a total of 1,000 kg of salt was procured from Ankur. This was ordered in split, 500 kg during pregnancy and 500 kg during postpartum period. This was done to avoid loss of iodine due to storage.

3.7 DETERMINATION OF URINARY IODINE

UIC estimation was performed at Molecular Diagnostics Laboratory, Lucknow. Prior to that 7 days practical training was availed from ICCIDD laboratory AIIMS, New Delhi under the guidance of Prof. M. G. Karmarkar.

Method: Simple micro plate method (Ohashi et al, 2000)

- Urine iodine estimation include initial step in which urine is digested in strong acid, ashed at high temperature or use of chemical agents such as ammonium per sulphate. Following digestion step, iodine is measures by its catalytic action on the reduction of Ceric ion (Ce^{4+}) to cerous ion (Ce^{3+}) coupled to oxidation of arsenite (As^{3+}) to (As^{5+}), this reaction is called Sandell-Kolthoff reaction.

- Ceric ion has yellow colour, while cerous is colourless. *(The colour disappearance is directly proportional to amount of iodine present in the system)*

- The initial step of digestion is necessary as it removes substances like nitrite, thiocyanate or ferrous ions which may interfere by reducing or oxidizing the ceric or arsenite reactants.

Principle

Urine is digested with ammonium per sulphate. Iodine is the catalyst in the reduction of ceric ammonium sulphate (yellow) to cerous form (colourless), and is detected by rate of colour disappearance (Sandell-Kolthoff reaction).

Reaction

$2Ce^{4+}$ (ceric) + $2I^-$ $\longrightarrow$ $2Ce^{3+}$ (cerous)   + I_2

I_2 + As^{3+} $\longrightarrow$ As^{5+} + $2I^-$
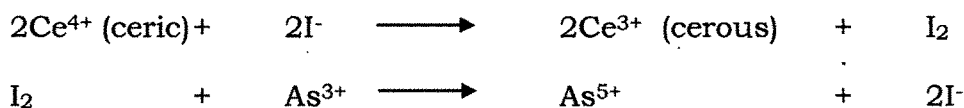
Equipments and apparatus required

1. Polypropylene SEROCLUSTER 96-well micro plates (Corning Costar Japan)

2. Standard oven (which can maintain temperature of 95°C)

3. Stainless steel cassette (sealing cassette, specially designed by Hitachi Chemical Techno-plant)

4. Microplate reader (ELISA reader)

5. Measuring devices (Micropipettes 0-100 μl, multichannel 8 plate micropipette 0-100 μl, standard pipettes- 10 ml, 5 ml, 1 ml)

6. Vortex mixer

7. Weighing machine

8. Micro tips

Chemicals Required

Ammonium Per Sulphate ($H_8N_2O_8 S_2$)

Arsenic Trioxide (As_2O_3)

Ceric Ammonium Sulphate [$Ce(NH_4)_4 (SO_4)4 .2H_2O$]

Concentrated Sulphuric Acid (H_2SO_4)

Sodium Hydroxide (NaOH)

Sodium Chloride (NaCl)

Potassium iodate (KIO_3)

(Glass distilled deionized water was used for preparation of reagent solution and dilution procedures)

Preparation of reagents

Ammonium per sulphate solution (1.31 mol/l) - Ammonium per sulphate (30 g) was dissolved in water to a final volume of 100 ml.

(Prepare fresh every day)

Arsenious acid solution (0.05 mol/l) - Arsenic trioxide (5 g) was dissolved in 100 ml of 0.875 mol/l sodium hydroxide solution. Concentrated sulphuric acid (16 ml) was then added slowly to the solution in an ice bath. After cooling, 12.5 g of sodium chloride was added to the solution, and the mixture was diluted to 500 ml with cold water and filtered.

(Filter and store in amber coloured reagent bottle, store at 4ºC)

***Ceric ammonium sulphate solution (0.019 mol/l)*-**Tetra ammonium cerium (IV) sulphate dihydrate (6 g) was dissolved in 1.75 mol/l sulphuric acid and adjusted to a final volume of 500 ml with the same acid solution.

Iodine calibrators - In a 100 ml volumetric flask, 168.6 mg of potassium iodate was dissolved in water to make a 7.88 mmol/l stock solution (1000 mg/l iodine). The stock solution was diluted 100- and 10 000-fold, and working solutions of 0.039 – 4.73 mmol/l (5– 600 mg/l iodine) were prepared.

Steps

1. In microplate pipette out 50 μl of standards known values samples and urine samples in the wall as shown below.

2. A1 (blank) - nothing was added.

3. In the first 2 columns standards were added (as shown in figure) and in H2 a known value sample was added.

4. From column 3 to 12, urine samples to be analyzed were added.

5. Add 100 μl of Ammonium Per Sulphate (freshly prepared) in each well (using multi-channel pipette).

6. Put the urine plate in heating cassette. Seal the cassette and put it in oven at 95ºC, for 90 minutes.

7. Take out the cassette from oven and allow to cool to room temperature.

8. Take out the digested microplate and take another microplate and transfer 50 μl of digested standards and urine samples in corresponding wells (using multi-channel pipette).

9. Add 100 μl of Arsenious Acid solution (using multichannel pipette). Wait for 15 minutes and then add 50 μl of Ceric Ammonium Sulphate in each well (using multi-channel pipette).

10. Measure optical density (OD) using Microplate reader at 405 nm. Plot the graph using log of absorbance on Y-axis vs iodine

concentration (µg/l) on X-axis. Extrapolate the values of urine samples from the graph.
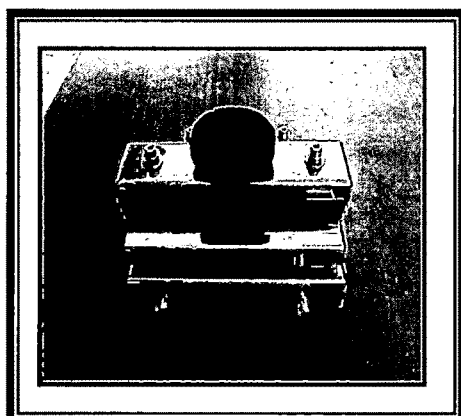

Figure 3.2: Sealing cassette (The cassette is used for digestion after a micro plate is placed inside) The sealing cassette includes a Teflon (fluorinated ethylene propylene)-laminated silicon rubber gasket and a handle to seal the wells of a micro plate

Table 3.3: Epidemiological criteria from the WHO for assessment of iodine nutrition in a population based on median or range of UI

UI (µg/L)	Iodine Intake
Pregnant women	
<150	Insufficient
150-249	Adequate
250-499	More than adequate
≥500[a]	Excessive
Lactating women[b]	
<100	Insufficient
≥100	Sufficient
Children <2 years of age	
<100	Insufficient
≥100	Sufficient

A The term excessive means in excess of the amount needed to prevent and control iodine deficiency.
b In lactating women, the numbers for median UI are lower than the iodine requirements because of the iodine excreted in breast milk.

Source: WHO/UNICEF/ICCIDD, 2007

Figure 3.3: Sample of microplate for urinary iodine analysis

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	100	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample
B	0	200	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample
C	0	200	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine Sample
D	10	300	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine Sample
E	10	300	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine Sample
F	20	400	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine Sample
G	20	400	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine Sample
H	100	Known value sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine sample	Urine sample	Urine Sample	Urine sample	Urine sample	Urine Sample

Standard curve for Urinary iodine

Concentration of UIE (µg/l)
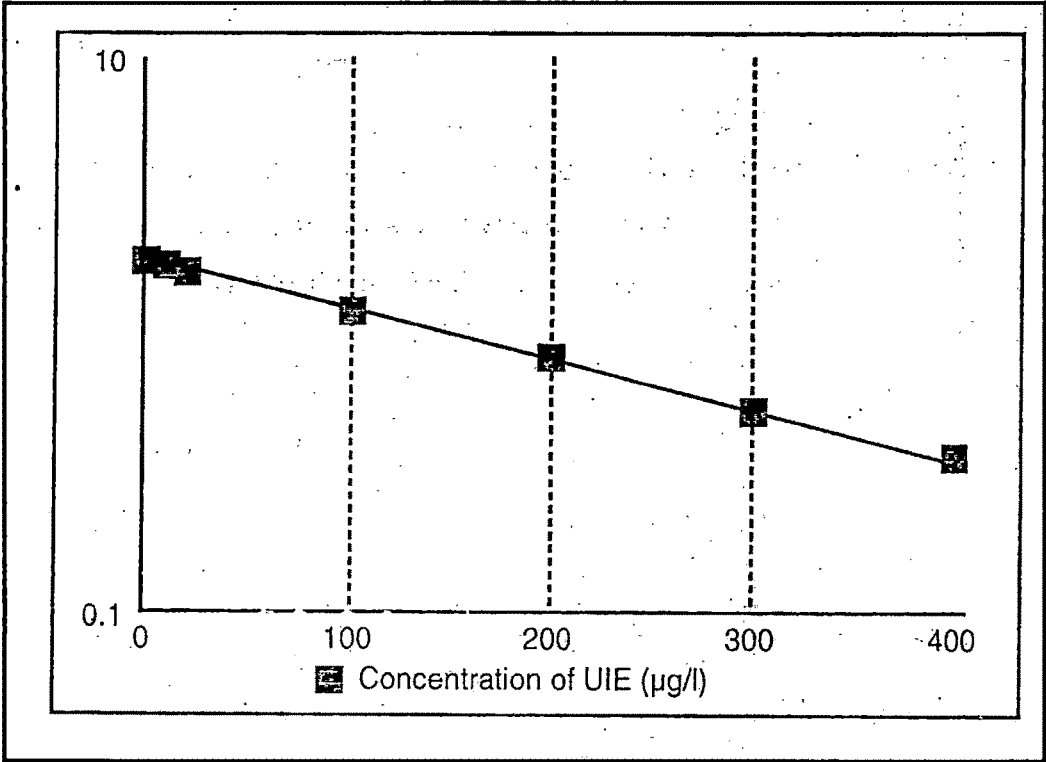
Standard	Concentration (µg/l)	Log of absorbance
S_1	0	1.8484
S_2	10	1.7459
S_3	20	1.2474
S_4	100	1.0597
S_5	200	0.7859
S_6	300	0.5062
S_7	400	0.3282

3.8 THYROID HORMONE ANALYSIS

Thyroid hormone analysis was performed at Radiation Medicine Centre, Bhabha Atomic Research Centre, Tata Memorial Cancer Hospital, Parel, Mumbai. Prior to this, six day training was availed from the same place under the guidance of Dr. M. G. R. Rajan and Mrs. Chandrakala S. Gholve.

Method: Radio Immuno Assays (RIA)

Principle: To perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma radioactive isotopes of iodine attached to tyrosine. This radiolabeled antigen is then mixed with a known amount of antibody for that antigen, and as a result, the two chemically bind to one another. Then, a sample of serum from a patient containing an unknown quantity of that same antigen is added. This causes the unlabeled (or "cold") antigen from the serum to compete with the radiolabeled antigen ("hot") for antibody binding sites. As the concentration of "cold" antigen is increased, more of it binds to the antibody, displacing the radio labeled variant, and reducing the ratio of antibody-bound radio labeled antigen to free radio labeled antigen. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigen remaining in the supernatant is measured using a gamma counter. Using known standards, a binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived.

Precautions

General remarks

- Bring all reagents to room temperature before pipetting

- Do not mix the reagents from kits of different lots.

- A standard curve must be included with each assay.

- The correct setting of the shaker is very important for the reproducibility of the assay.

- It is recommended to perform the assay in duplicate.

- Each tube must be used once only.

Basic rules of radiation safety

- No eating, drinking, smoking or application of cosmetics should be carried out in the presence of radioactive materials.

- No pipetting of radioactive solutions by mouth.

- Avoid all contact with radioactive materials by using gloves and laboratory overalls.

- All manipulation of radioactive substances should be done in an appropriate place, distant from corridors and other busy places.

- Radioactive materials should be stored in the container provided in a designated area.

- A record of receipt and storage of all radioactive products should be kept up to date.

- Laboratory equipment and glassware which are subjected to contamination should be segregated to prevent cross-contamination of different radioisotopes.

- Each case of radioactive contamination or loss of radioactive material should be resolved according to established procedures.

- Radioactive waste should be handled according to the rules established in the country of use.

Equipments and apparatus required

Precision micro pipettes

Semi-automated pipettes

Vortex mixer

Horizontal or orbital shaker

Aspiration system

Aspiration system

Gamma counter set for ^{125}I

Figure 3.4: Stratec SR300 Automated Radio Immunoassay Analyzer BARC Mumbai

STRATEC SR 300 is a fully automated radioimmunoassay analyzer containing the following modules:

1. Pipetting station
2. Washing station
3. Incubating station
4. Gamma counter

Determination of Thyroid Stimulating Hormone (TSH)

Principle

The Immuno Radio Metric Assay (IRMA) of thyroid stimulating hormone is a 'sandwich' type assay. Mouse monoclonal antibodies directed against two different epitopes of TSH (antigen) and hence not competing 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 added, 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 samples or calibrators are incubated in tubes coated with the first monoclonal antibody in the presence of the second monoclonal antibody labelled with ^{125}I. After incubation the content of tubes is aspirated and the tubes are rinsed so as to remove unbound ^{125}I labelled antibody. The bound radioactivity is then determined in a gamma counter. The TSH concentrations in the samples are obtained by interpolation from the standard curve. *(The concentration of TSH in the samples in directly proportional to the radioactivity)*

Kit: IMMUNOTECH, Beckman Coulter, Prague, Czech Republic

Performance characteristics of the kit

Analytical sensitivity:	0.025 mIU/l
Functional sensitivity:	0.141 mIU/l
Measurement range :	0.025-50 mIU/l

Reagents provided in kit:

Anti-TSH antibody-coated tubes: 100 tubes (ready to use)

^{125}I-labelled monoclonal anti-TSH antibody: 11 ml vial (ready to use)

Calibrators: seven 1 ml vials (ready to use), the calibrator vials contain from 0 to 50 mIU/l of TSH in bovine serum with sodium azide (0.1 %). The exact concentration in indicated on each vial label. The

calibrators were calibrated against the international standard, WHO 2nd IRP 80/558.

Control serums: two vials (lyophilized), the vials contain TSH lyophilized in bovine serum. The expected values are in the concentration range indicated on the vial label.

Wash solution (20x): one 50 ml vial, concentrated solution has to be diluted before use.

Reagent Preparation – let all the reagents come to room temperature.

Reconstitution of control samples – the content of the vials is reconstituted with the volume of distilled water indicated on the label. Wait for 30 minutes following reconstitution and mix gently to avoid foaming before dispensing. Store the reconstituted solutions at 2-8ºC for one day or aliquoted at <-18 ºC for a longer time, until the expiry date of the kit.

Preparation of the wash solution -pour the content of the vial into 950 ml of distilled water and homogenize. The diluted solution may be stored at <-18 ºC for a longer time, until the expiry date of the kit.

Standard curve for TSH
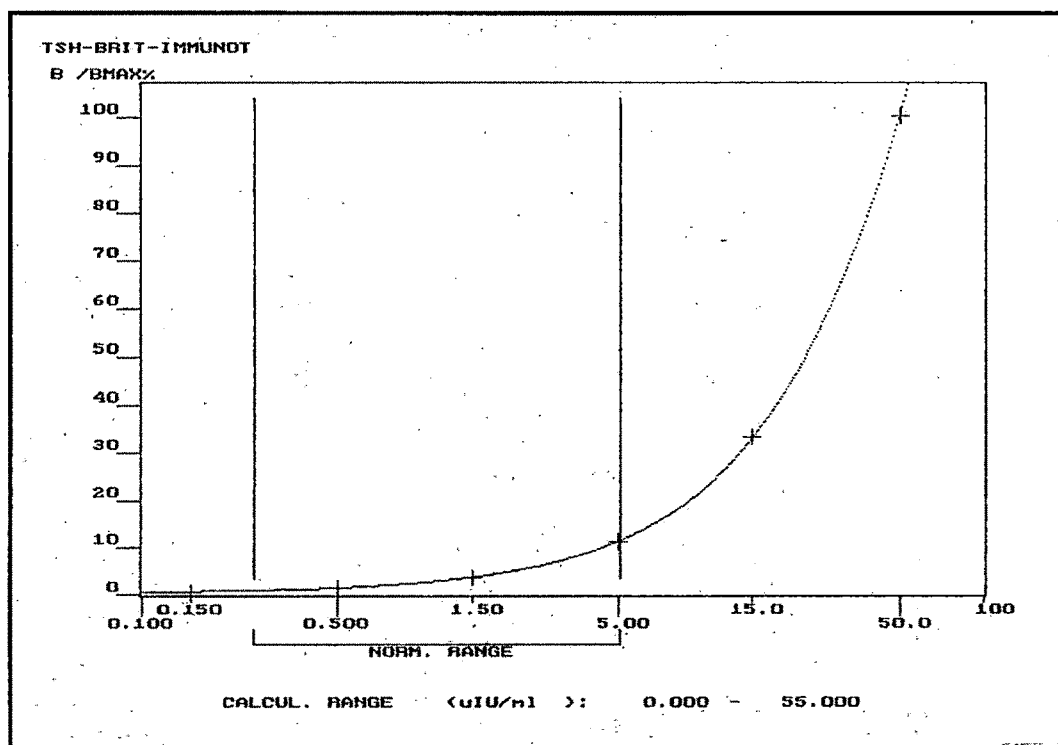
Normal value: 0.25-5.10 µIU/ml

Serial no.	Tube no.	Expected value (μIU/ml)	Actual value (μIU/ml)	Difference %	Percentage bound (% B)
1	S_1	0.000	0.000	-	0.1
2	S_2	0.150	0.150	-	0.4
3	S_3	0.500	0.500	-	1.2
4	S_4	1.500	1.500	-	3.5
5	S_5	5.000	5.000	-	11.0
6	S_6	15.000	15.000	-	32.9
7	S_7	50.000	50.000	-	100.0

Assay procedure

Step 1Additions*	Step 2Incubation	Step 3Counting
To coated tubes, add successively-

100 μl of calibrator, control or sample
100 μl of tracer

Mix | Incubate 1 hour at 18-25°C with shaking (>280 rpm) | Aspirate carefully the contents of tubes (except the 2 tubes <<total cpm>>)

Wash twice with 2 ml of wash solution

Count bound cpm (B) and total cpm (T) for 1 minute |

*add 100 μl of tracer to 2 additional tubes to obtain total cpm

Figure 3.5: Sandwich ELIZA method
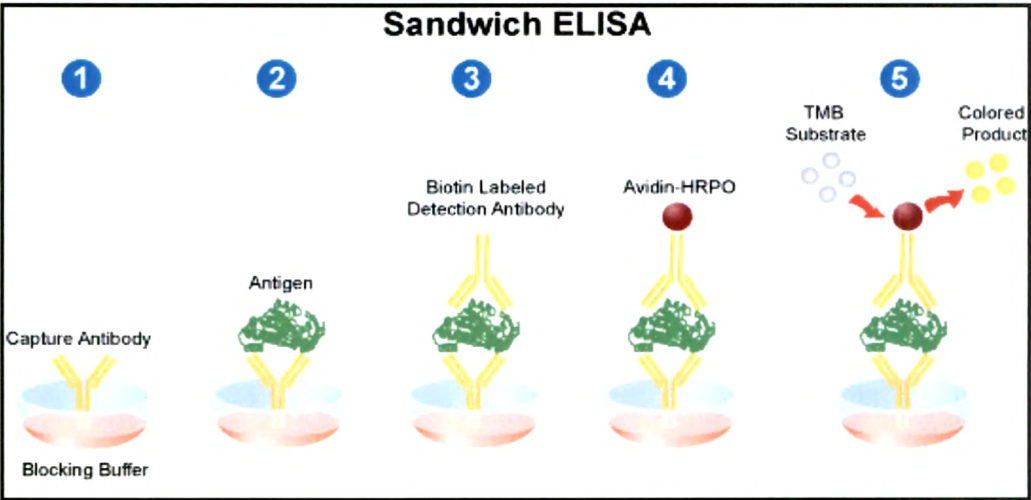
Determination of Free thyroxine (FT4)

Principle

The Radio Immuno Assay of free thyroxine (FT4) is a competition assay based on the principle of labelled antibody. Samples and calibrators are incubated with 125I labelled monoclonal antibody specific for T4, as tracer, in the presence of a biotinylated analog of thyroxine (ligand) in avidin-coated tubes. There is competition between the free thyroxine of the sample and the ligand for the binding to the labelled antibody. The fraction of antibody complexes with the biotinylated ligand binds to avidin-coated tubes. After incubation, the content of tubes is aspirated and bound radioactivity is measured. A calibration curve is established and unknown values are determined by interpolation from the curve.

Kit: IMMUNOTECH, Beckman Coulter, Prague, Czech Republic

Performance characteristics of the kit

Analytical sensitivity:	0. 5 pM
Functional sensitivity:	2.4 pM
Measurement range :	0.5-75 pM

Reagents provided in kit:

Coated tubes for the binding of ligand: 1000 tubes (ready to use)

125I monoclonal antibody: one 45 ml vial (ready to use), the vial contains 310 kBq, at the date of manufacture, of 125I-labelled immunoglobulins in liquid form with bovine serum albumin, sodium azide (<0.1%) and a dye.

Calibrators: five 0.5 ml vials (ready to use), the calibrator vials contain from 0 to 75 pM of free thyroxine in human serum and sodium azide (<0.1 %). The exact concentration is indicated on each vial label. The vials must be tightly capped immediately after pipetting and stored at 2-8°C. The evaporation of the calibrator solutions from

open vials may influence results of the further determination. Calibrators are verified to an internal reference standard.

Ligand: one 12 ml vial (ready to use), the vial contains a ligand solution which includes also bovine proteins and sodium azide (< 0.1 %).

Control serum: one vial (lyophilized), the vial contains T_4 in human serum. The expected values are in the concentration range indicated on the supplement. The vial must be tightly capped immediately after pipetting and stored according to #. The evaporation of the solution from open vial may influence results of the further determination.

Attention: all liquid reagents should be examined for the absence of precipitates; the antibody solution should be clear and blue-green, the calibrators may be opalescent and the ligand should be clear and colourless.

**Reagent preparation** – let all the reagents come to room temperature.

Reconstitution of control samples – the content of the vials is reconstituted with the volume of distilled water indicated on the label. Wait for 10 minutes following reconstitution and mix gently to avoid foaming before dispensing. Store the reconstituted solutions at 2-8°C for one week or aliquoted at <-18 °C for a longer time, until the expiry date of the kit.

Assay procedure

Step 1 Additions*	Step 2 Incubation	Step 3Counting
To coated tubes, add successively-		Aspirate carefully the contents of tubes (except the 2 tubes <<total cpm>>)
25 µl of calibrators, control or sample and 400 µl of tracer 100 µl of ligand Mix	Incubate 1 hour at 18-25°C with shaking (>350 rpm)	Count bound cpm (B) and total cpm (T) for 1 minute

*add 400 µl of tracer to 2 additional tubes to obtain total cpm

Standard Curve for FT4

FT4 Immunotech
B /BMAX%

CALCUL. RANGE (ng/dL): 0.000 - 6.495
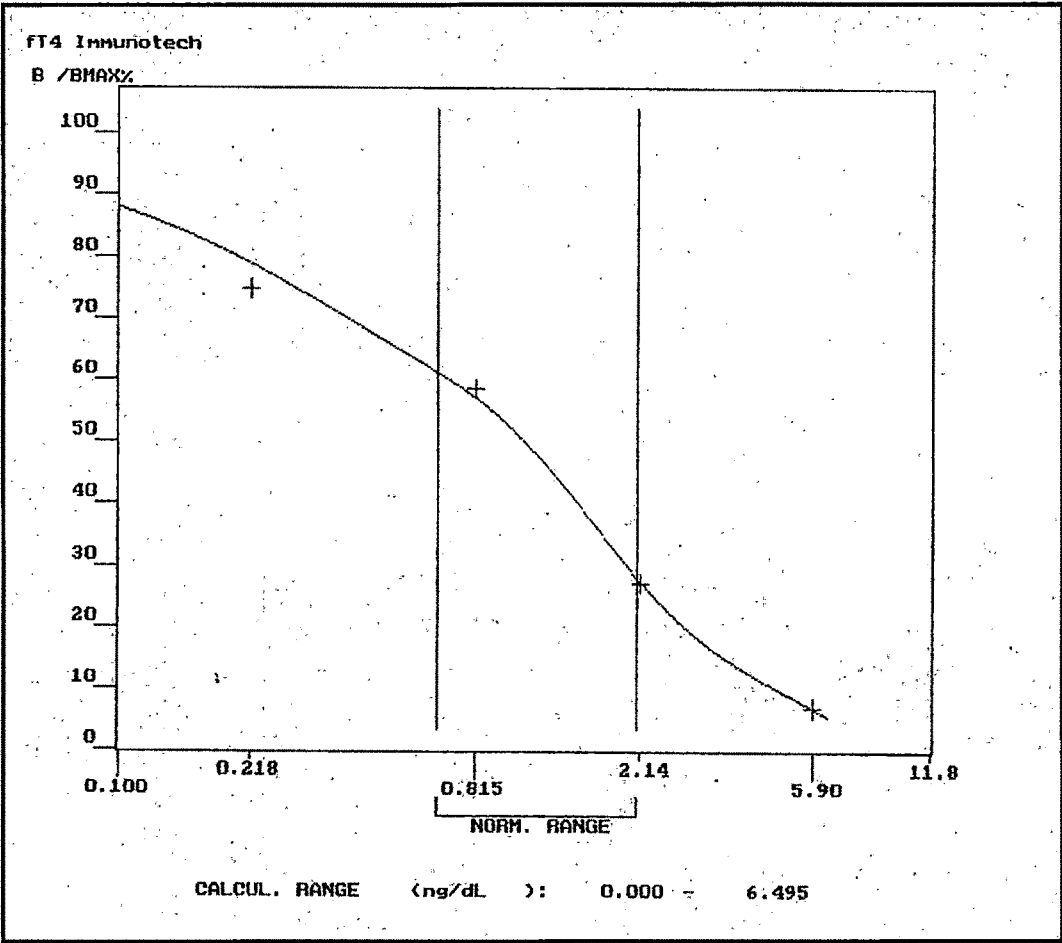
Normal Value: 0.65-2.10 ng/dl

Serial no.	Tube no.	Expected value (ng/dl)	Actual value (ng/dl)	Difference %	Percentage bound (% B)
1	S1	0.000	0.001	-	100.0
2	S2	0.218	0.281	-29.36	74.7
3	S3	0.815	0.760	6.81	58.1
4	S4	2.144	2.154	-0.48	27.0
5	S5	5.905	5.897	0.14	6.6

Determination of Thyroglobulin (Tg)

Principle

The Radio Immuno Assay of thyroglobulin (Tg) is a competition assay based on the principle of labelled antibody. Samples and calibrators are incubated with 125I labelled monoclonal antibody specific for Tg, as tracer, in the presence of a biotinylated analog of thyroxine (ligand) in avidin-coated tubes. There is competition between the thyroglobulin of the sample and the ligand for the binding to the labelled antibody. The fraction of antibody complexes with the biotinylated ligand binds to avidin-coated tubes. After incubation, the content of tubes is aspirated and bound radioactivity is measured. A calibration curve is established and unknown values are determined by interpolation from the curve.

Kit: BARC (In-house)

Reagents provided in kit:

1. Standard Tg- 8 vials containing 10 ml each (conc. 0, 12.5, 25, 50, 100, 200, 400, 800 ng/ml)
2. Tg free serum: 1 vial (10 ml), ready to use
3. r-antiTg antibody: 1 vial (10 ml)
4. Labelled Tg: 1 vial (10 ml), ready to use
5. Control: 2 vials (10 ml)
6. DAB-magnetic particles: 1 vial (10 ml)

Reagent preparation

PBS-EDTA: dissolve 35.49 g Na_2HPO_4 (0.25M); 8.76 g NaCl (0.15M); 3.72 g EDTA (0.01M) and 1 g sodium azide in 800 ml distilled water. Adjust pH 7 and raise the volume to 1000 ml.

Standard Tg: Thyroid tissue is collected and connective tissue is removed. It is then chopped into small pieces and extracted in normal saline. It is separated by salting-out method with ammonium sulphate. This is further purified by separation of Sephadex G-200 column chromatography followed by another column chromatography (Sepharose-6B). Purified native Tg is obtained. Tg obtained in this way is from a single specimen and is used in preparation of standards, labelled Tg and to produce anti-Tg antibodies in rabbits.

r-antiTg antibody: Normal rabbit is subcutaneously injected with 400 to 500 µg of purified native Tg in complete Freund's adjuvant. Following the initial dose, booster doses with the same amount of Tg was administered after 2-4 weeks respectively. The rabbits were than bled at fortnightly intervals after the final booster and the sera obtained were tested for its antibody activity in an Ouchterlony double diffusion system against purified Tg, thyroid extract and normal serum. The anti-sera gave a single intense precipitin line with purified Tg and thyroid extract. However, no precipitin line was visible in reaction with normal human serum. The antibodies were stored at -20°C till further use. Before use, diluted (1:75000) in PB-EDTA.

DAB-magnetic particles: Rabbit serum is separated by fractional precipitation using ammonium sulphate, followed by DEAE-Sephadez A-50 ion exchange chromatography and were used for immunizing a goat. The rabbit used here is a different one (not the one in which Tg was injected).

Assay procedure for Tg

Tube	Buffer (µl)	Free serum (µl)	Tracer 125I (µl)	Standard / Serum/ Control (µl)	Anti-serum (µl)		DAB-Magnetic Particles (µl)	
Total counts	-	-	100	-	-	Incubate at 40oC for 3 overnights	-	Add 0.4 ml assay buffer and leave it for 20 minutes on magnetic racks, decant and count
S1	100	100	100	100	100		100	
S2	-	100	100	100	100		100	
S3	-	100	100	100	100		100	
S4	-	100	100	100	100		100	
S5	-	100	100	100	100		100	
S6	-	100	100	100	100		100	
S7	-	100	100	100	100		100	
S8	-	100	100	100	100		100	
Control	100	-	100	100	100		100	
Sample	100	-	100	100	100		100	

Procedure

1. Following components were added to polystyrene tubes in sequence. The tubes were made in duplicates.

   a) Standard Tg (100 µl, range: 0-800 ng/ml)

   b) Tg free serum (100 µl) tubes containing standard Tg and 100 µl of PBS-EDTA buffer in the serum sample tubes.

   c) r-anti-Tg antibodies (100 µl, final dilution, 1:75000) in PBS-EDTA buffer.

   d) Labelled Tg (100 µl).

2. The tubes were incubated for 3 overnights at 4°C.

3. After incubation, 50 µl of DBA- magnetic particles were added to each tube and after incubated for 2 hours on shaker.

4. After 2 hours, 1 ml of PBS-EDTA buffer was added and left for 20 minutes on magnetic rack.

5. Decanted and dried, the tubes were counted for bound radioactivity.

Standard curve for Tg
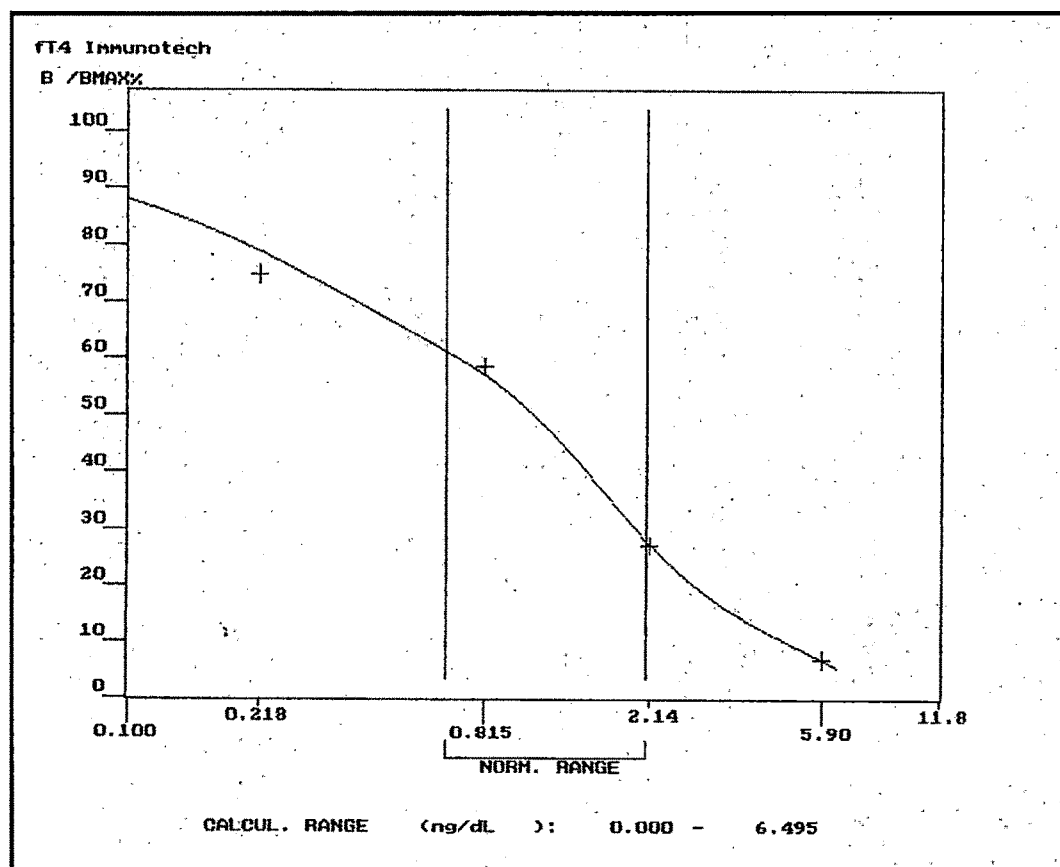
Normal value: 0.0-50.0 ng/dl

Serial no.	Tube no.	Expected Value (ng/ml)	Actual value (ng/ml)	Difference %	Percentage bound (% B)
1	S_1	0.15849	0.15849	-	47.84
2	S_2	12.50000	10.84695	-13.22	40.39
3	S_3	25.00000	25.34220	1.37	34.79
4	S_4	50.00000	52.40718	4.81	28.62
5	S_5	100.00000	109.94882	9.95	21.99
6	S_6	200.00000	202.87504	1.44	17.11
7	S_7	400.00000	368.38590	-7.90	13.32
8	S_8	800.0	736.12403	-7.98	10.19

Determination of Total Thyroxine (TT₄)

Principle

The Radio Immuno Assay of thyroxine (T₄) is a competition assay based on the principle of labelled antibody. Samples and calibrators are incubated with ^{125}I labelled monoclonal antibody specific for Thyroxine, as tracer, in the presence of a biotinylated analog of thyroxine (ligand) in avidin-coated tubes. There is competition between the thyroxine of the sample and the ligand for the binding to the labelled antibody. The fraction of antibody complexes with the biotinylated ligand binds to avidin-coated tubes. After incubation, the content of tubes is aspirated and bound radioactivity is measured. A calibration curve is established and unknown values are determined by interpolation from the curve.

Kit: BARC (In-house)

Reagents provided in kit:

Standard T₄- 5 vials containing 10 ml each (conc. 0, 2.5, 5, 10, 20, µg/dl), ready to use

Tracer: 1 vial (10 ml), ready to use

**Antibody-magnetic particles:** 1 vial (20 ml), ready to use

Control: 2 vials (10 ml), ready to use

Procedure

1. Bring all the reagents to room temperature.
2. Pipette 100 µl of standard, control or sample in the respective tubes.
3. Add 100 µl of tracer (^{125}I labelled T$_4$) to it followed by 1000 µl of antibody-magnetic particles.
4. Vortex all the tubes in vortex mixture.
5. Cover the tubes with aluminium foil and incubate the tubes in a 37°C water bath for 2 hours.
6. Keep the racks containing assay tubes over a magnetic rack for 15 minutes.
7. Decant the tubes into sink (used for liquid-radioactive discard) and leave the tubes inverted over absorbent sheet for 10 minutes.
8. Wipe the mouth of the tubes with a tissue paper wick while the rack is inverted and still in the magnetic field.
9. Count the tubes in a Gamma Counter for 1 minute; plot the standard graph of % bound and T4 concentration. Extrapolate % bound values of unknown samples to determine T4 concentration.

TSH IRMA kit   **FT4 RIA kit**
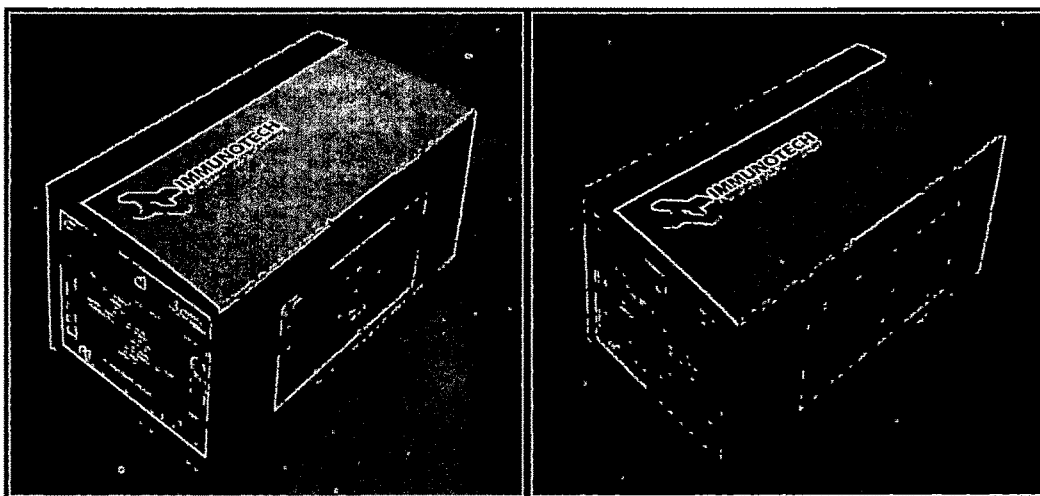
Assay procedure for TT4

Tube	Standard/control/ Sample (µl)	Tracer (µl)	Antibody Magnetic Particles (µl)
Standard 1	100	100	100
Standard 2	100	100	100
Standard 3	100	100	100
Standard 4	100	100	100
Standard 5	100	100	100
Control 1	100	100	100
Control 2	100	100	100
Control 3	100	100	100
Test Sample	100	100	100
Vortex all the tubes, Incubate all the tubes in 37oC water bath for 2 hours. Keep them on magnetic racks			
Decant the tubes and dry them			
Count the tubes on Gamma Counter for 1 minute			

Standard curve for TT4

TEST-TT4MAGDILSA
B /BMAX%

CALCUL. RANGE (ug/dL): 0.000 - 40.000
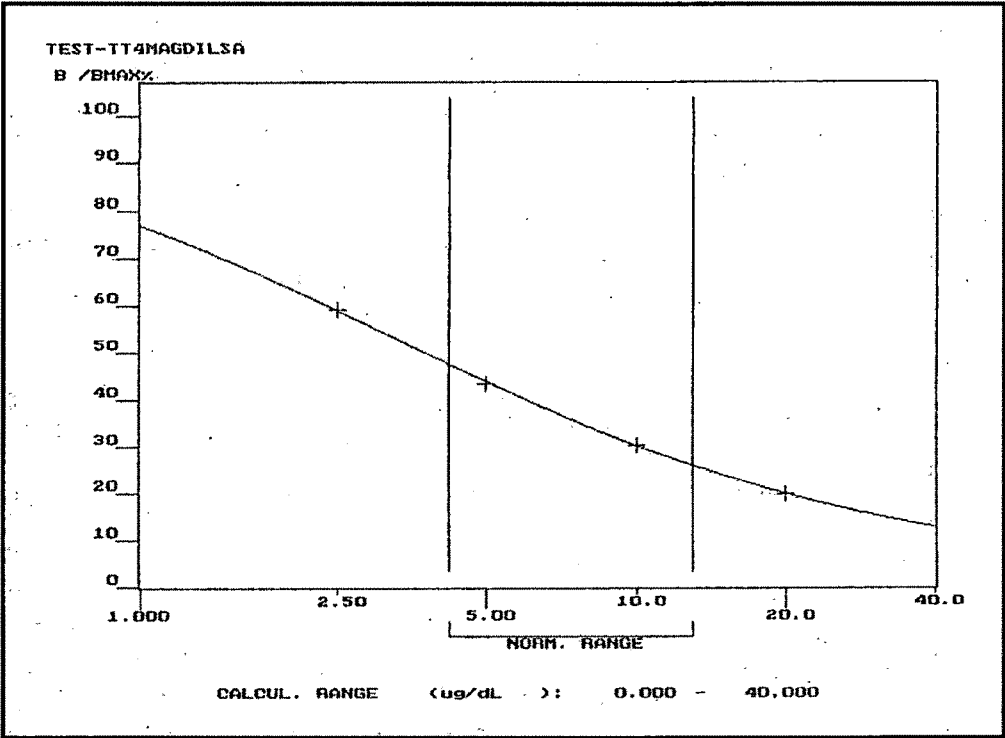
Normal value: 4.20-13.00 µg/dl

Serial no.	Tube no.	Expected value (µg/dl)	Actual value (µg/dl)	Difference %	Percentage bound (% B)
1	S_1	0.002	0.002	-	100.0
2	S_2	2.500	2.463	1.48	58.9
3	S_3	5.000	5.078	-1.56	43.2
4	S_4	10.000	9.957	0.43	30.0
5	S_5	20.000	19.997	0.02	19.5

3.9 DETERMINATION OF HEMOGLOBIN (HB) CONCENTRATION

Method: Sahli (Acid Hematin) method

Principle

When blood is added to 0.1 N Hydrochloric Acid hemoglobin is converted to brown coloured acid hematin. The resulting colour after dilution is compared with standard brown glass reference blocks of a sahli hemoglobinometer.

Specimen: Capillary blood or thoroughly mixed anti-coagulated (EDTA), venous blood (0.02 ml).

Requirements

Sahil hemoglobinometer: it consists of-

a. A standard brown glass mounted on a comparator
b. A graduated tube
c. Hb pipette (0.02 ml)

0.1 N HCL, distilled water and Pasteur pipettes

Method

1. By using a Pasteur pipette, add 0.1 N HCl acid in the tube upto the lowest mark (20 % mark).

2. Draw blood upto 20 µl mark in the Hb-pipette, adjust the blood column, carefully without bubbles. Wipe excess of the blood on the sides of the pipette by using a dry piece of cotton.

3. Transfer blood to the acid in the graduated tube, rinse the pipette well, mix the reaction mixture and allow the tube to stand for atleast 10 minutes.

4. Dilute the solution with distilled water by adding few drops at a time carefully add by mixing the reaction mixture, until the colour matches with the glass plate in the comparator.

5. The matching should be done up against natural light, the level of the fluid is noted at its lower meniscus and the reading corresponding to this level on the state is recorded in g/dl.

Remarks

This method is useful for places where a photometer is not available.

It can give an error of even 1 g/dl

Immediately after use rinse the Hb pipette by using tab water in beaker. This prevents blocking of the pipette.

Method: Cyanmethemoglobin method (Winkleman, 1974)

Principle

Hemoglobin is oxidized to methemoglobin. Met hemoglobin reacts with potassium cyanide to form cyanmethemoglobin, which is measured photometrically. The concentration of hemoglobin in the sample is directly proportional to the intensity of the coloured complex which is measured at 540 nm (520-560 nm or with green filter).

Hemoglobin + potassium ferricyanide $\xrightarrow{\textit{Oxidation}}$ Methemoglobin

Methemoglobin $\xrightarrow{\textit{Potassium Cyanide}}$ Cyanmethemoglobin

Kit: Reckon Diagnostics P. Ltd., Vadodara

Characteristics of the kit- linear upto 25 gm/dl

Regents provided

1. Drabkin's reagent (1 bottle/1000ml)

2. Hemoglobin standard (5ml, strength 60 mg/dl)

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Reagent storage and stability

The reagent provided is stable at room temperature until the expiry date printed on the label. Hemoglobin standard is stable at 2 to 8 °C until the expiry date printed on the label. Hemoglobin reagent contains potassium cyanide, hence use automated pipettes. Avoid ingestion.

Requirements

Test tubes, Pipettes (5 ml and 0.2 ml) and Spectrophotometer

Specimen: Capillary blood or thoroughly mixed anti-coagulated (EDTA), venous blood (0.02 ml).

Procedure

Pipette into test tubes	Test
Working reagent (ml)	5.00
Sample (ml)	0.02

Mix by inversion and read absorbance against distilled water after 3 minutes at 540 nm. The colour stability of the reaction mixture is 24 hours. Read the standard directly without dilution.

For a spectrophotometer or standard instrument (with regular calibrations) use of standard is not required. Use factor directly or read in the chart.

Details of calculation are given below-

For colorimeter-

Calculation Hb

$$(g/dl) = \frac{Absorbance\ of\ Test}{Absorbance\ of\ Standard} \times \frac{Dilution\ of\ Sample}{1} \times \frac{Strength\ of\ Standard}{1000}$$

Calculation Hb (g/dl) $= \frac{Optical\ Density\ of\ Test}{Optical\ Density\ of\ Standard} \times 15$

For spectrophotometer-

Calculation Hb (g/dl)$= Absorbance\ of\ test \times F\ (F = 36.77)$

Where $F = \frac{Mol.\ weight\ of\ Hb}{mmol.\ Ext.\ Coeff.} \times \frac{Dilution\ Factor}{1000} \times \frac{1}{10}$

Mol wt. of Hb= 64458

Mmolar Extinction Coefficient of Hb= 44.0

Dilution Factor= 251

Table 3.4: Hemoglobin cut-offs to define iron deficiency anemia during pregnancy

HB (g/dl)	Degree of IDA
<7	Severe
7-9.9	Moderate
10-10.99	Mild
≥11	Normal

Source: UNICEF, 2001

3.10 INFANT DEVELOPMENT TESTING

Method: Baroda Development Screening Tests for Infants (BDSTI)

BDSTI contains 54 items (32 mental and 22 motor) for evaluating child's development from 2-30 months, these items are selected from Bayley Scales of Infant development (BSID). BSID has a total of 230 items (163 mental and 67 motor) for testing development of a child from 2-30 months. Many items of the BSID use standardized equipment (cubes, pegboard, form-board etc.) and standardized techniques (certain performances timed by stop-watch). The BSID is a detailed test and has many items for testing the development of one skill-some of them very close in the development sequence. For instance, there are ten items related to the skill of sitting. Items like- sits with support, effort to sit, sits alone momentarily, sits alone 30 seconds or more and sits down require some experience and good judgment on the part of the health worker. Only those items which were simple and easy to administer and to asses and not requiring any special training, experience and equipment were selected. Since in present study, infants were followed till 12 months only. Hence, a total of 35 (20 mental and 15 motor) items out of 54 items from full scale of BDSTI (Baroda norms) have been used for present study (Appendix-iii).

Table 3.5: Phatak's Screening Test items and age-grouping

Months	Sr. No.	Items
1	1	Arms and legs thirst in play *
	2	Momentary regard
	3	Lateral head movement (prone) *
2	4	Responds to sound
	5	Follows moving person
	6	Free inspection of surroundings
3	7	Social smile/vocalizes
	8	Eye co-ordination
	9	Head erect and steady*
4	10	Holds head steady*
	11	Recognizes mother
	12	Elevates on arms*
5	13	Play with rattle/hand play
	14	Reaches for dangling ring
	15	Sits with slight support*
6	16	Turns head to sound
	17	Turns from back to side*
	18	Exploitive paper play
7	19	Discriminates strangers
	20	Pulls to sit*
8	21	Bangs to play
	22	Sits alone steadily*
9	23	Retails two things in two hands
	24	Pulls to stand*
	25	Playful response to mirror images
	26	Sits with good coordination*
10	27	Pulls string-secures toy
	28	Co-operates in play
	29	Crawling (pre-walking) *
11	30	Rings bell purposefully
	31	Fine prehension*
	32	Raises to sit*
	33	Stands by furniture*
12	34	Adjusts to words
	35	Says da-da

motor development items

Source: Phatak and Khurana, 1991

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Developmental curve

The 50 and 97 % level age placements of each item is placed against its serial number and then joined to have 2 smooth curves, the upper curve representing the 50 % pass level and the lower representing the 97 % pass level (Appendix-ix).

Table 3.6: Screening efficiency of BDSTI

Parameter	BDSTI vs BSID full scale
Sensitivity	65.62 to 93.33
Specificity	77.37 to 94.44
Over-referral	6.67 to 34.37
Under-referral	5.56 to 22.63
Screening validity	76.00 to 94.05

The screening validity, sensitivity and specificity of BDSTI are above 65 %, they can be used as valid for reliable screening tests for early detection of infants with delayed motor and mental development.

Ethical Approval

Permission for the study was obtained from concerned health authorities of the state and ethical approval was obtained from Baroda Medical College, Vadodara, Gujarat, India [ethical approval no: FCScFN ME67].

Statistical Analysis

Data was entered and saved into Microsoft excel (2007). For analyzing data, SPSS (Statistical Package for Social Sciences, version 14.0) and MedCalc (version 12.2.1.0) software were used.

Quantitative (parametric) data is represented as mean ± sd or mean (sd) and non parametric data is represented as median (95 % CI). Categorical data is represented as percentages. Before applying any test, data was tested for normality using Kolmogorov-Smirnov test,

histogram and Q-Q plot. Where indicated data was normalized using log transformations to facilitate the use of normal-theory analytic methods. Data was then back transformed and reported as geometric mean. Such transformation was performed for TSH.

In case of urinary iodine, where data showed skewness, non parametric tests were used.

For determining effect of 3 trimesters, repeated measures of ANOVA was used (paired samples) for parametric data and for non-parametric data Friedman test was used. Similarly for determining effect of pregnancy and postpartum period, one way ANOVA was used (unpaired samples) for parametric data and for non-parametric data Kruskal-Wallis test was used. After performing ANOVA, wherever required poct-hoc test (Bonferoni) was used for checking with-in group and between group differences.

To determine association between variables Pearson's correlation (parametric data) or Spearman's rank (non parametric data) correlation were calculated. Where data was found to be significantly associated further regression analysis (95% prediction level) was performed.

For paired samples paired t-test (parametric data) and Wilcoxon test (non parametric data) were used. Non independent samples, independent t test (parametric data) and Mann Whitney test (non parametric data) were used.

For comparing two diagnosis tests, diagnostic test was used as sensitivity, specificity, positive predictive value and negative values were calculated.

A two-tailed p value <0.05 was considered statistically significant, which was denoted as * [p<0.01 high significance denoted as **, p<0.001 very high significance denoted as *** and p<0.0001 very very high significance denoted as ****].

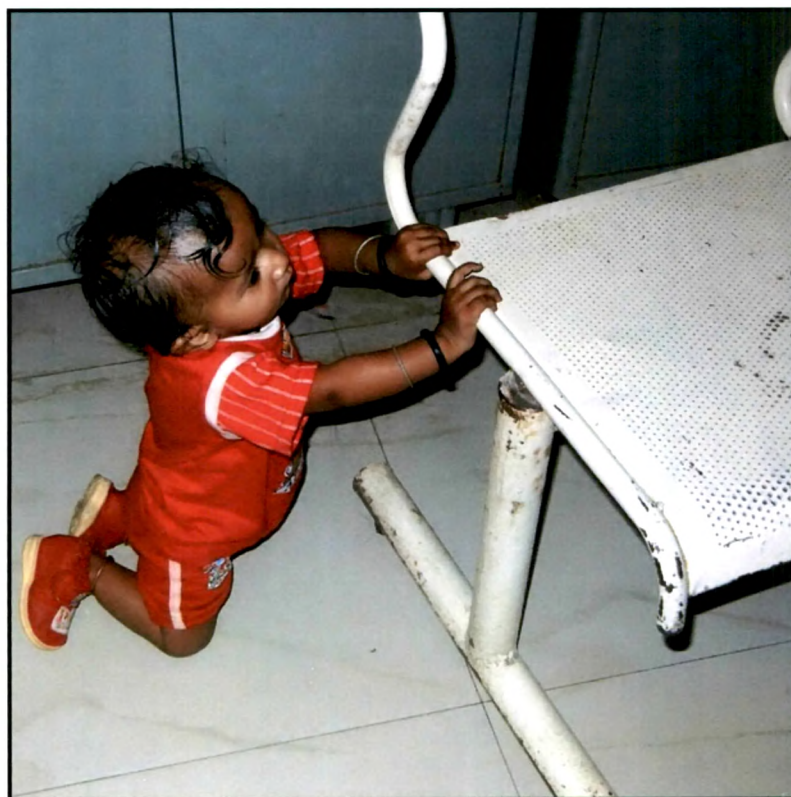**Picture 3.1: Enrolment of pregnant women and counselling**

Picture 3.2: Blood collection and DFS supplementation

Picture 3.3: Child sitting with good co-ordination and holding cubes [retain two things in two hands]

Picture 3.4: Child trying to stand up

Picture 3.5: Child using furniture for walking

Picture 3.6: Child stands up by furniture

Picture 3.7: Parents with their infants

Figure 3.8: Mother with her newborn baby