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## CHAPTER 4

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### *MATERIALS AND METHODS*

This project was executed at the Poultry Research Centre, Department of Poultry Husbandry, University of Agriculture, Faisalabad. Positioners and mechanical feed lifting system were fabricated to provide desired feeding treatments. Provision of 24 hours light was ensured by the fabrication of light sensors and light operated switches, which provided desired duration of light by the combination of natural and artificial light sources.

#### **4.1. Bird Procurement and Grouping**

Three hundred day-old broiler chicks (starbro) were purchased from a local hatchery M/S Al-Noor chicks and reared in a group for one week (adaptation period). After the adaptation period, all the birds were weighed individually and one hundred eighty chicks of middle-weight range ( $\mu \pm 1\sigma$ ) were selected to be used as experimental birds, whereas, the chicks on both the extremes were discarded. The chicks were randomly divided into eighteen experimental units (replicates) having ten chicks each. These replicates were further allotted to six treatments (A, B, C, D, E and F) i.e. 3 replicates per treatment at random.

#### **4.2. Feeding Plans**

Three feeding methods i.e., continuous feeding (CF, 24 hours feeding), intermittent feeding (IF, 1 hour feed and 3 hours off feed) and feed withdrawal (FW, no feed from 9:00 am to 5:00 pm) were used for the study. Under each feeding system birds were fed a ration either without supplemented fat (0%) or the ration with three percent supplemented fat (3%). The feeding schedule for intermittent feeding was:

<b>Feeding Hours</b>	<b>Feed Restriction Hours</b>
12.00 pm to 1.00 am	1.00 am to 4.00 am
4.00 am to 5.00 am	5.00 am to 8.00 am
8.00 am to 9.00 am	9.00 am to 12.00 am
12.00 am to 1.00 pm	1.00 pm to 4.00 pm
4.00 pm to 5.00 pm	5.00 pm to 8.00 pm
8.00 pm to 9.00 pm	9.00 pm to 12.00 pm

The treatments allotted to different groups were:

<i>Group A</i>	Continuous feeding (CF, 24 hour feeding) with commercial ration without fat supplementation (0 %)
<i>Group B</i>	Continuous feeding (CF, 24 hours feeding) with 3% fat supplemented commercial ration (3 %)
<i>Group C</i>	Intermittent feeding (IF, 1 hour feed and 3 hours off) with commercial ration without fat supplementation (0 %)
<i>Group D</i>	Intermittent feeding (IF, 1 hour feed and 3 hours off) with 3% fat supplemented commercial ration (3 %)
<i>Group E</i>	Feed withdrawal (FW, no feed from 9:00 am. to 5:00 pm) with commercial ration without fat supplementation (0 %)
<i>Group F</i>	Feed withdrawal (FW, no feed from 9:00 am. to 5:00 pm) with 3% fat supplemented commercial ration (3 %)

### 4.3. Housing

Each experimental unit was reared in a thoroughly cleaned and disinfected pen measuring 3'×4' and saw dust was used as litter material in the pens. The birds were kept under the same managerial conditions like floor space, light, temperature, ventilation, and relative humidity. Fresh and clean water was provided *ad libitum* throughout the experimental period and amount of water consumed was recorded by the use of automatic watering system specially deigned for experimental purpose.

During the adaptation period of one week, the birds were fed a commercial broiler starter mash. After this the birds were given experimental rations according to the plan as mentioned above, up to the age of six weeks (experimental period). A commercial ration (crumbs) was used during experimental period. The birds during the experiment were also vaccinated according to the following schedule.

#### 4.4. Vaccination Schedule

Age	Vaccine	Rout of vaccination
7 days	ND Mukteswar	Intra ocular
13 days	IBD (Gumboro) D78	Intra ocular
21 days	ND Lasota	Intra ocular
27 days	IBD (Gumboro) D78	Intra ocular

#### 4.5. Temperature (°C) and Relative Humidity (%)

Data regarding temperature and relative humidity inside the experimental room were recorded by using thermometers and hygrometer, respectively. The local atmospheric temperature and relative humidity data recorded at the Crop Physiology Department, Faculty of Agriculture, University of Agriculture, Faisalabad were also obtained to compare the housing conditions with the outside environment.

#### 4.6. Physiological Parameters

##### 4.6.1. Respiration Rate (No./min)

Respiration rate of three birds per replicate was recorded three times a day throughout the experimental period by observing the expansion and contraction of the ribcage exactly during one minute time. Stop watch was used for this purpose.

##### 4.6.2. Body Temperature (°F)

Body temperature of three birds per replicate was recorded three times a day (9:00 am, 4:00 pm and 10:00 pm) by inserting a digital thermometer into the rectum. The thermometer was kept in rectum until the temperature stabilized indicated by a beep sound, which usually took 1 minute per reading.

#### 4.7. Physical Parameters

##### 4.7.1. Body Weight (g)

The body weight of individual birds kept under each experimental unit was recorded weekly throughout the experimental period by subtracting initial body weight from the final weight.

#### **4.7.2. Feed Consumption (g)**

A weekly record of feed consumption was kept for each experimental unit during the experiment.

#### **4.7.3. Feed Conversion Ratio (FCR)**

Feed conversion ratio in terms of feed consumed per kg of live weight gain was calculated from the above data. The economics of different treatments was also worked out using the prevailing cost of inputs and income received from the sale proceeds.

#### **4.7.4. Water Consumption (mL)**

Water consumption was recorded daily along with pan evaporation in order to record actual water consumption per group. Special water tank were developed along with water measuring gauge and were attached to each automatic waterer in the experimental unit.

#### **4.8. Leg Abnormalities**

Gait scoring was conducted as described by (Kestin, 1992). The birds ability to walk was recorded on six point scale (0-5) by the judges. Zero with perfect gait while 5 with maximum abnormality (no walking at all), similarly hock burn and foot burn were scored from 0-3. Zero was taken as no signs of abnormality while three was taken as maximum abnormality (Su *et al.*, 1999).

#### **4.9. Mortality**

Record regarding mortality of experimental birds was maintained in order to find out the death losses under different experimental units and its impact on the economics of the broiler production.

#### **4.10. Slaughter Characteristics**

At the end of the experiment, two birds from each replicate were slaughtered randomly and excised for their dressing percentage and organ weights (liver, gizzard, heart, lungs, kidney, spleen, pancreas, bursa, thymus, adrenal, pituitaries and intestine). The gizzard and intestine of the slaughtered birds were emptied,

whereas the rest of the organs were defatted before weighing. The data thus recorded were used to calculate relative organ weight using the formula:

$$\text{Relative organ weight} = \text{organ weight} / \text{live weight of birds} \times 100$$

Data regarding the length of intestine and weight of abdominal fat pad were also recorded.

## **4.11. Laboratory Analysis**

### **4.11.1. Feed and Meat Analysis**

Feed required for the study was procured from a single batch and then three feed samples were taken for proximate analysis. Whereas, meat samples from the thigh and breast muscles of the slaughtered birds were taken, at the end of experiment. These samples were frozen till analysed. The feed samples were grounded whereas the meat samples were minced thoroughly at the time of their proximate analysis and then further used for analysis.

### **4.11.2. Determination of Dry Matter (%)**

One gram of the sample was placed in hot air oven maintained at 100-105°C. The sample was dried to a constant weight and weight sample was recorded after cooling the sample to room temperature in a desiccator. The DM content was calculated by the following formula:

$$\text{DM (\%)} = (W_2/W_1) \times 100$$

*Where*

$W_1$  is the mass (g) of sample before drying

$W_2$  is the mass (g) of the sample after drying.

### **4.11.3. Crude Protein (%)**

Crude protein was estimated by Kjeldahl's method. The sample was digested with concentrated  $H_2SO_4$  in the presence of catalyst mixture containing  $K_2SO_4$  &  $HgSO_4$  (9:1). The digested sample was diluted to a definite volume with water. A known aliquot of the diluted sample was mixed with 40 percent NaOH solution to an excess alkaline reaction and mixture was distilled with steam in micro-kjeldahl

apparatus. The ammonia so liberated was collected in 10 ml N/20 HCl having a few drops of methyl red as an indicator. The excess of the acid was measured by titrating against standard N/20 NaOH solution. The amount of N/20 NH<sub>3</sub> liberated was determined by difference. Nitrogen (N<sub>2</sub>) and CP contents were worked out by the following formula:

$$N_2 (\%) = \text{ml N/20 NH}_3 \times 0.0007$$

$$\text{CP} (\%) = \% N_2 \times 6.25$$

#### 4.11.4. Ether Extract (%)

Two gram of dried sample was transferred to asbestos thimble. The mouth of the thimble was plugged with fat free absorbent cotton. The thimble was placed in the glass jacket and 150 ml diethyl ether was taken in the receiving flask of Soxhlet's apparatus. The apparatus was placed in the heating assembly maintained at specific temperature. The extraction continued for about 8 hours. The ether was allowed to evaporate under hood and the extract was completely dried in an oven for thirty minutes at 105 °C. The weight of the extract was recorded after cooling the beaker in the desiccator. Percentage of the EE was calculated by the following formula:

$$\text{EE} (\%) = (\text{wt of ether extract} / \text{wt of sample}) \times 100$$

#### 4.11.5. Total Ash (%)

In a clean and tared crucible two grams of sample was taken. The crucible was placed on a hot plate and heated gradually until the substance was carbonized. The crucible was then placed in a muffle furnace at 650 °C until the white or light gray ash was obtained. Weight of ash was recorded after cooling the crucible in a desiccator. The ash percentage was calculated by the following formula:

$$\text{Total ash} (\%) = \{(a-b)/w\} \times 100$$

Where

a = mass (g) of crucible with ash

b = mass (g) of dried tared crucible

w = mass (g) of dried sample

## **4.12. Haematology**

### **4.12.1. Collection of Blood**

A five ml of blood was collected by direct heart puncture from three birds per replicate by using 24-gauge needle at 4:30 am (before morning feeding) in separate screw type tubes for obtaining plasma and serum. Blood collecting tubes having 0.2 ml heparin as an anti-coagulant were used to collect the blood plasma. Blood samples containing anticoagulant were used for the study of various haematological parameters. However, samples of blood for getting the serum were collected in centrifuge tubes without anticoagulant.

These blood samples were centrifuged in a centrifuge machine (Beckman TJ-6) for 5 minutes at 2000 rpm. Plasma and serum thus obtained were taken out of the centrifuge tubes and stored in eppendorf at  $-4^{\circ}\text{C}$  until analysis.

### **4.12.2. pH**

Blood pH was recorded at the time of slaughtering of the birds. The blood was collected into a container and the probe of pH meter (Beckman H-5) was immediately dipped into the container for the measurement of pH.

### **4.12.3. Haemoglobin (g/dL)**

Haemoglobin concentration was determined by cyanmethemoglobin method. A 20- $\mu\text{L}$  blood of each bird was mixed in 5 ml Drabkin's solution (potassium phosphate 52 mmol/L, potassium ferricyanide 30.4 mmol/L and potassium cyanide 38.4 mmol/L). Colour was developed by mixing of blood and Drabkin solution. The absorbance was measured on a spectrophotometer (Hitachi U-2001) at 540 nm (Benjamin, 1978).

### **4.12.4. Packed Cell Volume (%)**

Packed cell volume (PCV) was determined by the method described by (Benjamin, 1978). Microhaematocrit tube was filled upto the mark with the well mixed heparinized blood and the open end was sealed by heating the end of tube in a flame. Then the sealed haematocrit tube was placed in the capillary centrifuge

with the sealed end away from the center, taking care to balance it against another tube of the same size. Placed the cover screw guard and centrifuged for 6 minutes at 12000 rpm. Tubes were then removed and the percent values of PCV were recorded directly from the graphic haematocrit tube such that the tube was held against the liner chart, with the top of the liquid exactly at the top line and the bottom of the tube against the bottom line.

#### 4.12.5. Erythrocyte Sedimentation Rate (mm/hr)

Erythrocyte sedimentation rate was determined by westergen method by drawing 1.6 mL of blood into a test tube containing 0.4 mL of anticoagulant and gently shaking the tube. Further the blood was sucked into westergen tube upto the mark zero and then the tube was placed vertically in ESR stand for one hour. At the end of one hour the reading was taken at the boundary between the clear liquid above and red color citrated blood below.

#### 4.12.6. Erythrocyte (No.10<sup>6</sup>/μL) and Total Leukocyte Counts (No./cu mm)

Erythrocyte counts were done with the help of a haemocytometer as described by Natt and Herrick (1952). The composition of Natt and Herrick solution used as diluents for blood is given below:

NaCl	3.88 gm
Na <sub>2</sub> SO <sub>4</sub>	2.5 gm
Na <sub>2</sub> HP <sub>4</sub> .12H <sub>2</sub> O	2.91 gm
KH <sub>2</sub> PO <sub>4</sub>	0.25 gm
Formalin (37%)	7.5 ml
Methyl Violet	0.10 gm
Distilled water to make	
Volume upto	1000 ml

The blood was diluted 1:200 with above-mentioned diluent using an erythrocyte-diluting pipette. After mixing the contents of the pipette first drop was discarded and the next drop was placed on the edge of cover slip of the counting chamber. Erythrocytes were counted at 40 X in the large middle square (1mm<sup>2</sup>) of

chamber. In the middle square, four corners and one middle small square (each subdivided into 16 small squares) were used for counting. The erythrocyte numbers were calculated by the following formula:

$$(X/80) \times 400 \times 200 \times 10 = \text{erythrocytes} / \mu\text{L}$$

**Where**

X = total cells counted in 80 small squares

80 = subdivision of five small squares (4×4×5)

400 = total number of small squares in the large middle square

200 = dilution 1: 200

10 = 0.1 mm depth of the chamber

Same charged chamber was used for leukocyte count. Leukocyte counts were made in all small squares (400) of the middle large square (1 x 1 mm). The leukocyte number was determined by the following formula:

$$Z \times 200 \times 10 = \text{leukocytes}/\text{mm}^3$$

Where z = leukocytes counted

200 = dilution factor

10 = 0.1 mm depth of chamber

#### **4.12.7. Differentials Leukocytes Count**

Blood smears were made by using a drop of blood, air dried, fixed with methanol and stained with gimsa stain as described by Benjamin (1978). One hundred leucocytes were counted under oil immersion and categorized as, lymphocytes, monocytes, eosionophils and heterophils.

### **4.13. Blood Biochemistry**

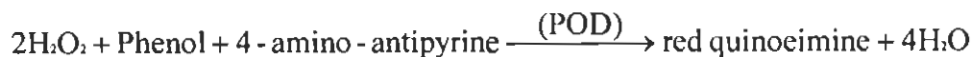
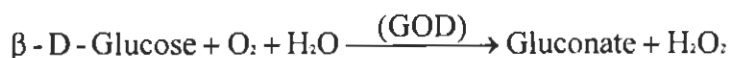
#### **4.13.1. Glucose (mg/dL)**

Enzymatic colorimetric kit was used for plasma glucose determination.

**Principle:**

Enzymatic colorimetric test on the basis of Trinder – Reaction.





### Reagents:

#### Buffer enzyme reagent

Phosphate Buffer, pH 7.5	05 mol/L
Phenol	7.5 mmol/L
Glucose oxidase (GOD)	12000 $\mu$ /L
Per oxidase (POD)	660 U/L
4-amino antipyrine	0.40 mmol/L

### Procedure:

Three test tube were taken, in one of these test tubes, 1 mL working reagent was taken and labeled as blank, in second test tube added working reagent and standard reagent and labeled as standard. Then in the third test tube added working reagent and sample and labeled as sample. Incubated these tubes for 15 minutes at 37°C then read the absorbance of samples against reagent blank at 546 nm.

### Calculation:

Glucose concentration was determined by following formula:

$$\text{Glucose in mg/dL} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Standard concentration}$$

Concentration of standard = 100 mg/dL (5.56 mmol/L)

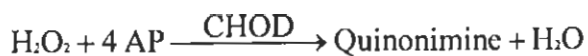
### 4.13.2. Cholesterol (mg/dL)

Plasma cholesterol was determined by using commercially available kit.

#### Principle:

In the presence of cholesterol esterase, the cholesterol esters in sample are hydrolyzed to cholesterol and free fatty acids. The cholesterol produced hydrogen peroxide which was detected using POD.





**Reagents:**

**Reagents 1:**

Buffer pH 6.9	90 mmol/L
Phenol	26 mmol/L

**Reagent 2:**

Cholesterol esterase (CHE)	300 U/L
Cholesterol oxidase (CHOD)	300 U/L
Per oxidase (POD)	1250 U/L
4-aminoantipyrine (4-AP)	0.4 mmol/L

**Procedure:**

Taken three test tubes as blank, unknown and standard. Then added 1 mL working reagent in each test tube. Taken 10 µL sample in sample test tube, added 10 µL standard in standard test tube. Incubated at 37°C for 5 minutes. Measured the absorbance of unknown and standard against reagent blank at 546 nm.

**Calculation:**

$$\text{Cholesterol mg/dL} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{concentration of standards}$$

**4.13.3. Total Proteins (g/dL)**

Plasma protein was determined by using commercially available kit Bio Rays Cat No. 1405.

**Principle:**

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex, the intensity of colour is proportional to the total proteins.

**Reagents:**

K. Na. tartrate	32 mmol/L
Potassium iodide	18 mmol/L
Copper sulphate	12 mmol/L
Sodium hydroxide	32 mmol/L

**Standard Solution:**

Total protein 5 g/dL

**Procedure:**

Three test tubes were taken and labeled as blank, standard and sample respectively. Added 1 mL biuret reagent in three test tubes. Then taken 20  $\mu$ L distilled water in blank and 20  $\mu$ L standard solution in standard test tube. Then taken 20  $\mu$ L sample in the sample test tube. Mixed and incubated at 20-30  $^{\circ}$ C for 25 minutes. Measured the absorbance of the standard and sample against the reagent blank at 540 nm.

**Calculation:**

$$\text{Conc. of total proteins (g/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of Standard}$$

**4.13.4. Albumin (g/dL)**

Concentration of plasma albumin was measured by using commercially available kit (Bio Rays Cat No. 1465).

**Principle:**

Measurement of plasma albumin is based on its quantitative binding to the indicator bromocresol green. The albumin-BCG-complex absorbs maximally at 578 nm.

**Reagent:**

<b>BCG Reagent:</b>	Succinate buffer	75 mmol/L pH 4.2
	Bromocresol green	0.15 mmol/l L
	Brij 35	
	Albumin standard	5 g/dL

**Procedure:**

Three test tubes were taken and labeled as blank, standard and sample respectively. Added 1 mL BCG reagent in these three test tubes. Finally added 10  $\mu$ L distilled water in the blank test tube and 10  $\mu$ L standard in the standard test tube. Then added 10  $\mu$ L sample in the sample test tube. Mixed thoroughly and incubated

for 15 min. at 20-30 °C. Measured the absorbance at 630 nm against the reagent blank.

**Calculation:**

$$\text{Albumin concentration (g/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

**4.13.5. Globulins (g/dL)**

The globulin was measured by calculations as follow:

$$\text{Globulin (g/dL)} = \text{Total protein} - \text{Albumin}$$

**4.13.6. Urea (mg/dL)**

**Principle**

Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. the ammonia produced in the first reaction combines with  $\alpha$ -oxoglutarate and NADH in the presence of glutamate-dehydrogenase (GLDH) to yield glutamate and  $\text{NAD}^+$ .



**Procedure:**

Urea in the plasma was estimated by enzymatic kit method described in the Randox Kits following the UV method (Kassirer, 1971).

Wavelength	340nm (Hg 334 nm or Hg 365 nm)
Cuvette	1 cm light path
Temperature	37°C
Measurement	against reagent blank

Pipetted 10  $\mu\text{l}$  of each standard solution and plasma into test tubes of standard and sample. Working reagent 1.0 ml was added in each tube (Reagent blank, standard, sample). Shaked and incubated the above mentioned solutions for at least 3 minutes at 37°C. Added 200  $\mu\text{l}$  reagent 2 in reagent blank, standard and

sample, respectively. Shaked and incubated for at least 5 minutes at 37°C. Measured the absorbance of standard (A° standard) and the sample (A° sample) against the reagent blank within 2 hours.

Urea concentration was determined by the formula.

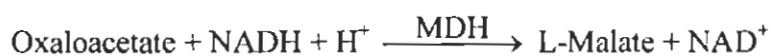
$$\text{Urea Conc. (mg/dl)} = \frac{A^\circ \times \text{standard}}{A^\circ \times \text{sample}} \times 50$$

#### 4.14. Enzymes

GOT and GPT in the plasma were estimated by using photometric methods.

##### 4.14.1. Glutamate Oxaloactate Transaminase (GOT; U/L)

###### Principle:



###### Reagent:

<b>R1:</b>	TRIS	pH 7.64	110 mmol/l
	L-Aspartate		320 mmol/l
	MDH (malate dehydrogenase)		≥ 800 U/l
	LDH (lactate dehydrogenase)		≥ 1200 U/l
<b>R2:</b>	2-Oxoglutarate		65 mmol/l
	NADH		1 mmol/l

###### Assay Procedure:

Wavelength	340 nm
Optical path	1 cm
Temperature	37°C
Measurement	Against air
Sample	50 µl
Monoreagent	500 µl

After mixing absorbance readings were taken after 1 min. Then again absorbance was recorded after 1, 2 and 3 min. and average reading was taken.

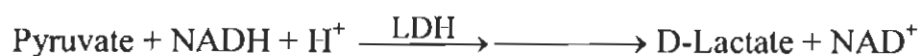
**Calculation:**

From absorbance readings  $\Delta A/\text{min}$  was calculated and then value was multiplied by the Standard factor (1745).

$$\text{GOT (U/L)} = (\Delta A/\text{min}) \times \text{Factor}$$

**4.14.2. Glutamate Pyruvate Transaminase (GPT; U/L)**

**Principle:**



**Reagents:**

**Components and Concentrations**

<b>R1:</b>	TRIS	pH 7.15	100 mmol/l
	L-Alanine		500 mmol/l
	LDH (lactate dehydrogenase)		$\geq 1700$ U/l
<b>R2:</b>	2-Oxoglutarate		15 mmol/l
	NADH		0.18 mmol/l

**Assay Procedure:**

Wavelength	340 nm
Optical path	1 cm
Temperature	37°C
Measurement	Against air
Sample	50 $\mu$ l
Monoreagent	500 $\mu$ l

After mixing absorbance readings were taken after 1 min. Then again absorbance was recorded after 1, 2 and 3 min. and average reading was taken.

**Calculation:**

From absorbance readings  $\Delta A/\text{min}$  was calculated and value was multiplied by the Standard factor (1745).

$$\text{GOT (U/L)} = (\Delta A/\text{min}) \times \text{Factor}$$

**4.15. Minerals Estimation (ppm)**

(Feed, Meat and Plasma)

All samples were subjected to wet digestion by following method of Richards (1968).

**Digestion Method:**

1 ml of plasma and 1 gram of feed and meat sample was taken into a digestion flask, 10 ml of concentrated nitric acid for plasma, feed and meat was added into it. Mixture was then heated at 60 to 70°C for about 15 min. After cooling, 5 ml of perchloric acid ( $\text{HClO}_4$ ) was added in the flask. The contents of the flask were heated vigorously till volume was reduced to 1-2 ml. The contents were diluted up to 25 ml by adding redistilled water. The digested and diluted samples were used for the estimation of minerals in the samples.

**Digested Blank:**

For the preparation of digested blank, 10 mL of concentrated nitric acid was taken in digestion flask. On digestion apparatus this sample was placed for 30-40 min on digestion apparatus in order to evaporate all the fumes from the flask. Then added 5 mL perchloric acid ( $\text{HClO}_4$ ) in it after cooling. This solution was again placed on digestion apparatus in order to evaporate till the volume remained 1.5 to 2 mL. Redistilled water was added in order to made desired volume of blank solution.

**Preparation of Stock Solution:**

Stock solution was prepared by calculating the availability of the element in the respective salt and then salt was diluted into the redistilled water in order prepared the stock solution of a known concentration. One gram of element in one liter water gave the concentration of 1000 ppm.

### **Preparation of Standard Solution:**

Standard solution for desired concentration were made from stock solution by using the formula:

$$C_1 V_1 = C_2 V_2$$

**Where**

- $C_1$  = Concentration of stock solution
- $V_1$  = Volume of stock solution
- $C_2$  = Concentration of standard solution
- $V_2$  = Volume of standard solution

*Sodium and potassium* were estimated by using flame photometer. Whereas *calcium, magnesium, zinc, copper, iron, manganese, and selenium* were determined by using atomic absorption spectrophotometer. (AOAC, 1990)

#### **4.15.1. Chloride (ppm)**

Chloride was determined by silver nitrate method of Mour's titration (Sawyer et al., 1994).

#### **Reagents:**

##### ***Silver nitrate standard solution (AgNO<sub>3</sub>):***

Dissolved 4.791 g silver nitrate (AgNO<sub>3</sub>) in redistilled water and diluted upto 1 liter.

##### ***Potassium chromate indicator:***

5 mg of potassium chromate was dissolved in 100 ml distilled water.

#### **Procedure:**

##### ***Sample:***

One ml of digested sample (1g or 1ml of basic sample diluted up to 25 ml for plasma and meat and 100 ml for feed) was taken in china dish. Then it was made basic by adding calcium carbonate about 0.05-0.08 g. Then one drop of potassium chromate indicator was added. The contents were titrated against silver nitrate drop wise. End point was precipitate of brick red colour.

**Blank:**

1 ml of digested sample of nitric acid and perchloric acid was taken. Titrated it against silver nitrate solution. Indicator used was potassium chromate, one drop of indicated was added. End point of blank was the precipitate of brick red colour.

**Calculation:**

$$\text{Chloride as Cl}^- = \frac{25(V_1 - V_2)}{\text{Vol. used for sample}} = \text{Amg}/25\text{mL}$$

**Where**  $V_1 =$  Volume used for sample

$V_2 =$  Volume used for blank

25 = Dilution of digested sample

25 ml of solution contains chloride = Amg

1g of sample contains Cl = Amg

1000 g of sample contain Cl = Amg  $\times$  100 = Bppm

**4.15.2. Phosphorus (ppm)**

To estimate phosphorus in the sample, Aminonaphthol Sulphonic acid method was used under the principle of Colorimetric Estimation.

**Reagent**

- 2.5% Ammonium Molybdate:** 2.5 gms ammonium molybdate + 3 mL concentration  $\text{H}_2\text{SO}_4 \rightarrow$  100 mL by D.W.
- Aminonaphthol Sulphonic acid:** 0.5 gms Ammonaphthol sulphonic acid + 195 mL 15%  $\text{NaHSO}_3$  (sodium bisulphate solution) + 5 mL 20%  $\text{Na}_2\text{SO}_4$  and 1 mL sodium sulphate was added.
- Standard Solution of Phosphorus:** 0.351 gms of  $\text{KH}_2\text{PO}_4$  (potassium dihydrogen phosphate + 1 mL concentration D.W. – make upto 100 mL. It will give concentration 0.8 mg/mL.

**Procedure:**

One gram of sample was digested by wet digestion method and diluted up to 25 ml.

Three test tubes were prepared as follows:

Reagent	Standard	Sample	Blank
Sample Solution	-	0.1 mL	-
Standard Phosphorus Solution	0.1 mL	-	-
Ammonium Molybdate (2.51%)	1 mL	1 mL	1 mL
Aminonaphthol Sulphonic Acid	0.4 mL	0.4 mL	0.4 mL
D.W.	8.5 mL	8.5 mL	8.6 mL

Solution was shaken, allowed to stand for 5 minutes to develop colour. Zero absorbance was set by blank at 720 nm wavelength.

#### Calculation:

Absorbance of standard phosphorus

Solution = X

Absorbance of sample solution = Y

X absorbance is due to = 0.08 mg P/0.1mL

Y absorbance is due to =  $0.08 \times Y/X = A$  mg P

0.1 ml sample contain P = A mg

1 ml sample contain P =  $A/0.1$  mg

25 ml sample contain P =  $A \times 25/0.1 = B$  mg

1g of sample contain P = Bmg

1000 g sample contain P =  $Bmg \times 1000 = C$  ppm

## 4.16. Hormones

### 4.16.1 Triiodothyronine ( $T_3$ ; ng/mL)

For the quantitative determination of the triiodothyronine ( $T_3$ ) concentration in the plasma was determined by using DSL Triiodothyronine Enzyme immunoassay kit.

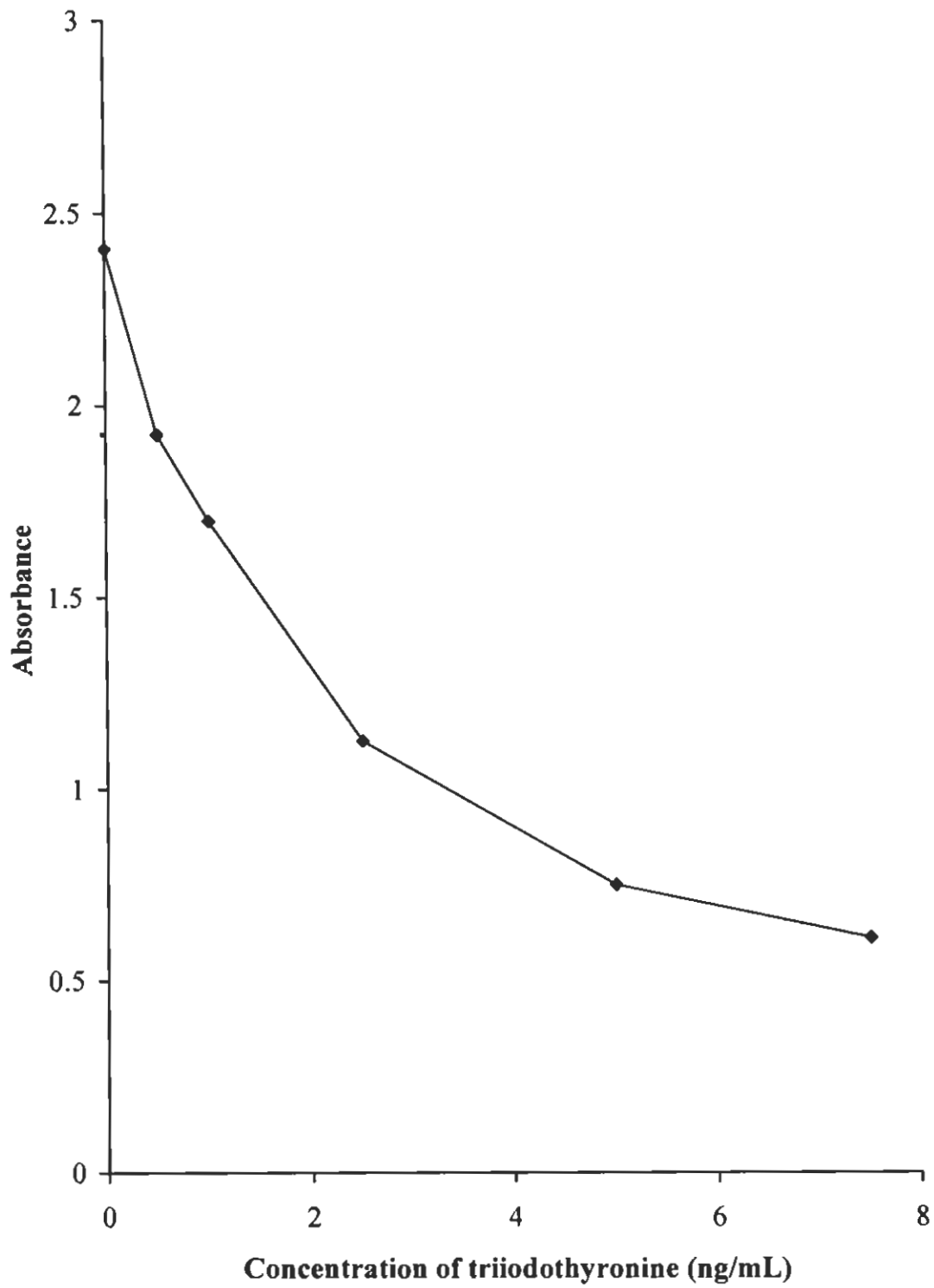
**Principle:**

It is a solid phase competitive binding enzyme immunoassay. Triiodothyronine in the sample that compete with the  $T_3$  enzyme conjugate for antibody binding sites on a well coated with a  $T_3$  antibody. The amount of  $T_3$  in the plasma sample is inversely proportional to amount of  $T_3$  enzyme conjugate bound to the well. The enzyme intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled  $T_3$  standards assayed in the same way. The concentration of  $T_3$  in the unknown sample was then calculated.

**Procedure:**

All the specimens and reagent were allowed to reach room temperature ( $25^\circ\text{C}$ ) and mixed thoroughly by gentle inversion before use.

Secured the desired number of coated well in the holder. Made date sheet with sample identification. Pipetted  $50\mu\text{L}$  of standard, samples and controls in the appropriate wells. Dispensed  $50\mu\text{L}$  of the antibody reagent into each well. Mixed thoroughly for 30 minutes. Added  $100\mu\text{L}$  of working conjugate reagent into each well. Mixed thoroughly for 30 seconds. Incubated at room temperature for 60 minutes. Removed the incubation mixture by flicking plate contents into a waste container. Rinsed and flicked the wells 5 times with distilled water. Struck the wells on to absorbent paper to remove any water from the wells. Dispensed  $100\mu\text{L}$  of tetramethyl benzidine (TMB) reagent into each well. Gently, mixed for 5 seconds. Incubated at room temperature in the dark for 20 minutes without shaking. Stopped the reaction by adding  $50\mu\text{L}$  of stop solution to each well. Gently mixed for 30 seconds. Read absorbance at  $450\text{nm}$  with an ELISA reader within 15 minutes. The standard curve was obtained by plotting absorbance values of standard against appropriate  $T_3$  concentrations (Fig. 4.1).



**Fig. 4.1: Standard Curve for triiodothyronine**

**Calculations:**

Calculated the average absorbance value for each set of reference standard, control and samples. Constructed a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/mL on linear graph paper with the absorbance on Y-axis and concentration on X-axis. Used the mean absorbance value for each sample, determined the corresponding concentration of  $T_3$  in ng/mL from the standard curve.

**4.16.2 Thyroxine ( $T_4$ ;  $\mu\text{g/dL}$ )**

For the quantitative determination of the total thyroxine ( $T_4$ ) concentration in the serum was determined by using DSL  $T_4$  enzyme immunoassay.

**Principle:**

It is a solid phase competitive binding enzyme immunoassay. In the  $T_4$  enzyme immune assay (EIA), a certain amount of anti- $T_4$  antibody is coated on microtiter wells. A measured amount of patient serum, and a constant amount of  $T_4$  conjugated with horseradish peroxidase are added to the microtiter wells. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled  $T_4$  in the sample. By reference to a series of  $T_4$  standards assayed in the same way, the concentration of  $T_4$  in the unknown sample is quantified.

**Procedure:**

Secured the desired number of coated wells in the holder. Pipetted 25 $\mu\text{L}$  of standard, specimens, and controls into appropriate wells. Dispensed 100 $\mu\text{L}$  of working conjugate reagent into each well. Thoroughly mixed for 30 seconds. Incubated at room temperature 25°C for 60 minutes. Removed the incubation mixture by flicking plate contents into a waste container. Rinsed and flicked the microtiter wells 5 times with distilled water. Struck the wells against absorbent paper to remove all residual water. Dispensed 100 $\mu\text{L}$  of tetramethyl benzidine (TMB) reagent into each well. Gently mixed for 5 seconds. Incubated at room temperature in the dark for 20 minutes. Stopped the reaction by adding 100 $\mu\text{L}$  of

stop solution (1N H<sub>2</sub>SO<sub>4</sub>) to each well. Gently mixed for 30 seconds. Read absorbance at 450nm with ELISA reader within 15 minutes. Standard curve was obtained by plotting absorbance values of standards against appropriate T<sub>4</sub> concentrations (Fig. 4.2).

**Calculations:**

Calculated the average absorbance value (A<sub>450</sub>) for each set of reference standards, control and samples. Constructed a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in µg/dL on linear graph paper with absorbance on Y-axis and concentration on X-axis. Used the mean absorbance value for each sample, determined the corresponding concentration of T<sub>4</sub> in µg/dL from the standard curve.

**4.16.3 Cortisol (µg/dL)**

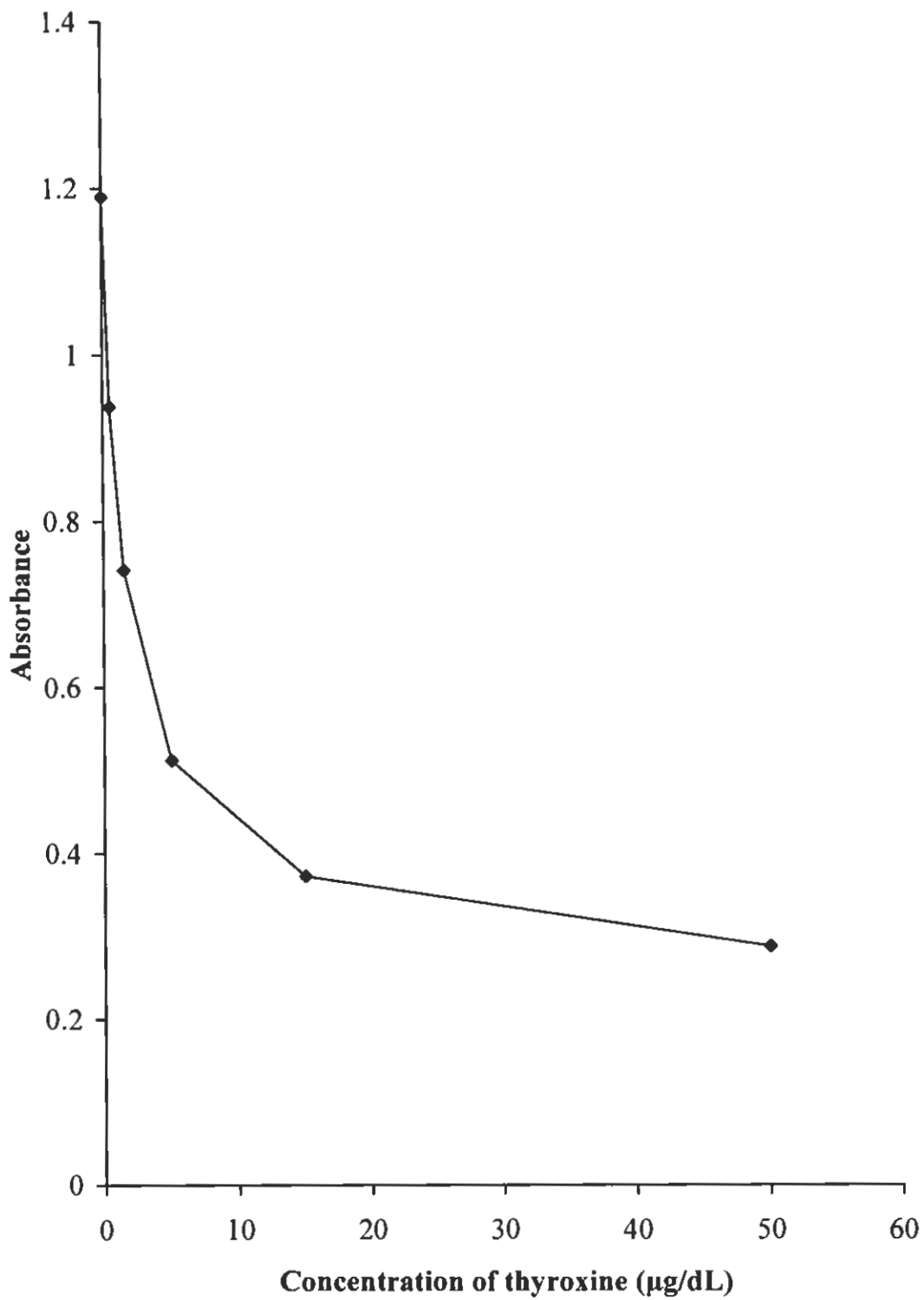
For the quantitative measurement of cortisol in the plasma DRG solid phase enzyme immunoassay (DSL) was used.

**Principle:**

The solid phase enzyme immunoassay for the cortisol is a competitive type immunoassay. Wherein horse radish peroxidase-labeled cortisol (HRP-cortisol) competes with cortisol present in the patient sample for a fixed and limited number of antibody sites immobilized on the wells of the microstrip. As a result of immuno reaction the HRP-cortisol fraction bound to the antibody in the solid phase is measured by adding a chromogen/substrate solution, which is converted to a blue compound. After incubation, use of hydrochloric acid (HCl) stops the enzymatic reaction and solution become yellow. The absorbance of the solution, photometrically measured at 450nm is inversely related the concentration of cortisol present in the sample.

**Procedure:**

The entire reagents were brought to the room temperature. In the strip holder a number of strips were left to make the running of calibrator, controls and samples in duplicate. Placed the exceeding strip and desiccant into the transparent plastic



**Fig. 4.2: Standard Curve for thyroxine**

pouch and sealed it properly. Pipetted 25µL of calibrators and samples into wells of the strip. Added 200µl of HRP cortisol conjugate to each well in sequence. Incubated for 60 minutes at room temperature without converting the plate. Discarded the incubation solution, rinsed the wells (three times) with the washing solution and removed the residual. Promptly pipetted 100 µL of the chromigen/substrate mixture into the rinsed wells. Incubated for 15 minutes at room temperature. Pipetted 100 µL of stop solution into the wells. Shacked the plate gently and carefully. Read at 450nm with in 1 hour from stopping (Fig. 4.3).

**Calculation:**

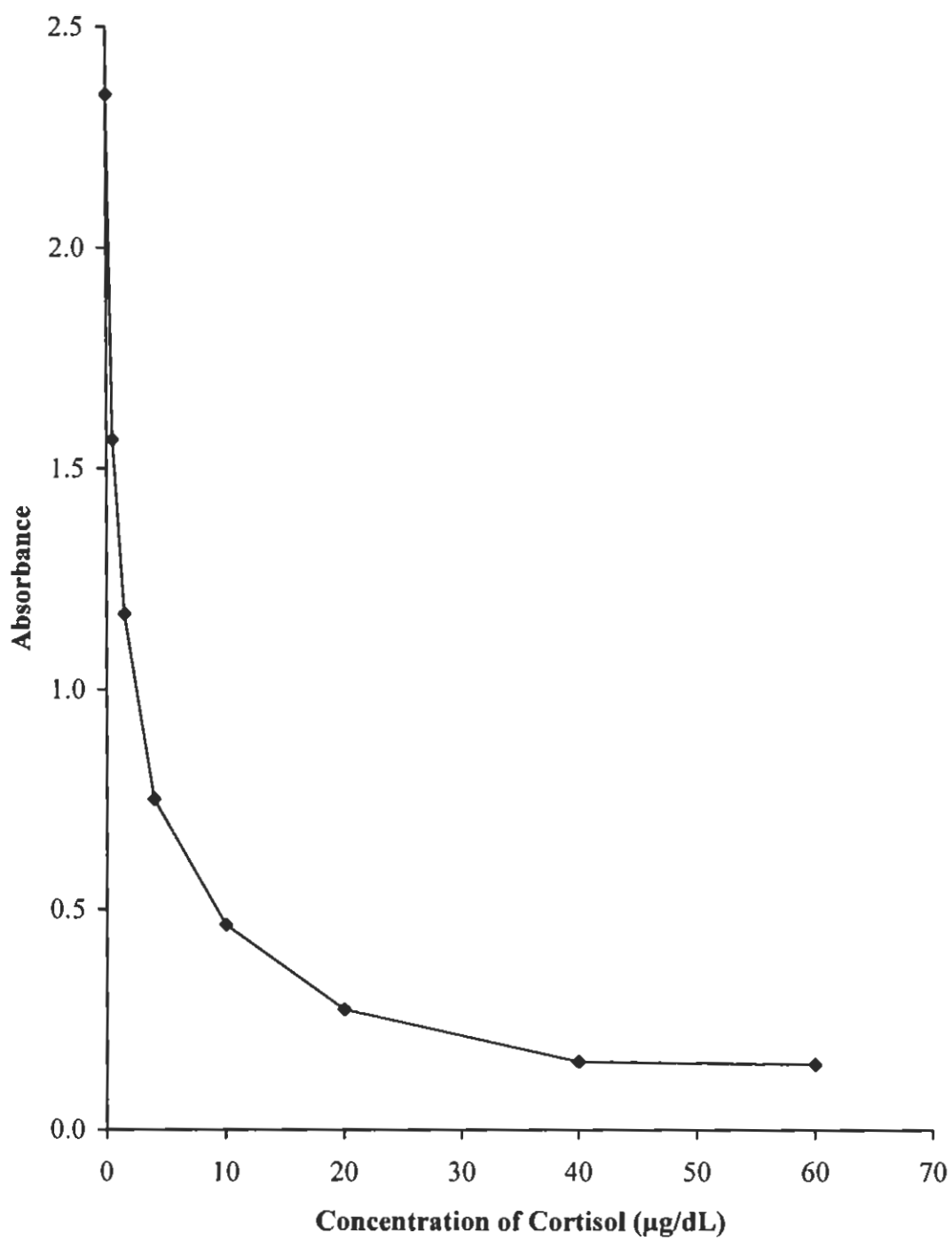
Calculated the average absorbance values ( $A^{450}$ ) for each set of reference standard's control and sample. Constructed a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration µg/dL on a linear graph paper with the absorbance value on vertical axis (y-axis) and concentration at horizontal axis (x-axis). Mean absorbance value for each sample was used to determine the corresponding concentration of cortisol in µg/dL from the standard curve.

**4.17. Immune Response**

**Haemagglutination Inhibition Test:**

Haemagglutination inhibition (HI) titer of each serum sample was determined as described below.

1. Immunoplates (8 rows and 12 columns of round wells) were used. With the help of multichannel microdiluter, all the wells of the plate were filled with 50 µl of the normal saline solution.
2. Serum sample (50 µl) was placed in the first well of the row A. Similarly, 50 µl of each of serum sample number 2, 3, 4, 5, 6, 7 and 8 was added to first well of row B, C, D, E, F, G and H, respectively.



**Fig. 4.3: Standard Curve for Cortisol**

3. With the help of multichannel microdiluter, the mixture in the first well of each row was mixed and 50 µl of that was transferred to 2nd well of the respective rows thus diluting each sample as 1:4. This procedure was repeated until the 10th well and at the end 50µL was discarded.
4. The ND virus antigen (50 µl of 4 HA unit) was added to each well from 1 to 11 of each row. The plate was incubated at room temperature (25°C) for 30 minutes.
5. Washed suspension of chicken RBC (50 µl of 0.5 percent suspension) was added into each well of the plate from well 1 to 12 of each row.
6. The plate was agitated to ensure even suspension of the erythrocytes in the mixture.
7. The plate was kept undisturbed at room temperature until a clear pattern of haemagglutination or haemagglutination inhibition (button formation) was seen.
8. The highest dilution of each serum sample causing 50% inhibition of haemagglutination was taken as end point. The HI titer of each serum sample was expressed as reciprocal of the serum dilution.

### **Determination of Antibody Titers Against IBDV**

Antibody titers against infectious bursal disease virus (IBDV) were measured by indirect Haemagglutination (IHA) test, briefly described as follows:

#### **Washing of erythrocytes:**

Human blood (5.0 ml) of group “O-ve was collected aseptically in a disposable syringe and was transferred to glass tube containing 1 ml of 4% sodium citrate. The blood was centrifuged at 1500 RPM for 5 minutes. The plasma and buffy coat were pipetted out while packed erythrocytes were resuspended in sterile phosphate buffered saline (PBS). The erythrocytes were given three subsequent washings. The packed erythrocytes were used for sensitization with antigen.

### **Composition of phosphate buffered saline (PBS) pH 7.2**

Sodium Chloride (NaCl)	8.0 gms
Potassium dihydrogen Phosphate (KH <sub>2</sub> PO <sub>4</sub> )	0.34 gms
Dispotassium hydrogen phosphate (K <sub>2</sub> HPO <sub>4</sub> )	1.21 gms
Distilled water (H <sub>2</sub> O)	upto 1.0 liter

### **Sensitization of erythrocytes**

An amount of 1 ml of packed erythrocytes was mixed with 2 ml of antigen and 2 ml of phosphate buffer saline. The contents were thoroughly mixed and kept at 37°C for an hour. The treated erythrocytes were washed three times in PBS to wash out excessive antigen. Finally, the packed erythrocytes sensitized with antigen were used at one percent concentration for conduction of indirect Haemagglutination (IHA) test.

### **Inactivation of serum samples:**

All the serum samples were inactivated in a water bath at a temperature of 56°C for 30 minutes. Then the inactivated serum samples were subjected to IHA test for determination of antibody titers against IBDV.

### **Procedure for IHA:**

The test carried out in round bottom micro titration plates (Flow Laboratories), each having 96 wells, arranged in 8 rows and 12 columns designated as A-H and 1-12 respectively. All wells were dispensed with 5 µL of PBS using 4-channel micropipette. Then 50 µl of each test serum was added in first well of each row.

After 3 times mixing of the sample in first well, 50 µL was transferred from first well to the second well of the row. Likewise, serial transfer of the serum dilutions were maintained to the last well, discarding 50 µl from the last well. Thus, two fold serial dilutions of each test serum was made. The last two columns (11<sup>th</sup> and 12<sup>th</sup>) were maintained as positive and negative controls. The wells of column 11<sup>th</sup> and 12<sup>th</sup> were dispensed respectively with 50 µl of known positive and known negative anti-IBD serum.

After diluting the serum samples, 50 µl of sensitized human group O, RBC's (1%) were dispensed in each well. The plates were gently tapped to ensure even

dispersion of erythrocytes and were kept at 37°C for 30 minutes. Degree of agglutination in each row was recorded. The serum samples that caused a distinct erythrocytic agglutination resulting in characteristic reticulum settling of erythrocytes throughout the bottom of the well were considered positive and the end point dilution was recorded.

The samples showing peculiar central button-shaped settling of erythrocytes were considered as negative. The IHA antibody titer of each serum was narrated as the reciprocal of its endpoint dilution. Thus, the IHA antibody titers of all serum samples were recorded. (Cunningham, 1966; Buxton and Fraser, 1977)

#### **Data Analysis:**

Data of IHA titers were analyzed statistically. Geometric mean titers (GMT) of each sample were calculated. Finally cumulative GMT of each group was calculated.

#### **Calculation of Geometric mean titers (GMT):**

For the conversion of base two logarithmic mean titers to geometric mean titers following formula was used (Thrusfield, 1999).

1. Log of each reading was taken.
2. Summation of log.
3. Mean of log was taken.
4. Finally antilog of mean log was taken

$$\text{GMT} = (\text{Anti } \Sigma \log/n)$$

#### **4.18. Statistical Analysis**

The data thus collected was analyzed by analysis of variance technique using Completely Randomized Design with 3 × 2 factorial arrangement of treatments (Steel *et al.*, 1997). The differences in means of the treatments were compared by Duncan's Multiple Range test (Duncan, 1955).