EFFECT OF CAPTOPRIL
GLYCATION INHIBITION IN NORMAL AND DIABETIC PLASMA BY CAPTOPRIL

ORIGINAL
PROF-1755

MISS GULL-E-FARAN
Demonstrator
Department of Biochemistry
Independent Medical College, Faisalabad

DR. NIGHAT ASLAM
Assistant Professor of Biochemistry
Independent Medical College
Faisalabad

DR. MUHAMMAD ANJUM ZIA
Assistant Professor
Department of Chemistry & Biochemistry
University of Agriculture, Faisalabad

ABSTRACT... Objectives: (1) To investigate the inhibitory effect of Captopril on level of glycation (in vivo). (2) To study glycation inhibition in vivo. **Study design:** Case study. **Period:** Sep. 2006 to March. 2008. One year seven months. **Setting:** Department of Biochemistry University of Agriculture, Faisalabad. **Methods:** Different parameters like fluorescence, total proteins, TBA (thiobarbituric acid) method, periodate borohydride assay were used to check the effect of inhibitor on glycation. Thirty two combinations were made and all these combinations were placed at 37˚C, at same time for five weeks. 3mL of blood sample was drawn after 1st, 3rd and 5th week of incubation to perform the experiments for glycation and glycation inhibition. Along with the same temperature (37˚C), different combinations of glucose and inhibitor were used. **Results:** Effective concentration of inhibitor helped to decrease the level of glycation. All concentrations of glucose (G_1, G_2 and G_3) showed glycation with protein. The inhibitor Captopril (all concentrations) showed variations in inhibition of glycation at one temperature (37˚C) with different parameters (Fluorescence, TBA and Periodate) but the most effective concentration of inhibitors at each condition is I_3 (1mM) but I_1 (10 mM) and I_2 (5 mM) were also equally effective after I_3. Periodate borohydride Assay is more effective for glycation determination than thiobarbituric acid assay. **Conclusions:** Captopril can be used as glycation inhibitor in future. As it enhances the activity of transketolase, it can produce 3DG compound which can block the AGEs. However, more experimentations should be done on animal or on large scale before its application in diabetic patients.

Key words: Glycation, Inhibition, Captopril.

INTRODUCTION

Diabetes mellitus is a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. The effects of diabetes mellitus include long term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristic symptom such as thirst, polyuria, blurring of vision, and weight loss. In its most severe forms, ketoacidoses or a non-keto hyperosmolar state may develop and lead to stupor, coma and death in absence of effective treatment[1].

Glycation (also called as non-enzymatic glycosylation) is the result of a sugar molecule, such as fructose or glucose, bonded to a protein or lipid molecule without controlling action of an enzyme. Much of the early laboratory research work on fructose glycations used inaccurate assay techniques that led to drastic underestimation of the importance of fructose in glycation[2].

It has been known for a long time that human blood proteins like hemoglobin and serum albumin[3] may undergo a slow non-enzymatic glycation, mainly by formation of a Schiff base between - amino groups of lysine (and sometimes arginine) residues and glucose molecules in blood. This reaction can be inhibited in the presence of antioxidant agents although this reaction may happen normally elevated glycoalbumin is observed in diabetes mellitus[4].

Glycation has the potential to alter the biological structure and function of the serum albumin protein[5]. Moreover, glycation end products (AGE) result in abnormal biological effects that leads to tissue damage via alteration of the structure and functions of tissue proteins, stimulation of cellular responses through receptor specific for AGE-proteins and via generation of

reactive oxygen intermediate[6].

A number of synthetic and natural compounds as inhibitors/activator of non-enzymatic glycation products are aminoguanidine, benfotamine, aminosalicyclic acid, thiamine, ascorbic acid, and captopril. As in this research work the inhibitory effect of captopril against glycation products was studied by using two different glycation assays.

In this research work accuracy and efficacy of TBA and periodate methods was compared. Most active concentrations of glucose and inhibitor were also optimized.

MATERIALS AND METHODS

The aim of the project was to study the inhibition of glycation with captopril against glycation products in vivo with diabetic and normal plasma (Plasma obtained from normal and diabetic individuals).

Selection of Conditions and Concentrations

To study the inhibitor's effect on glycation or glycation inhibition in vivo, thirty two combinations were made and placed at 37˚C for five weeks. Plasma was used as protein. Three milliliter of samples was drawn after 1st, 3rd and 5th week of incubation to perform the experiments for glycation and glycation inhibition. Different concentrations of glucose and inhibitor were used at temperature 37˚C.

Inhibition with Normal and Diabetic Plasma

Blood samples of diabetic patients (insulin dependent) who were clinically diagnosed were collected from D.H.Q. Hospital Faisalabad, National Hospital Faisalabad, Chiniot Dialysis Centre Faisalabad, and Allied Hospital Faisalabad.

Blood samples from each patient were collected by using sterilized disposable syringes by venopuncture. These samples containing anticoagulant were mixed gently by tapping then centrifuged at 8000rpm and Plasma fractions were collected and stirred at 20˚C.

All plasma was pooled and mixed together to make volume of 200ml which was taken as one plasma sample for experiment. Normal plasma was pooled from healthy male and female volunteers blood samples.

In vivo Inhibition of glycation in plasma

In vivo Glycation of plasma (Preparation of Plasma-AGE) Plasma was incubated with all glucose concentrations with and without inhibitor in Phosphate buffer saline (containing Sod.Azide) at 37˚C for 1-5 weeks[7].

Three different concentrations of inhibitor and glucose were incubated with Plasma for detecting glycation inhibition.
Concentration of glucose, Inhibitor and protein.

Glucose Concentrations

Glucose 1: 500mmol
Glucose 2: 250mmol
Glucose 3: 5.5 mmol

Inhibitor Concentrations

Inhibitor 1: 10 mmol
Inhibitor 2: 5 mmol
Inhibitor 3: 1 mmol

Protein Concentration

Plasma: 20 mg/ml
Samples were drawn after 1st, 3rd and 5th week of incubation to perform the experiments for glycation and glycation inhibition. In above samples, each week 3ml stock solution was taken from 9ml and remaining was again placed at 37˚C.

From this 3ml, 40µL was taken and then added 2ml buffer in it and made volume 2.40 ml. Then absorbance was taken at 370nm and 280nm by using this dilution.

Estimation of Advanced Glycation End Products

Advanced glycation end products (AGEs) were estimated by taking absorbance at 370nm using spectrophotometer.

Glucose Estimation by Kit Method (mg/dL)

Enzymatic (Kinetic) colorimetric test (GOD-PAP) Glucose in all samples was measured by Glucose

oxidase method as provided by Biocon kit.

Total protein Estimation (g/dL)
Total protein in all samples after dialysis was determined by Biuret method using biuret reagent.

Dialysis
As free glucose is the major hindrance in estimation of glycation level, so free glucose was removed by using dialyzing membrane. Glycated albumin was dialyzed against dist.H2O for twenty-four hours at constant stirring at room temperature. After dialysis, samples were again placed at 20˚C until use.

Measurement of Glyation Level
After dialysis two methods were used for measurement of glycation level.These were Thiobarbituric Acid (TBA) and Periodate Borohyride Colometric Techniques.

Standard Preparation
Fructose standard 0.1M (1.8g/100ml) ranged from 10nmol/ 0.7 ml to 50nmol/ 0.7 ml[7].

Procedure
In 0.7 ml sample (2mg protein) added 20µL HCl and 0.1 ml $NaIO_4$, mixed the reagents and placed the sample at room temperature for 30 minutes. Cooled on ice for 5 minutes and added 0.3 ml ice cold ZnSO4 + 0.1ml $NaOH_4$ in each tube.

Centrifuged for 10 minutes at 5000rpm.Supernatant (almost 1.1 ml) was taken and added 1ml F.D.R (Formaldehyde Detection Reagent).Incubated at 37˚C for one hour and reading was taken at 510nm.

TBA Method
TBA technique was used for the determination of both enzymatic and non-enzymatic glycation. The standard curve was made by using fructose standard solution.

Non-enzymatic Glycation
0.5 ml plasma (or protein conc. 10mg) sample was taken. Added 0.1ml of NaOH in non-reduced samples. Left the tubes for 30 minutes at 37˚C.Added one drop of 1N HCl and 0.25ml of oxalic acid (2N) in each tube. Capped the tubes and autoclaved for 15 minutes. After autoclaved, cooled the tubes and put them in ice box. Then added 0.5 ml of chilled TCA. Centrifuged the samples for 15 minutes at 15000 rpm (in appendorff). After centrifugation, supernatant was taken 1.5ml,and added 0.5ml of TBA(or some amount).Incubated the samples at 37˚C for 15 minutes and reading was taken at 443nm.

Enzymatic Glycation
0.5ml plasma (or protein conc. 10mg) sample was taken. Added 0.1ml of NaBH4 in reduced samples. Left the tubes for 30 minutes at 37˚C. Added one drop of 1N HCl and 0.25ml of oxalic acid (2N) in each tube. Capped the tubes and autoclaved for 15 minutes. After autoclaving, cooled the tubers and put them in ice box. Then added 0.5ml of chilled TCA.Centrifuged the samples for 15 minutes at 15000rpm (in appendroff). After centrifugation, supernatant was taken 1.5ml and added 0.5ml of TBA.Incubated the samples at 37˚C for 15 minutes and reading was taken at 443nm.

Non-enzymatic glycation
Non-enzymatic glycation was determined as follows:

NE glycation	=	Total glycation- E glycation
NE	=	Non-enzymatic
E	=	Enzymatic

Note: Duplicate reaction was made respectively for both reduced and non-reduced Glycation estimation.

Statistical Analysis
Data was analyzed by using average, mean and standard factor to see the effect of captopril on glycation.

RESULTS AND DISCUSSION
The study was initiated to investigate the inhibitory effect of captopril on level of glycation (in vivo). Different parameters like Fluorescence, Total proteins, Dialysis, TBA (tribarbituric acid) method, Periodate Borohydrate Assay were being used to check the effect of inhibitor on glycation.

Glycation Level
Glycation is a non-enzymatic reaction between reducing sugar (glucose, fructose and galactose etc.) and free

amino groups on proteins, lipids and nucleic acid. In inhibition of glycation, glycation is inhibited because chemical reaction of glycation is inhibited at any stage of chemical reaction.

Captopril prevents the "cross linking" (or glycosylation) of proteins which may cause many of the problems of old age. It has been suggested that inhibition of the Maillard reaction may prevent the progress of diabetic complications and slow the aging process. Captopril reduces the antiproliferative effect of hyperglycemia, advanced glycation end products and glycated basic fibroblast growth factors in cultured bovine aortic endothelial cells in comparison with aminoguanidine.

We checked and compared the glycation level by using different concentrations of glucose (G_1, G_2 and G_3) and inhibitor (I_1, I_2 and I_3) at 37˚C. We also checked and compared the effect of inhibitor on level of glycation at 37˚C.

Fluorescence

Fluorescence shows cross-linking between glucose and protein. In disease conditions e.g. diabetes cross linking of glucose increases due to persistent hyperglycemia, which in turn increases fluorescence. Fluorescence was also determined by using different concentrations of glucose (G_1, G_2 and G_3) and inhibitor (I_1, I_2 and I_3) at 37˚C. The results obtained are shown.

Fig. 4.1. shows that in case of normal plasma and buffer, G1 (500 mM) shows no fluorescence. But G_1 + Protein (500 mM conc. of glucose) increased glycation up to third week but after that the curve dropped because the glycation level decreased. I_1 (10mM conc) did not work up to 3rd week and glycation level tend to increase but it worked in 5th week and decreased the glycation level. I_2 (5 mM conc.) also not worked upto 3rd week but worked in 5th week and caused decrease in glycation level. I_3 (1mM conc.) worked up to 3rd week and decreased the glycation level but it did not work in 5th week and glycation level increased at 37˚C for normal plasma.

Fig. 4.2. shows that in case of normal plasma and buffer, glucose concentration of G_2 (250 mM) shows no florescence. But G_2 + Protein (250 mM conc. of glucose) increased glycation up to third week but after that the curve dropped because glycation level decreased. I_1 (10 mM conc.) did not worked up to 3rd week but worked in 5th week and caused decrease in glycation level to some extent. I_3 (1mM conc.) not worked up to 3rd week but worked in 5th week and caused decrease in glycation level at 37˚C for normal plasma.

Fig. 4.3 shows that in case of normal plasma and buffer glucose concentration of G_3 (5.5. mM) shows no fluorescence at any level of incubation but G_3+ protein (5.5. mM conc. of glucose) increased glycation level up to third week but after that the curve dropped because glycation level decreased. I_1 (10 mM conc.) did not work up to 3rd week and glycation level increased but it worked in 5th week and decreased the glycation level to some extent. I_2 (5 mM conc.) also not worked upto 3rd week but worked in 5th week and caused decrease in glycation level. I_3 (1mM conc) did not work up to 3rd week and glycation level increased but it worked in 5th week and decreased the glycation level to great extent at 37˚C for normal plasma.

The results of fluorescence at 37˚C showed that in case of normal plasma simple protein and glucose did no show any glycation but all concentration of glucose (G_1, G_2 and G_3) with protein showed high glycation level at 37˚C. All concentrations of inhibitor (I_1, I_2 and I_3) showed inhibition of glycation with all concentrations of glucose except I_3 which did not work with G_1 concentration of glucose.

The results of fluorescence at 37˚C for diabetic plasma showed that simple protein and glucose did not show any glucation but all concentrations of glucose (G_1, G_2 and G_3) with protein showed high glycation level at 37˚C. All concentrations of inhibitor (I_1, I_2 and I_3) showed inhibition of glycation with all concentrations of glucose except I_2 which did not worked to decrease glycation level with G_2 concentration of glucose at 37˚C for diabetic plasma.

Dolhofer et al. (1990)[8] illustrated that incubation of human immunoglubin G with glucose in vitro leads to covalent incorporation of the sugar concomitant with marked changes in molecular structure. After six to ten days of glucose incubation, absorption at 350 nm and fluorescence at 440 nm upon excitation at 370 nm markedly increased, indicating formation of non enzymatic browning products.

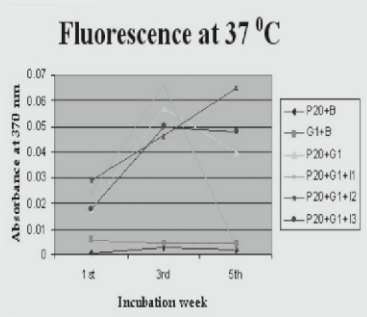

Fig-4.1

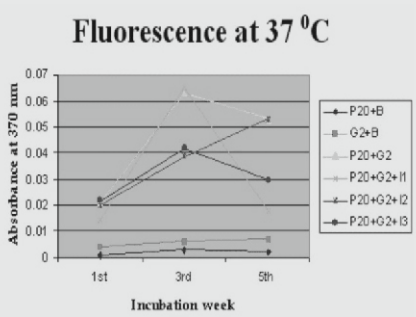

Fig-4.2

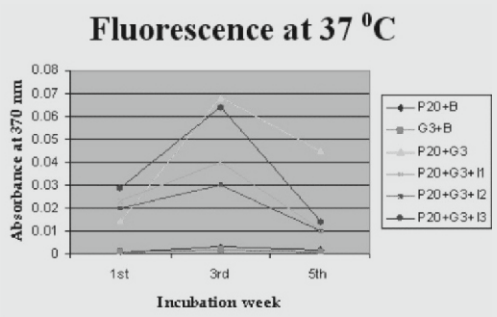

Fig-4.3

Thiobarbituric acid (TBA) Assay

It is used to measure the glyation level. TBA technique (Furth, 1988) was used for the determination of both enzymatic and non enzymatic glycation.

Thiobarbituric acid test is used to measure the glycation level. Here glycation levels were checked by this test for different concentrations of glucose (G_1, G_2 and G_3) and inhibitor (I_1, I_2 and I_3) at 37˚C.

Fig. 4.4 shows that G_1 (500 mM conc.) increased glycation upto third week but after that the curve dropped because these glycation products converted into AGEs. I_1 (10 mM conc.) did not worked in 5th week and caused decrease in glycation level. I_3 worked through out and decreased glycation level at 37˚C for normal plasma.

Fig. 4.5 shows that G_2 (250 mM conc.) increased glycation level upto 5th week to a great extent. I_1 (10 mM conc.) did not work throughout and glycation level increased. I_2 (5 mM conc.) did not work upto 3rd week but worked in 5th week and caused decrease in glycation level. I_3 (1mM conc.) did not work upto 3rd week and glycation level increased but it worked in 5th week and decreased the glycation level to some extent at 37˚C for normal plasma.

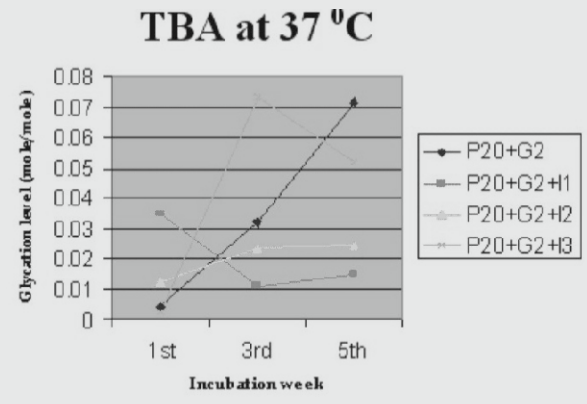

Fig-4.4

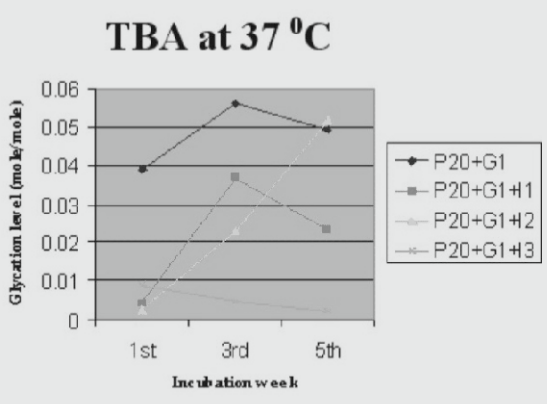

Fig-4.5

The results of TBA at 37˚C for normal plasma showed that all concentrations of glucose (G_1, G_2 and G_3) with protein showed high level of glycation throughout incubation period. I_1 did not worked with G_1 and G_2 but it inhibit glycation with G_3 to some extent, 1_2 showed inhibition with G_1 and G_3 but did not show inhibition with G_2 and I_3 showed inhibition with G_1 and G_3, but did not show inhibition with G_2.

Periodate Borohydride Assay

It is a colormetric assay for determination of degree of glycation of serum protein that is unaffected by glycoslyation residues.

Fig. 4.6 shows that G_1 (500 mM conc.) increased glycation upto third week but after that the curve dropped because these glycation products converted into AGEs. I_1 (10mM conc.) did not work to 3rd week and glycation level increased but it worked in 5th week and decreased the glycation level. I_2 (5 mM conc.) also did not work up to 3rd week and glycation level increased but it worked in 5th week and decreased the glycation level. I_3 (1mM conc.) did not work upto 3rd week and glycation level increased but it worked in 5th week and decreased the glycation level at 37˚C for normal plasma.

Fig. 4.7 shows that G_2 (250 mM conc.) increased glycation upto third week but after that the curve dropped because the glycation products converted into AGEs. I_1 (10 mM conc.) did not work upto 3rd week and glycation level increased but it worked in 5th week and decreased the glycation level. I_3 (1mM conc.) did not worked up to 3rd week and glycation level increased but it worked in 5th week and decreased the glycation level at 37˚C for normal plasma.

Fig. 4.8 G_3 shows that (5.5. mM conc.) increased glycation upto third week but after that the curve dropped because these glycation products converted into AGEs. I_1 (10 mM conc) worked through out and decreased glycation level. I_2 (5mM conc.) worked through out to decrease glycation level. I_3 (1mM conc.) worked throughout and decreased glycation level at 37˚C for normal plasma.

The results of periodate assay at 37˚C showed that all concentrations of glucose (G_1, G_2 and G_3) with protein showed high glycation level during incubation time. Here I_1, I_2 and I_3 showed inhibition with all four different concentrations of glucose. It shows that there is no effect of inhibitor concentration on extent of inhibition of glycation at 37˚C for normal plasma.

The results of periodate assay at 37˚C showed that all concentrations of glucose (G_1, G_2 and G_3) with protein showed high glycation level during incubation time. Here I_1, I_2 and I_3 showed inhibition with all four different concentration of glucose. It shows that there is no effect of inhibitor concentration on extent of inhibition of glycation at 37˚C for diabetic plasma.

Eric et al. (1999)[9] worked on Periodate Borohydride Assay for glycation inhibition in rats.

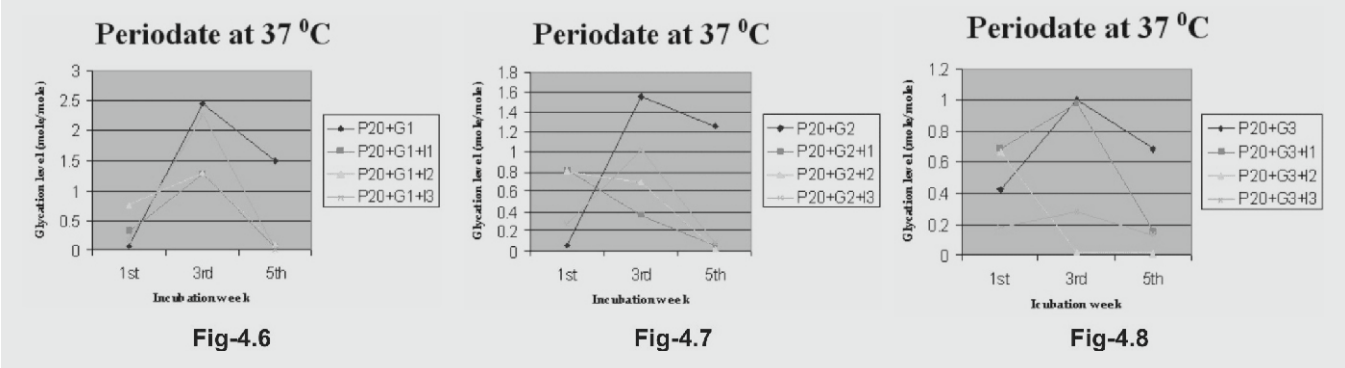

Fig-4.6 Fig-4.7 Fig-4.8

Correlation between Thiobarbituric acid (TBA) and Periodate Borohydride Assays for Glycation at 37˚C (for normal plasma).

In this research two glycation assays i.e. Thiorbarbituric acid assay (TBA) and periodate borohydride assay are correlated.

Fig. 4.9 shows that TBA and Periodate Borohydride assays both have negative correlation (r = -0.26076) during 1st week at 37˚C.

Fig. 4.10 shows that TBA and periodate Borohydride assays shows positive correlation (r = 0.664633) at 37˚C in 3rd week.

Fig. 4.11 shows that TBA and Periodate Borohydride assays shows positive correlation (r= 0.421261) at 37˚C in 5th week.

Above results shows that at 37˚C TBA and Periodate assay have more positive correlation (r = 0.664633) in 3rd week as compare to 5th week (r = 0.421261) and in 1st week it showed negative correlation (r = 26076).

Correlation between Thiobarbituric Acid (TBA) and Periodate Borohydride Assays for Glycation at 37˚C (for diabetic plasma).

In this research work two glycation assays i.e. Thiobarbituric acid assay (TBA) and Periodate borohydride assay are correlated.

Fig. 4.12 shows that TBA and Periodate Borohydride assays both have negative correlation (r= - 0.27057) at 37˚C during 1st week.

Fig. 4.13 shows that TBA and Periodate Borohydride assays shows positive correlation (r = 0.09864) at 37˚C in 3rd week.

Fig. 4.14. shows that TBA and Periodate Borohydride assays shows positive correlation (r = 0.44901) at 37˚C in 5th week.

Above results shows that at 37˚C TBA and Periodate

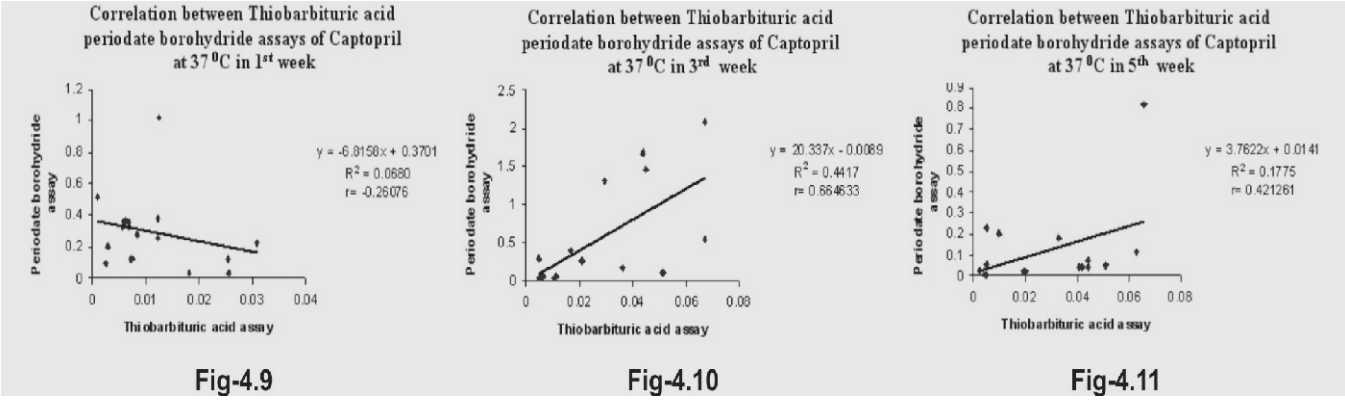

Fig-4.9 Fig-4.10 Fig-4.11

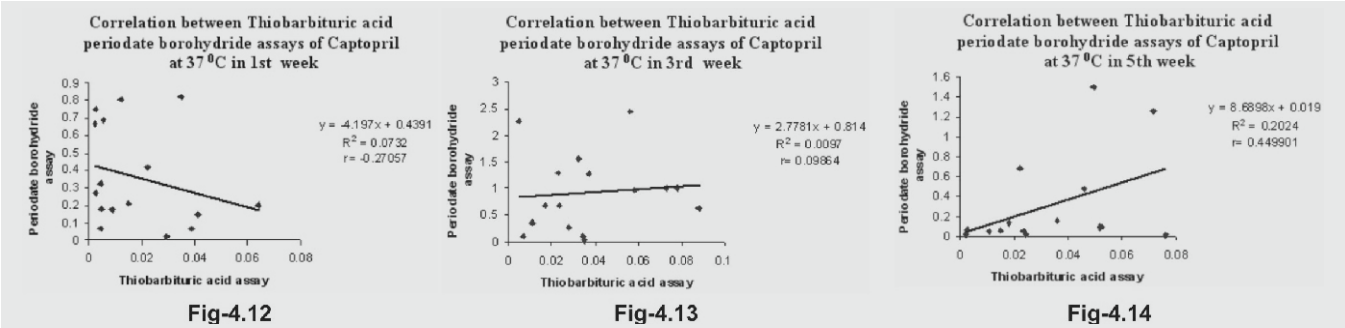

Fig-4.12 Fig-4.13 Fig-4.14

assays have more positive correlation (r = 0.449001) in 5th week as compare to 3rd week (r = 0.09864) at in 1st week it showed negative correlation ®= -27057).

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Correspondence Address:
Miss. Gull-e-Faran
Department of Chemistry & Biochemistry
University of Agriculture, Faisalabad
Demonstrator
Department of Biochemistry
Independent Medica College
Faisalabad.

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