CHAPTER 3: METHODOLOGY

3.1 Selection criteria for patients and control subjects

The patients and controls included in the study were non smokers, non alcoholics and not using any vitamin and are not any antibiotic supplements. They were not on any corticosteroid treatment or antioxidant supplement. The authors' Institutional Research and Ethics Committee approved this study. Informed consent was obtained from all the participants in the study.

Forty patients with ED were enrolled in this study. ED was diagnosed on the basis of the following criteria, periphlebitis of the retina, neovascularization and vitreous haemorrhage not associated with anterior uveitis, choriditis, parsplanitis or other retinal vascular diseases which mimic ED

Patients in the age group of 15- 40 years, with active vasculitis of unknown etiology, diagnosed as ED after detailed fundus examination by an ophthalmologist, and twenty healthy adult volunteers were recruited in the study based on the inclusion criteria. Active vasculitis in the ED is characterized by the presence of serous exudates rounds the retinal veins with retinal edema. In addition to the above, there was venous occlusion, neovascularization, fibrovascular scar formation with or without vitreous haemorrhage observed in all patients.

Among the forty (40) patients, 20 of them along with age matched healthy controls in the group I are used for the study of amino acids involved in the metabolism of homocysteine, the protein bound homocysteine modification and to study the correlation between the homocysteine with glutathione synthesis and turnover in Eales' disease.

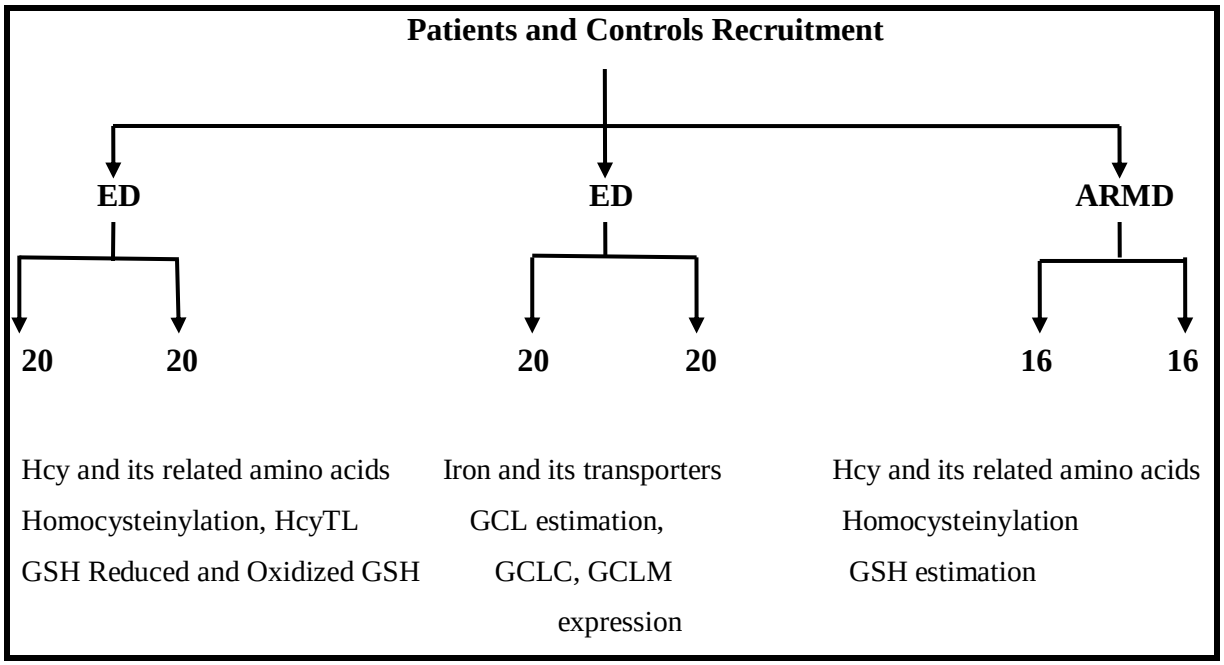
Another 20 patients along with age matched healthy controls in group II are used to study the association of elevated homocysteine with trace element iron. The demographic details of the patient group I and group II are given in table I and table II as an appendix.

Patients with ARMD 16 study subjects along with age matched healthy controls were enrolled in the study. The demographic details of the patients group III are presented in table III as an appendix**.** Patients recruited for the study were diagnosed with ARMD and were maintaining vegetarian diets at the time when their samples were collected. The grading of exudative ARMD was based on the age-related eye disease study group (AREDS) recommendations. Subjects (both patients and controls) with a history of diabetes mellitus, renal disease, hypertension, cardiovascular disease, smoking, alcohol consumption and those on antioxidant supplements were excluded from the study. The ophthalmologist ruled out any systemic/ophthalmic diseases from the control subjects before recruitment after a detailed examination. Informed consent was obtained from all the participants in the study. The author's Institutional Research and Ethical Committee approved the study. All procedures pertaining to human subjects strictly adhered to the tenets of the Helsinki declarations.

Participants enrolled in the group III are used for the study of amino acids involved in homocysteine, the protein bound homocysteine modification and to study the correlation between the homocysteine with glutathione synthesis are listed in table 5.

The representative consent letter, clinical proforma of both the vascular diseases are included in the appendix.

Table 5: Patients and controls recruitment in the study



3.2 Blood tests

Ten mL of blood was drawn from the participants after overnight fasting into EDTA containing tubes. Plasma and Serum were separated immediately from blood cells by centrifugation at 3000 g at 25˚C for 10 min and stored at -80˚C up to 2 weeks for other investigations. Haemoglobin, TBARS and GSH were estimated on the same day of the sample collection. Hcy, protein homocysteinylation on samples from the test and control subjects were performed simultaneously in the ED and ARMD. mRNA and protein expression were done in the peripheral blood mononuclear cells isolated from heparinised blood samples by histopaque - density gradient elution method.

3.3 Separation of peripheral blood mononuclear cells

Ten mL of heparinised venous blood sample was used for separation of mononuclear cells. 5 mL of blood was carefully overlaid with 5 mL of histopaque density gradient solution (Sigma, USA) and centrifuged at 2,700 rpm for 30 min at RT. After centrifugation, the middle layer which contains (both lymphocytes and monocytes) were carefully aspirated and washed thrice with 1X Phosphate buffered saline (PBS) pH 7.4. After the final wash, the pellet was carefully aspirated and resuspended. 10 µL of the cell was used to assess the viability of the cell by trypan blue dye exclusion test. Then the cells were processed for further investigations.

3.4 Determination of Homocysteine and its related amino acids by High performance liquid chromatography:

3.4.1 Principle of Precolumn derivatization of homocysteine and its related amino acids using Ortho-phthaldehyde (OPA):

Homocysteine and its sulfur containing amino acids such as cysteine, methionine, taurine were treated with iodoacetic acid (IAA) for alkylation of – SH group at pH 11.5 and other amino acids such as glutamic acid and glycine react with OPA in the presence of β-mercaptoethanol to form an isoindole derivative. This is detected using a fluorescent detector at Excitation 340 nm and Emission 450 nm by the method of

Tcherkas and Denisenko 2001 with slight modifications [125]. OPA derivatization procedures involve a rapid reaction and high sensitivity.

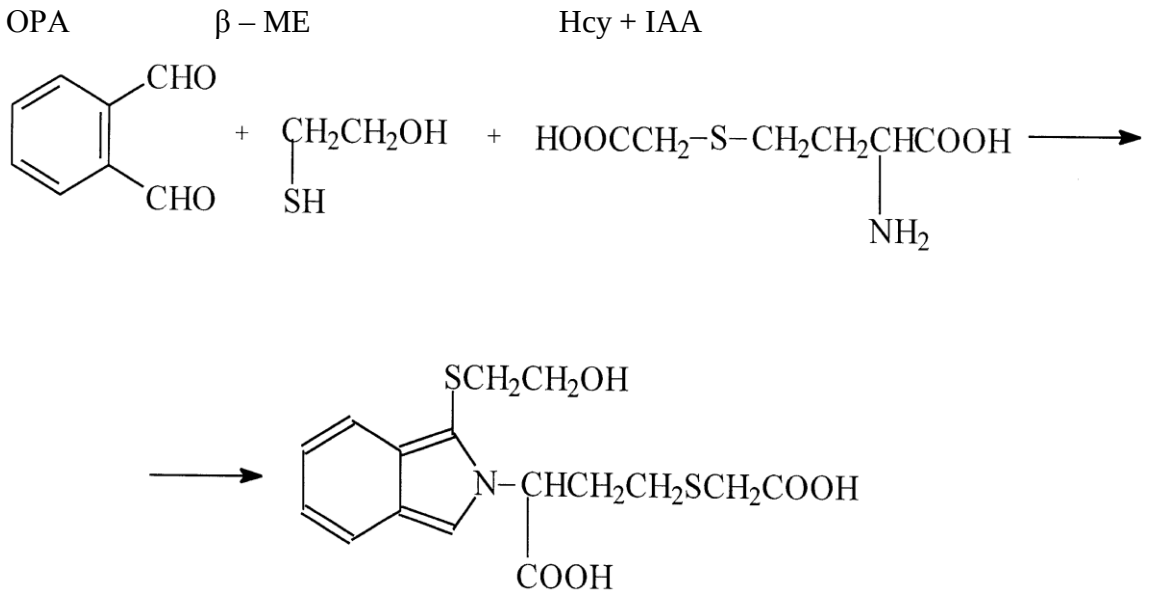


Figure 8: OPA and β-mercatoethanol react with an amino acids

The HPLC system (model 1100; Agilent Technologies, Palo Alto, CA) consisted of two pumps fitted with 50 µl loop rheodyne injection valve and fluorescence detector. Separation was performed on a 150 X 4.6 mm column (internal diameter, 5 mm; ODS; Phenomenex, Torrance, CA). The elution procedure was performed with 0.05 M acetate buffer (pH 7.0) containing 80 % methanol in linear gradient, at 26˚C with a flow rate of 1.0 mL / min. A fluorescence detector with excitation at 340 nm and emission at 450 nm was used for detection. Before analysis the system was calibrated with standards in the range of 25 to 100 ng. The method is linear up to 25 ng. The detection limit is upto 100 pg.

3.4.2 Processing of Standards :

Stock solutions of Homocysteine, Cysteine, Methionine, Taurine, Glutamic acid, Glycine and norvaline (1 mg /1 ml) were prepared in double distilled water and then dissolved in 0.05 M Perchloric acid and stored at -20˚C. Working solutions of standards were prepared freshly before use and is as follows;

3.4.3 Processing of standard DL – Homocysteine and its related amino acids:

Working intermediate standard**:** From the stock 1 mg/ml, working intermediate standards (S1 to S4) ranging from 5 – 100 µg/ml is prepared as given in the flow chart below and is processed for derivatization.

Table 6: Processing of Intermediate Standards of Homocysteine and its related amino acids

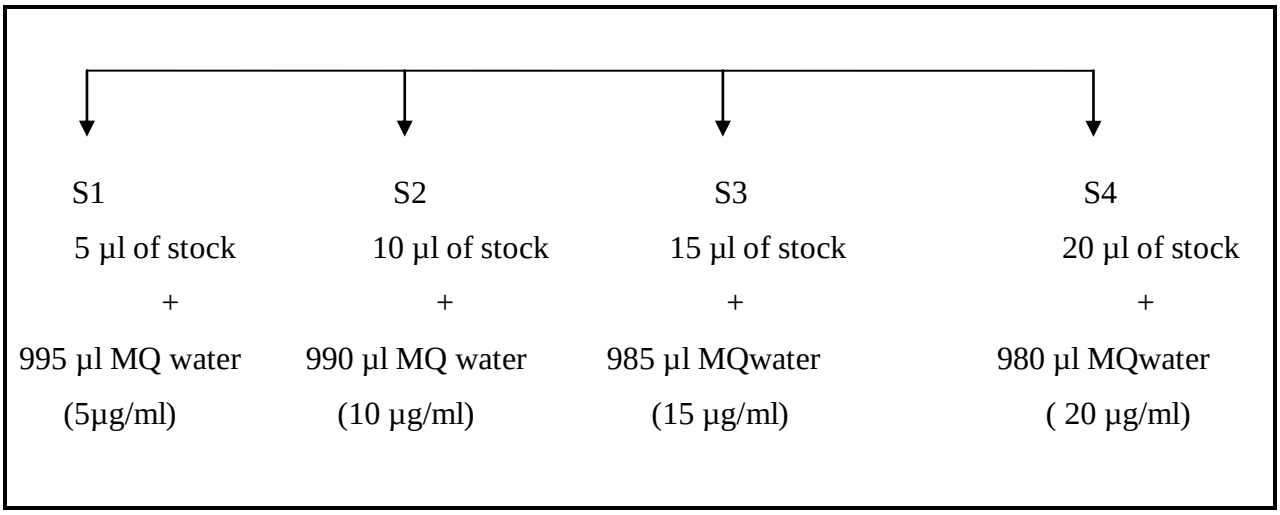
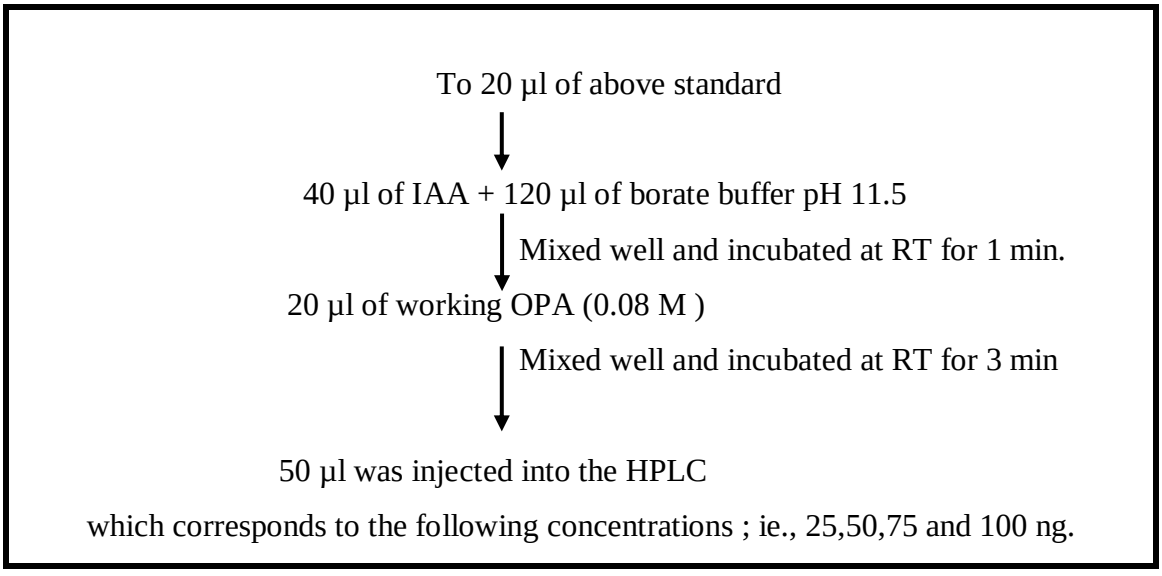


Table 7: Processing of working Standards of Homocysteine and its related amino acids

The above intermediate working standards were proceeded for derivatization.



Liquid chromatography parameter:

Pressure - 400 bar	Temperature - 26ºC
Flow rate – 1 ml/min.	Detector – Fluorescence
Excitation – 340 nm	Emission – 450 nm
Injection volume – 50 µl	

3.4.4 Processing of plasma samples:

Plasma sample was treated with β- mercaptoethanol to reduce the disulphide to sulphydryl group. The sample is deproteinised using methanol. This is then treated with iodoacetic acid for alkylation of – SH group containing amino acid and then treated with the OPA to form an isoindole derivative. This is detected using a fluorescent detector with a Excitation of 340 nm and Emission at 450 nm. The processing of the sample was given in the flow chart below and is processed for derivatization as follows;

Table 8: Processing of plasma Samples for Homocysteine and its related amino acids

100 µl of plasma + 20 µl of β- mercaptoethanol

↓ kept at 30 sec at RT

380 µl of ice cold 100% methanol

↓ centrifuged in cooling centrifuge at 10,000 rpm for 5 min

20 µl of the supernatant was taken for the assay

+

40 µl of IAA + 120 µl of borate buffer pH 11.5

↓ Mixed well and incubated at RT for 1 min

20 µl of working OPA

↓ Mixed well and incubated at RT for 3 min.

50 µl is injected into HPLC

3.4.5 Method standardization:

Calibration: 25 – 100 ng concentrations of Glutamic acid, DL-Cysteine, DL-Homocysteine, Glycine, Methionine, Taurine and Norvaline standard were injected into HPLC and were used as calibrators.

Inter-assay variations - This was done by repeating the analysis of standard, three times in similar conditions on the same day.

Intra-assay variations – This was done by injecting the standards on different days under different conditions.

Percentage recovery – The mean percentage recovery was calculated by spiking the sample with known concentrations of the standards.

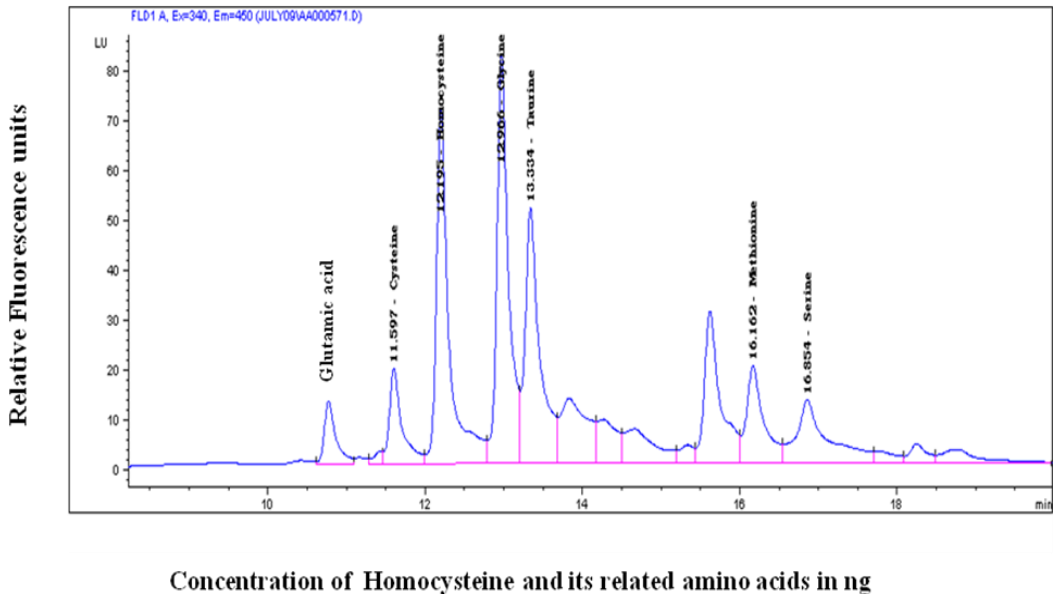
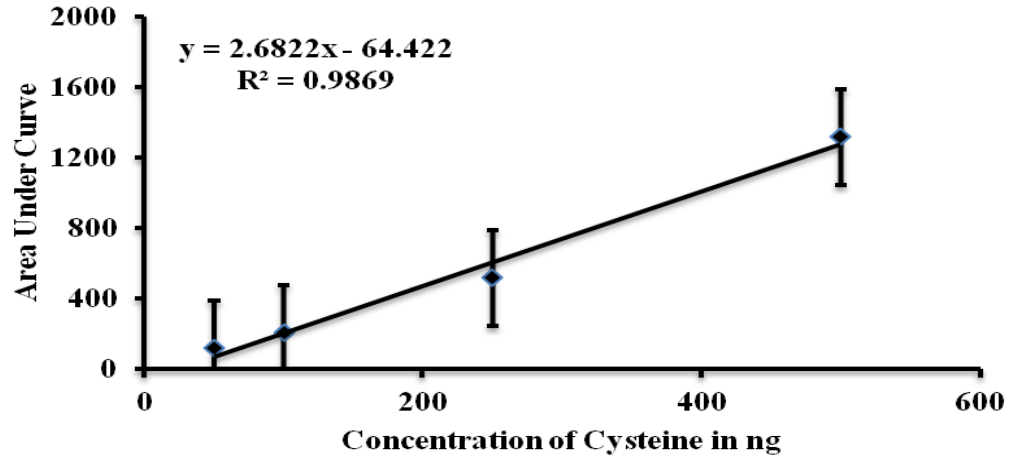
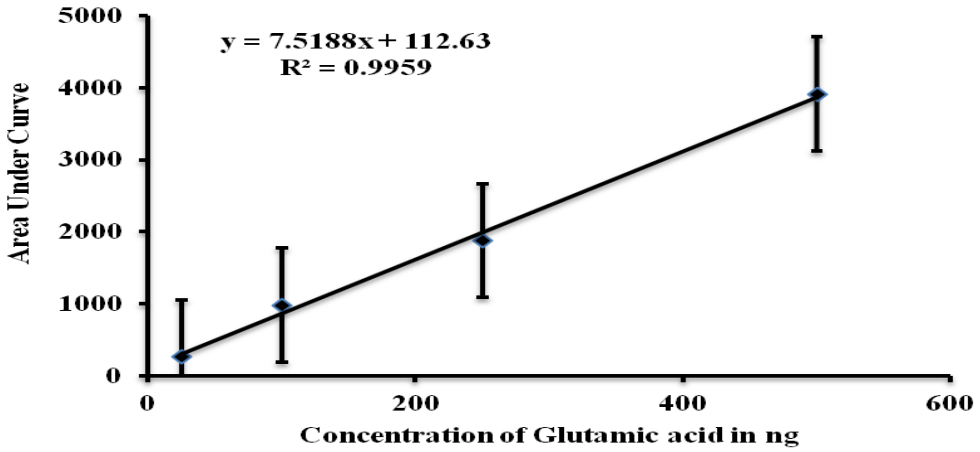


Figure 9: HPLC Chromatogram of standards using FLD

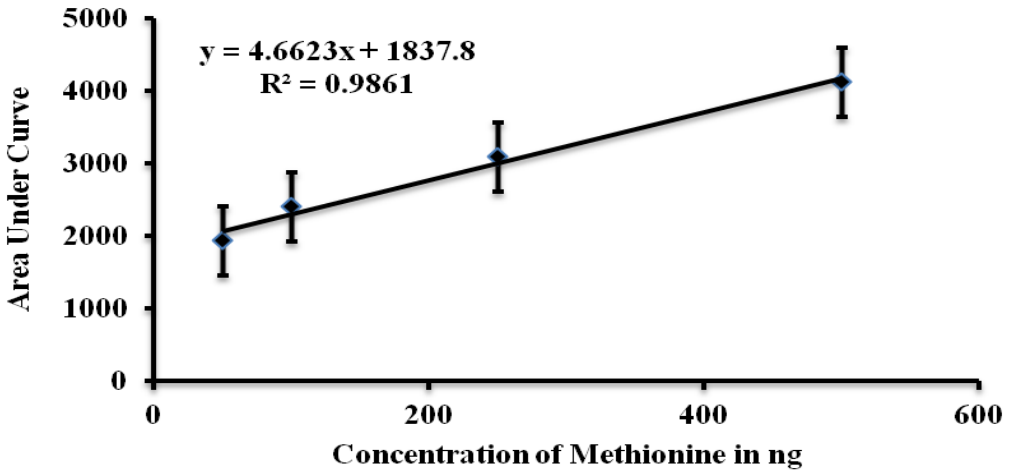
[A]


y = 2.6822x - 64.422
R² = 0.9869
Area Under Curve
2000
1600
1200
800
400
0
0
200
400
600
Concentration of Cysteine in ng

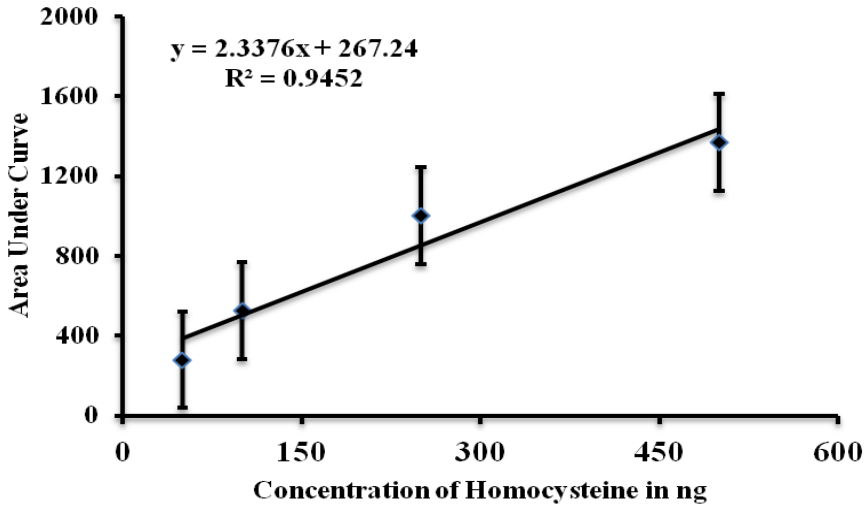
[B]


y = 7.5188x + 112.63
R² = 0.9959
Area Under Curve
5000
4000
3000
2000
1000
0
0
200
400
600
Concentration of Glutamic acid in ng

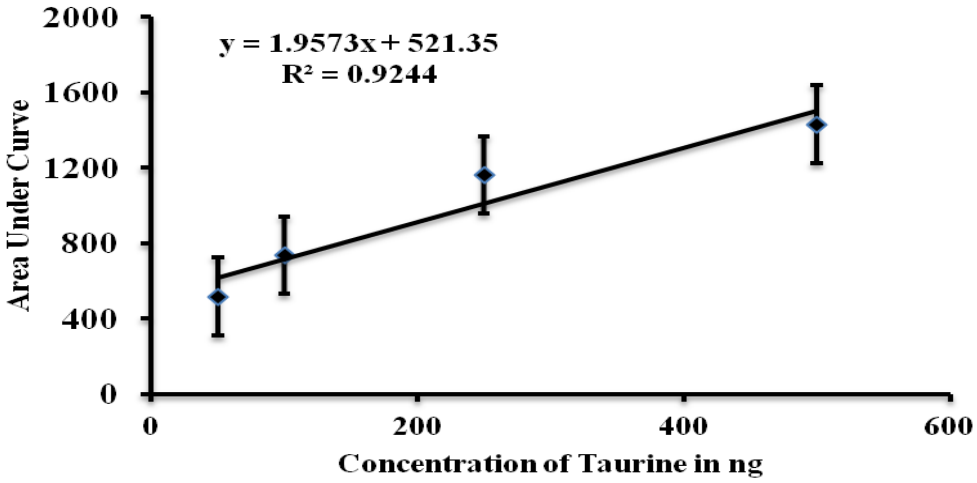
[C]


y = 4.6623x + 1837.8
R² = 0.9861
Area Under Curve
5000
4000
3000
2000
1000
0
0
200
400
600
Concentration of Methionine in ng

[D]



[E]



(F)

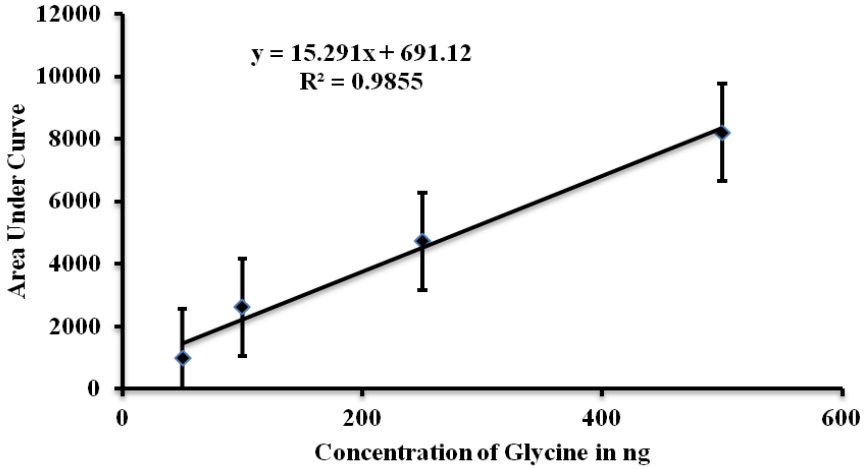


Figure 10 (A, B,C, D, E, F): HPLC calibration curve of standards using FLD

3.5 Determination of Plasma Homocysteine-Thiolactone (HcyTL)

3.5.1 Principle:

Amino acids whose α-amino groups have a pKa is 9.5, the α-amino group of HcyTL has a pKa is 7.1. Because its pKa is 7.1 and it lacks a free carboxyl group, HcyTL is chiefly neutral at pH near 8 and mostly positively charged at pH near 6. The selective extraction of HcyTL from human plasma, first adjusted the pH of samples to 8.0 to convert HcyTL to a neutral form. The hydrophobicity of the neutral form of HcyTL is sufficient to facilitate its transfer to the organic phase upon extraction with chloroform/methanol. Subsequent conversion of the neutral form to a positively charged form facilitates re-extraction of HcyTL from the organic phase with diluted HCL.

Because of its positive charge at pH values below its pKa, HcyTL is expected to be retained on a cation exchange column, such as polysulfoethyl aspartamide. HcyTL was selectively retained on the column equilibrated with 10 mM sodium phosphate buffer, pH 6.6, containing 5 mM NaCl, in contrast to Hcy, which was not bound.

3.5.2. Processing of standards:

Stock Standard: 1 mg/mL of Homocysteine-thiolactone in Milli Q Water.

Intermediate Standard: 4 µL made up to 100 µL. i.e.,4000 ng/L concentration.

Working standard:

Conc (ng)	Intermediate Standard (µL)	Milli Q Water (µL)
20	25	1000
40	50	1000
60	75	1000
80	100	1000
100	125	1000

From this 50 µl injected into HPLC.

3.5.3 Sample preparation for the determination of Hcy-thiolactone:

Sample preparation for the determination of HcyTL was described by Jakubowski et.al.,[126]. Plasma Hcy-Thiolactone was determined by HPLC using a cation-exchange Polysulfoethyl aspartamide column was described by Jakubowski et.al., [127]. The detection limit was 10 pmol. The levels of plasma HcyTL was expressed as nM.

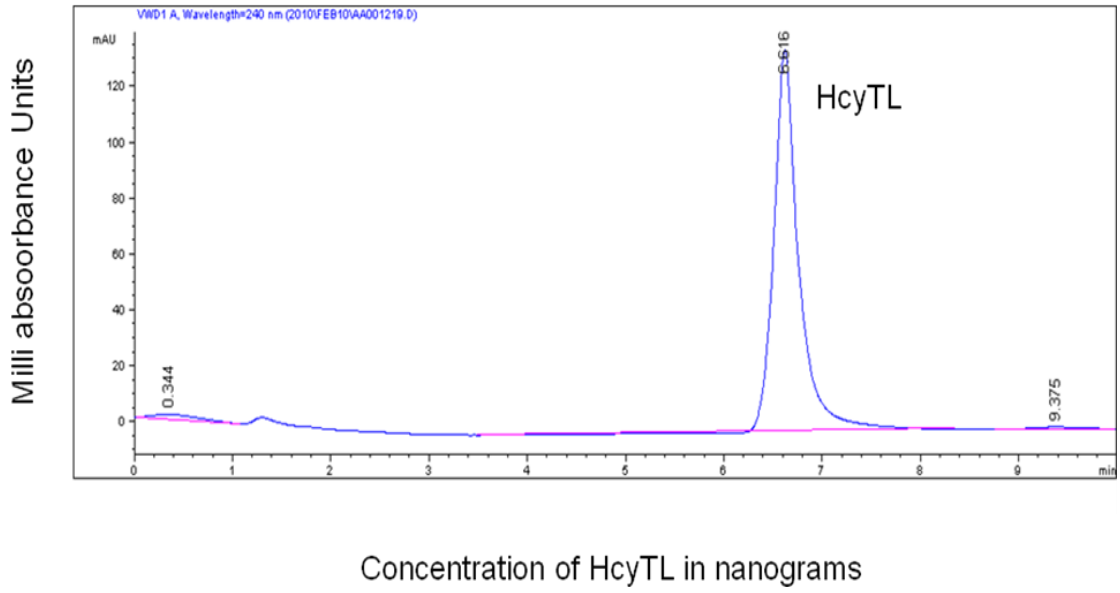


Figure 11: HPLC chromatogram of standards using Variable wavelength Detector

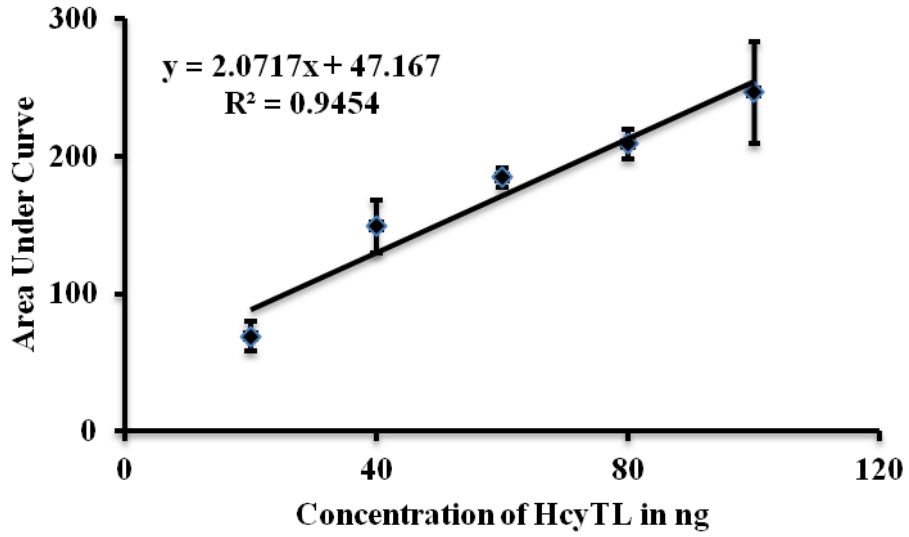


Figure 12 : HPLC calibration curve of standards using Variable Wavelength Detector

3.6 Determination of protein-Cys bound homocysteine and protein-Lys bound homocysteine by High performance liquid chromatography:

Processing of plasma samples for protein-Cys bound Hcy and protein-Lys bound homocysteine :

Plasma samples were subjected to dialysis against water for 12 h to remove free Hcy. After dialysis the samples were aliquoted in to two separate vials, first one was treated with 6 N HCl and second one was treated with 50 mM DTT for 30 min in boiling water bath and centrifuged at 2,500 rpm for 5 min in order to release lysine bound or cysteine bound Hcy by the method of Jakubowski et.al., [24] with the slight modifications. Following the release of Hcy, 100 µL of plasma samples were processed similar to that of Hcy given in the above procedure.

3.7 Determination of Iron, Iron binding capacity and haemoglobin levels in serum by spectrophotometer :

Blood haemoglobin was determined by automated analyzer (Beckman Coulter) and serum haemoglobin was performed by the spectrophotometric scanning technique described by Blakney et.al., [128]. Serum iron was determined by fully automated analyzer followed by the method of smith et.al., [129] (Dade Behring, Siemens, USA) and serum total iron binding capacity was determined by fully automated analyzer followed by the method of yamanishi et.al., (Dade Behring, Siemens, USA) [130].

Principle of serum haemoglobin assay:

The fractional absorption of a portion of the 578 nm band of oxyhaemoglobin is used in conjunction with the absorption coefficient of oxyhaemoglobin to relate absorption to haemoglobin concentration. The fractional absorbance of oxyhaemoglobin at 578 nm is proportional to the haemoglobin concentration.

Processing of samples:

Read the serum sample absorbance at 520 nm, 578 nm with water as a blank and set the wavelength at 598 nm with water as a blank.

Calculation:

OD 562 nm – OD 598 nm / 2.25 = X

OD 562 nm – X = Y

(OD 578 nm – Y) * 155 = serum haemoglobin (mg/dL)

Y = baseline correction at 578 nm

(OD 578 nm – Y) = Fractional absorption of Oxyhaemoglobin.

3.8 Determination of aminolevulinic acid synthase levels in serum and peripheral blood mononuclear cells:

Principle: Aminolevulinic acid synthase (ALAS) (EC 2.3.1.37) catalyzes the pyridoxal phosphate-dependent succinyl coenzyme A and glycine to Aminolevulinic acid. Further ALAS reacts with acetyl acetone at pH 4.7 to form an ALA-pyrrole. In the presence of modified Ehrlich's reagent, a coloured complex of ALA-pyrrole is formed which is quantitated at 556 nm in the Beckman DU 800 spectrophotometer.

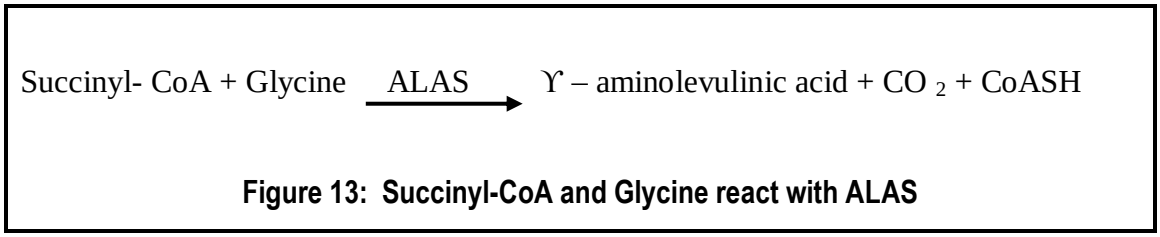


Figure 13: Succinyl-CoA and Glycine react with ALAS

Aminolevulinic acid synthase activity in serum and peripheral blood mononuclear cells was determined by Hunter et.al., [131]. The serum level of ALA is expressed in the form of unit activity (U/L) whereas in PBMC the ALA value is expressed in the form of specific activity of enzyme (unit/min/mg protein). The minimum detection limit of ALA is up to 2.5 µg.

Table 9: Protocol for Standards

S.NO	Reagent Preparation	Blank	S1	S2	S3
1	Milli q water (µL)	300	____	____	____
2	ALA Standard (µL)	____	300	300	300
3	Concentration (µM)	____	2.5	10	40
4	Sodium acetate (µL)	400	400	400	400
5	Acetyl acetone (µL)	35	35	35	35
Incubated the tubes at 80º C for 15 min					
6	Ehrlich's Reagent (µL)	700	700	700	700
Incubated at RT for 10 min and measured the absorbance at 556 nm					

Table 10: Processing for samples

S.NO	Reagent Preparation	Blank	Control	Test
1	Milli q water (µL)	300	480	480
2	Enzyme control (µL)	____	350	____
3	Enzyme Substrate (µL)	____	____	350
4	Cofactor Cocktail (µL)		150	150
Incubated at 37 ºC for 20 min. From the above extraction, 300 µL used for the assay				
5	Sample (µL)	____	300	300
6	10 % TCA (µL)	____	150	150
Centrifuged at 13,000 rpm for 5 min; transfer the supernatant in to a fresh tube.				
7	Supernatant (µL)	____	300	300
8	Sodium acetate (µL)	400	400	400
9	Acetyl acetone (µL)	35	35	35
Incubated at 80 ºC for 15 min and allowed to cool the tubes at RT				
10	Ehrlich's Reagent (µL)	700	700	700
Incubated at RT for 10 min and measure the absorbance at 556 nm				

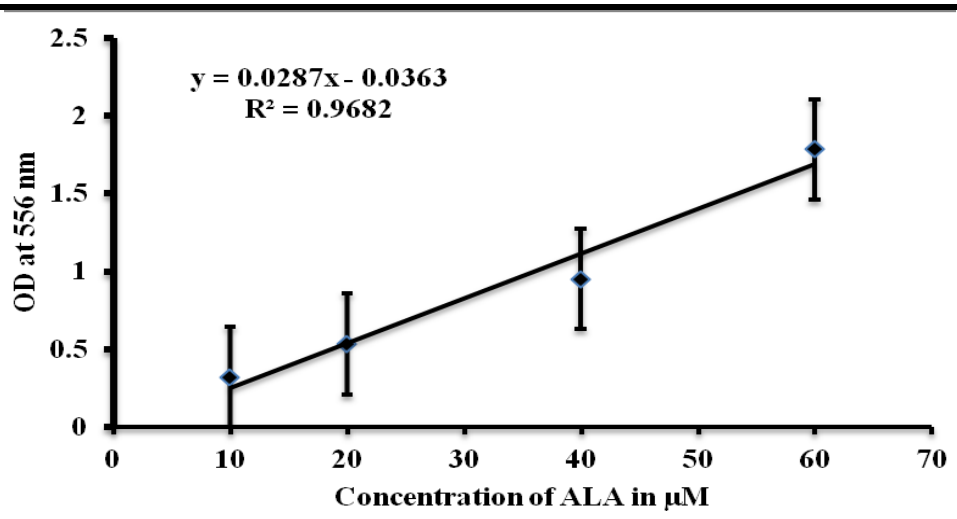


Figure. 14 Standard graphs of aminolevulinic acid by spectrophotometer

3.9 Determination of heme in serum and peripheral blood mononuclear cells:

Heme content in serum and peripheral blood mononuclear cells was determined using a QuantiChrom Heme assay method followed by Berry et.al., [132] (Bio Assay Systems, Hayward, CA, USA).

Principle: Heme assay is based on an improved aqueous solution method, in which the heme is converted into a coloured form. The intensity of colour, measured at 400 nm is directly proportional to the heme concentration in the sample.

Procedure: Pipette 50 µL water (blank) and 50 µL of calibrator was added in to 96 flat bottom well plates. 50 µL of serum and peripheral blood mononuclear cells was added directly to the well. Then 200 µL of chromogen was added to all the wells and incubated for 5 min at RT. After incubation, the plate was read at 400 nm using a plate reader (Molecular devices Spectramax M2e).

Calculation:

$$\text{Heme} = \frac{\text{OD sample} - \text{OD Blank}}{\text{OD calibrator} - \text{OD Blank}} \times 62.5\ \mu M.$$

The detection limit of heme is up to 0.6 µg.

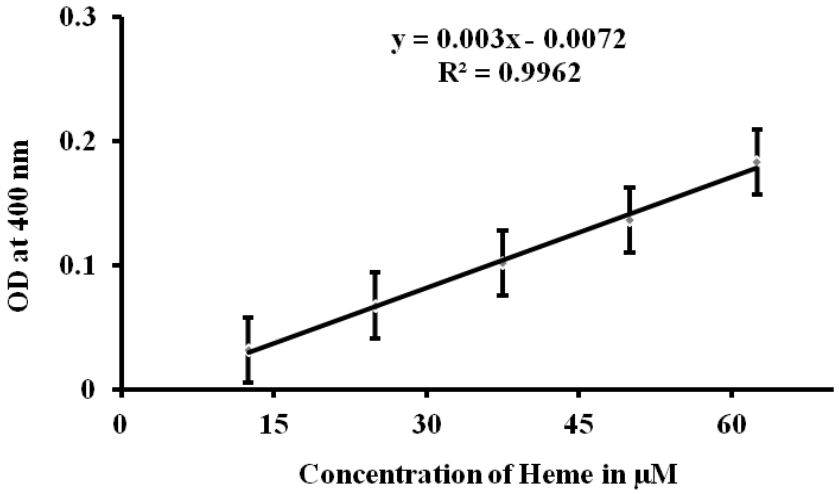


Figure 15: Standard graphs of Heme by spectrophotometer

3.10 Determination of Ferritin, Transferrin and serum transferrin receptors in serum and peripheral blood mononuclear cells:

Ferritin in serum and monocytes was determined by the method of Bernard [133]. Transferrin and serum transferrin receptor in serum were determined by Kreutzer [134] and Samuelson et.al., [135] (ELISA, BioVendor, Canada, USA).

3.10.1 Ferritin

Principle: Serum ferritin causes agglutination of latex particles coated with anti-human ferritin antibodies. The agglutination of the latex particles is proportional to the ferritin concentration which is measured spectrophotometrically.

Procedure: Pipette 6 µL of saline (blank) and 6 µL of ferritin standard at varying concentration diluted with normal saline in to the corresponding 96 flat bottom well plates. 6 µL of serum and peripheral blood mononuclear cells was added directly to the well apart from blank and the standards well. Then 200 µL of chromogen was added to all the wells and was incubated for 5 min at 37ºC. After incubation, the plate was read at 540 nm using a plate reader (Molecular devices Spectramax M2e). The detection limit is up to 4 µg.

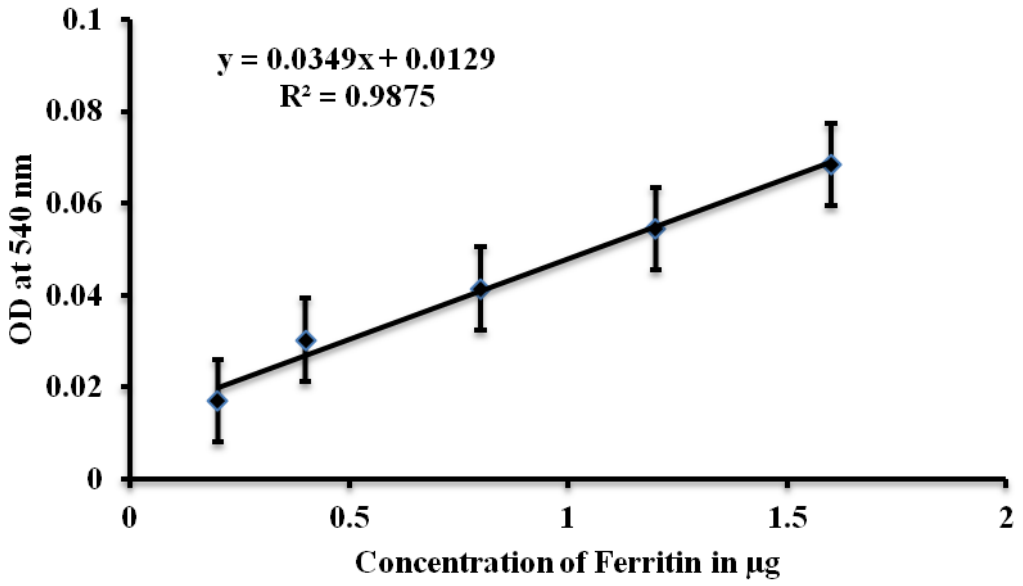


Figure 16: Standard graphs of Ferritin by spectrophotometer

3.10.2. Transferrin

Principle : Transferrin in the sample precipitates in the presence of anti-human transferrin antibodies. The light scattering of the antigen-antibody complexes is proportional to the transferrin concentration which is measured by spectrophotometrically.

Procedure :

Pipette 2 µL of water (blank) and 2 µL of transferrin standards into the corresponding 96 flat bottom well plates. 2 µL of serum and peripheral blood mononuclear cells was added directly to the well apart from blank and the standards well. Then 200 µL of chromogen was added to all the wells and incubated for 5 min at 37ºC. After incubation is over, the plate was read at 540 nm. The detection limit of transferrin is up to 4.8 mg.

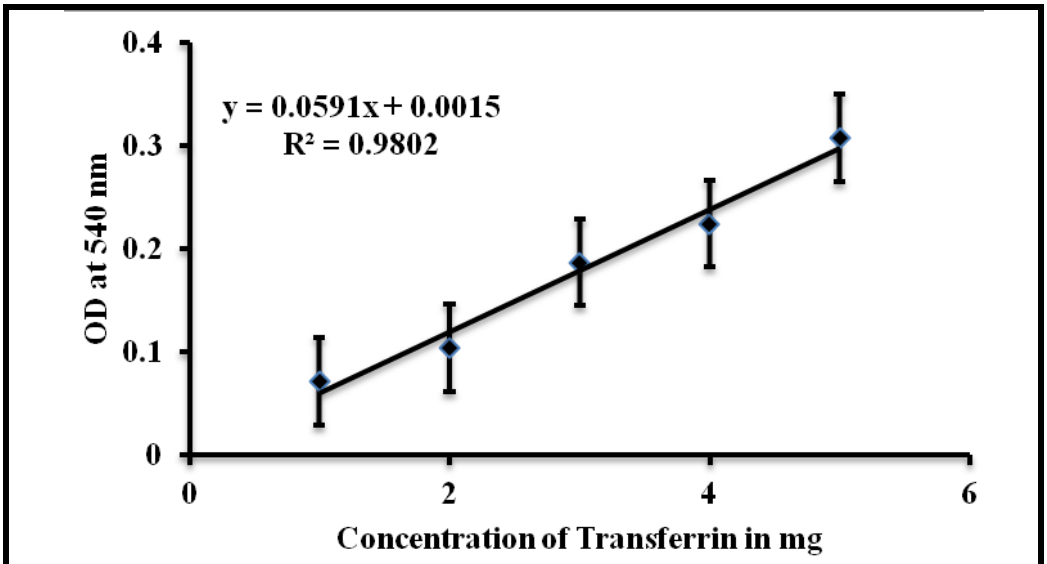


Figure 17: Standard graphs of Transferrin by spectrophotometer

3.10.3 Serum transferrin receptors

Principle: 100 µl of human serum TFR Standards and samples are incubated in microplate wells pre-coated with monoclonal anti-human serum transferrin receptors (sTfR) antibody. After 60 min of incubation and washing, monoclonal anti-human sTfR antibody- conjugated with horseradish peroxidase (HRP) was added to the wells and incubated for 60 min with captured sTfR. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by the addition of acidic solution and absorbance of the resulting yellow product was measured. The absorbance is proportional to the concentration of sTfR.

Procedure: 100 µl of diluted Standards, dilution Buffer (Blank) and samples were added into the appropriate wells. The plate was incubated at RT for 1 h, in rocker at 300 rpm. The wells were washed 3 times with 300 µl of wash solution. After the final wash, 100 µl of conjugate solution was added into each well. The plate was incubated at RT for 1 h, in rocker at 300 rpm. The wells were washed 3 times with 300 µl of wash solution. Then 100 µl of substrate solution was added into each well and incubated for 10 min at room temperature. The colour development was stopped by adding 100 µl of Stop Solution. The absorbance was read at 450 nm. The detection limit of s TFR is up to 2 ng.

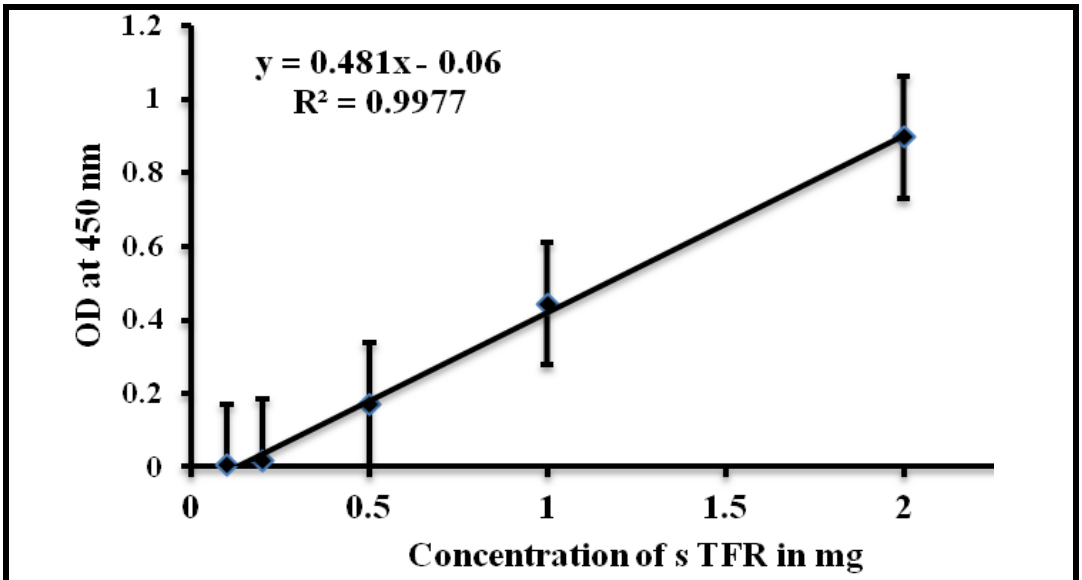


Figure 18: Standard graphs of serum transferrin receptors by spectrophotometer

3.11 Determination of Heme oxygenase activity in serum and peripheral blood mononuclear cells :

HO activity (EC 1.14.99.3) assay in serum and peripheral blood mononuclear cells was performed using the method Bussolati et.al., [136] in which bilirubin, the product of heme degradation, was extracted with chloroform and its concentration was determined in a fluorescent plate reader and calculated using the difference in absorbance at wavelength λ from 464 and λ 530 nm using an extinction coefficient of 40 mM^{-1} cm^{-1}. The total specific activity of HO was expressed as pmoles of bilirubin formed / mg of protein / h. The protein concentration was performed using Bovine serum albumin (BSA) as a standard by Biuret method for serum sample [137] and in PBMC cells by Bradford method [138].

Table 11: Processing for samples

Reagents required	Blank	standard	Test
Samples (µL)	____	_____	50
Standard (µL) 10 µg concentration	____	10	____
2 mg cytosol (µL)	25	25	25
0.8 mM NADPH (µL)	_	25	25
1 mM G-6-P (µL)	25	25	25
0.2 U G-6-P-D (µL)	2.5	2.5	2.5
10 (µg) / mL Hemin (µL)	10	10	10
100 mM phosphate buffer pH 7.4 (µL)	197.5	162.5	162.5
Incubated at 37˚ C for 1 h in the dark.			
Chloroform (µL)	250	250	250
Fluorescence intensity was measured at 464 and 530 nm			

Calculation : The extinction coefficient (Δ 464 -530) of 40 mM/cm was used for calculation of bilirubin concentrations.

Heme oxygenase ativity was expressed in picomoles of bilirubin per mg of protein per hour.

$$\text{Enzyme activity} = \frac{\Delta\ 464 - 530}{40} \times \frac{\text{total volume}}{\text{sample volume}} \times \frac{10^{6}}{\text{protein (mg/ml)}} \times \frac{1}{\text{time (min).}}$$

3.12 Determination of Vascular endothelial Growth Factor

VEGF in serum and peripheral blood mononuclear cells was determined by using Quantikine ELISA kit (R&D, USA).

Principle: VEGF is the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are added into the wells and VEGF present is bound by the immobilized antibody. While washing, unbound substances are removed; an enzyme-linked polyclonal antibody specific for VEGF is added to the wells. Following a wash

to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of VEGF present in the sample. The colour development is stopped and the intensity of the colour is measured by spectrophotometrically at 450 nm.

Procedure: Prepare all reagents, working standards, and samples are freshly prepared.100 µL of standards, samples and buffer (blank) are added to the corresponding wells, to this, 100 µL of assay diluents are added and incubated at RT for 2 h. The wells were washed 3 times with 300 µl of wash Solution. After the final wash, 200 µL of VEGF conjugate was added to each well and incubate for 2 h at RT. The wells were washed 3 times with 300 µl of wash Solution. After the final wash, 200 µL of substrate solution was added to each well and incubate for 25 min at RT. Then 50 µL of stop solution was added to each well and the absorbance was read at 450 nm using a plate reader (Molecular devices Spectramax M2e). The detection limit of VEGF is up to 5.0 pg.

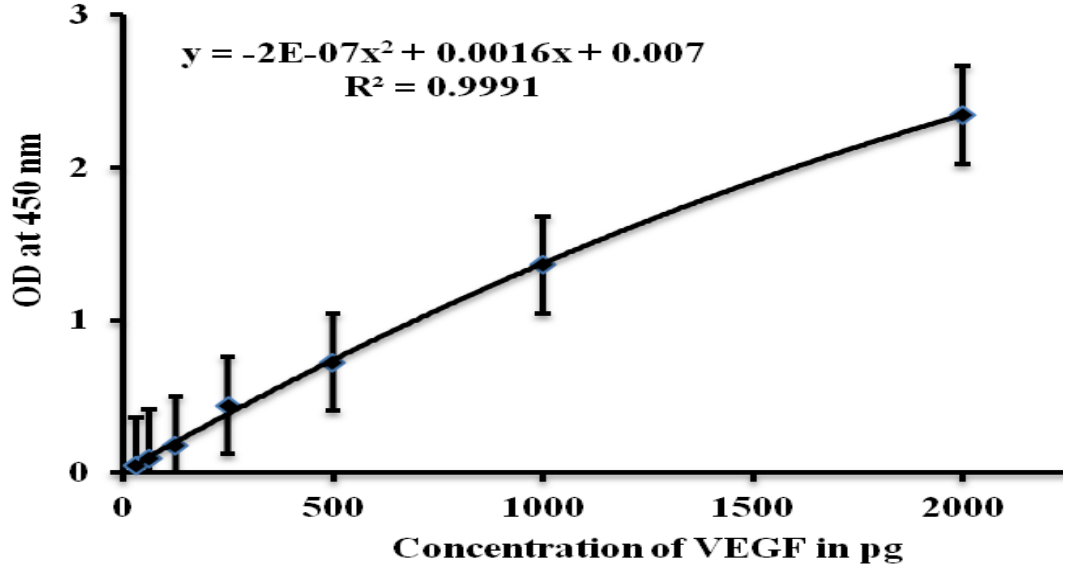


Figure. 19 Standard graphs of VEGF by spectrophotometer

3.13 RNA ANALYSIS

Total RNA extraction:

Cell samples were harvested and washed with PBS and the total RNA was extracted by TRI method. To the cell pellet (PBMC cells), ARPE-19 cells and HUVECs 1 ml of trizol reagent was added and the pellet was resuspended. This was incubated for 5 min

at RT. To this 200 µL of chloroform was added and mixed well (without vortex) and kept at RT for 3 min. This was centrifuged at 12,000 rpm for 15 min at 4ºC and the aqueous layer was carefully transferred to a new vial. To this 500 µL of isopropanol was added and incubated at RT for 10 min. After incubation, it was centrifuged for 12,000 rpm for 10 min at 4ºC and to the pellet 1 ml of 75% alcohol was added and centrifuged at 14,000 rpm for 5 min. The RNA pellet was dried and reconstituted with 20 µl of DEPC treated water and stored at -80ºC. The RNA was quantified using the nanodrop (Thermo scientific, Wilmington, DE). For all samples 1µg of total RNA samples was used for the cDNA conversion (Bio-rad cDNA synthesis kit). The processing of samples for cDNA conversion is as follows;

Table 12: The processing of samples for cDNA conversion is as follows;

S.NO	Reagents required	Test sample (µL)	Control sample (µL)
1	5 X reaction mixture	4	4
2	RNA (1 µg) concentration	2.2	2.0
3	Reverse transcriptase enzyme (200 units / µL)	1	1
4	Water	12.8	13

The cDNA was quantified using Nanodrop1000 and diluted for real time amplification or semiquantitative RT-PCR. Quantitative real-time PCR assays developed using RNA Master SYBR Green Kit according to the manufacture's protocol using Applied Bio systems (Foster City, CA). Levels of ϒ-glutamate-cysteine ligase catalytic unit (GCLC), ϒ-glutamate-cysteine ligase modifier unit (GCLM), Nuclear erythroid related factor 2 (Nrf2), hepcidin, ferroportin and hypoxia inducible growth factor HIF 1α, HIF 2 α were determined and the details of primers given below. The $2^{-\Delta\Delta Ct}$ method was used to estimate relative transcript levels with GAPDH and 18 s rna as endogenous control. Relative quantification data were obtained with ABI prism 7000 SDS software. Each sample was studied in triplicate. The primer sequences for all the genes are given below:

Table 13: The primer sequences for all the genes are listed below;

S. NO	Gene Nam	Accession number	Forward primer	Reverse primer
1	GCLC	NM_001197115.1	5'CTGTTGCAGGAAGGCATTGAT 3'	5' TTCAAACAGTGTCAGTGGGTCTCT3'
2	GCLM	NM_005270754.1	5'ATGGCCTGTTCAGTCCTTGG 3'	5'CTCGTGCGCTTGAATGTCAG 3'
3	Nrf2	NM_001145413.2	5'CGGTATGCAACAGGACATTG 3'	5'GTTTGGCTTCTGGACTTGGA 3'
4	18 s rna		5' GTGGAGCGATTTGTCTGGTT 3'	5' GGACATCTAAGGGCATCACAGA3'
5	Hepcidin	NM_021175.2	5′GACACCCACTTCCCCATCTG 3′	5′ GCAGGGCAGGTAGGTTCTAC3′
6	Ferroportin	NM_014585.5	5′ TTGGGGAGATCGGATGTGGC 3′	5′ GTCACCGATGATGGCTCCCA 3′
7	HIF 1α	NM_001530.3	5′CAGTCGACACAGCCTGGATA 3′	5′ TGTCCTGTGGTGACTTGTCC 3′
8	HIF 2 α	NM_001430.4	5′AGGTGGAGCTAACAGGACATAG3	5′GCTGACTTGAGGTTGACAGTAC 3′
9	GAPDH	NM_002046	5′GCCAAGGTCATCCATGACAAC 3′	5′GTCCACCACCCTGTTGCTGTA 3′

3.14 Western blot:

3.14.1 Sample preparation:

The PBMC cell was extracted using 100 µl of Hepes buffer (pH7.0) with proteinase inhibitor cocktail and sonicated for 2 min. The lysed cells were centrifuged at 10,000 rpm for 5 min at 4ºC and the protein was estimated by Bradford method [138].

3.14.2 Polyacrylamide gel electrophoresis:

The protein samples of PBMC for blotting were resolved using SDS-PAGE electrophoresis. For this purpose 10 % separating gel was prepared and poured in the glass plate sealed with 2 % agarose gel and left it without disturbing. The separating gel was covered with saturated butanol and left for polymerization. After the separating gel was polymerized 4 % stacking gel was prepared and poured above the separating gel by removing the butanol layer and the comb was placed carefully without any air bubble and left it aside for solidification. After the gel has been solidified the comb was removed carefully. The protein sample was mixed with 5x sample loading buffer to final volume of 50 µl and incubated in boiling water bath for 5 minutes. Then the sample was loaded on the gel and run till the dye reaches the bottom of the gel. The gel was taken carefully.

3.14.3 Immunodetection:

The proteins were transferred to a nitrocellulose membrane at 100 V for 1 h in cold transfer buffer. Once transferred the blots were blocked with 5 % blocking solution (skimmed milk powder in PBST). The primary antibodies were added according to standardized dilutions and incubated for overnight at 4ºC. The blots were washed 3 times with PBST buffer 5 min each. Then the secondary antibodies were added according to standardized dilutions and incubated for 2 h at RT. The blots were washed 3 times with PBST and 1 time with PBS buffer 5 min each. The presence of specific proteins was then detected by using Amersham chemilumniscence detection reagents (Fluorochem3-Protein simple).

3.15 Determination of Glutathione by spectrofluorometer :

Plasma glutathione was determined by the method of Miuo-Lin-Hu [139] with slight modifications as follows; equal volume of plasma was precipitated with equal volume of 10% Trichloroacetic acid, vortexed and kept in ice for 10 min, followed by centrifugation to remove the protein. 0.2 ml of the supernatant was added to 1.7 ml of 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 8.0 and 0.1 ml of OPA. The mixture was incubated at RT for 15 min and the fluorescent of the OPA derivative of glutathione in plasma was determined by measuring the excitation at 350 nm and emission at 420 nm using a plate reader (Molecular devices Spectramax M2e).

3.16 Estimation of reduced and oxidized glutathione by high performance liquid chromatography using Electrochemical Detector

3.16.1 Principle

The electrochemical detector responds to substances that are either oxidizable or reducible and the electrical output results from an electron flow caused by the chemical reaction that takes place at the surface of the electrodes.

3.16.2 Processing of Standards

A stock standard for reduced glutathione and oxidized glutathione dissolved in buffer and stored at -20˚C.Working solution of standards was prepared daily as follows;

3.16.3 Processing of reduced Glutathione

Intermediate standard**:** From the stock 1 mg/ml, Intermediate standards (10 µl in 1000 µl of buffer) corresponding the following concentrations ranges from 20 – 100 ng/ml is prepared. From this, 20 µl is injected into the HPLC.

3.16.4 Processing of oxidized Glutathione – Conversion of GSSG to GSH

Intermediate standard : From the stock 1 mg/ml, Intermediate standards (10 µl in 1000 µl of buffer) were prepared. For the conversion of GSSG to GSH, reducing agent 50 mM DTT was added to the above standard.

The above intermediate standards where proceeded for derivatization .

Table 14: Processing of standards

To 50 µl of above standard

+

5 µl of DTT

↓ Mixed well and incubate at RT for 10 min.

20 µl is injected into the HPLC

Which corresponds to the following concentrations such as 50,100,250,500,750 and 1000 ng.

Liquid chromatography parameter:

Pressure - 400 bar

Temperature – 27.7ºC

Flow rate – 1 ml/min

Detector – Electrochemical detector (Glassy carbon electrode)

Run time - 10 min

Range - 1 µA FS

E cell – 0.90

Volt– 1.16 v

Injection volume – 20 µl.

3.16.5 Analyzing plasma samples:

One ml of plasma sample was collected, aliquoted into five vials and stored at – 80º C till the analysis. Plasma sample (100 µl) is deproteinised using methanol and injected into an HPLC CBM 20A (Shimadzu, Japan), a Rheodyne Model injection valve with a 20 µl sample loop and ECD Antec Leyden electrochemical detector equipped with an analytical cell. Isocratic separation was achieved using the flow rate of 1.0 mL/min at the temperature of 27.7 ºC in an ODS, hypersil C-18 column, particle size 5 µm, 150 X 4.6 mm and the signal generated by the electrode was detected by electrochemical detector by the method of Sakhi et.al., [140].

Table 15: Processing of samples

GSH 100 µl of plasma + 500 µl of methanol

↓ cyclomixed and centrifuged in cooling centrifuge at 2,500 rpm for 10 min

20 µl of the supernatant is injected into the HPLC for analysis of GSH

GSSG

100 µl of plasma + 500 µl of methanol

↓ cyclomixed and centrifuged in cooling centrifuge at 2,500 rpm for 10 min

50 µl of the supernatant + 5 µl of DTT

↓ Mixed well and incubate at RT for 10 min

20 µl was injected into HPLC for the analysis of GSSG

Standard graphs of GSH by ECD

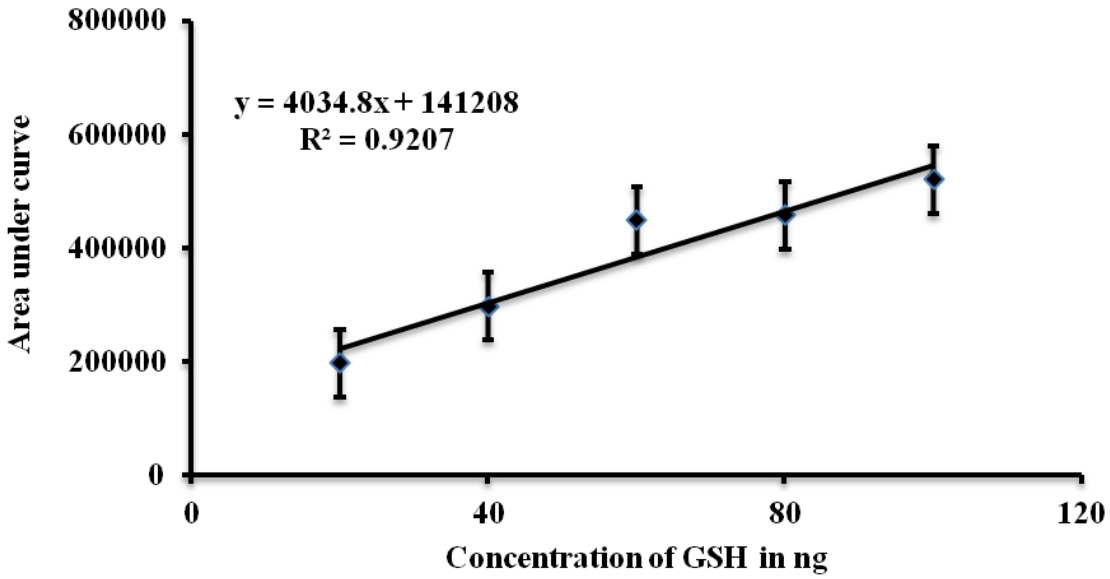


Figure.20 HPLC calibration curve of standards using ECD

Standard graphs of GSSG by ECD

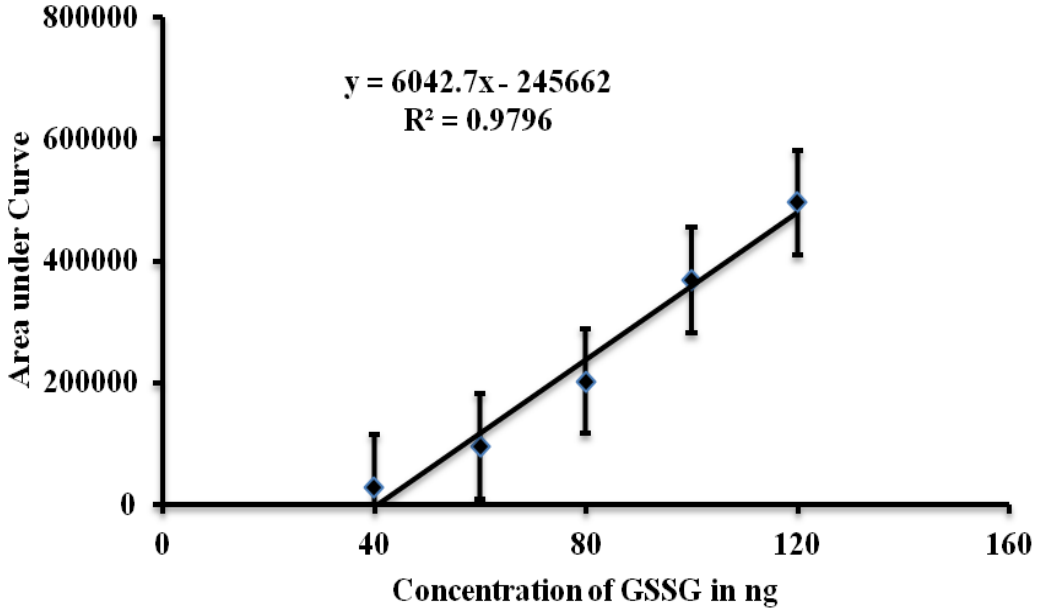


Figure 21: HPLC calibration curve of standards using ECD

3.17 Determination of activity of ϒ-glutamate-cysteine ligase by spectrofluorometer:

Glutathione is synthesized by two enzymes ϒ-glutamate-cysteine ligase (EC 6.3.2.2) a rate limiting enzyme and glutathione synthetase (EC 6.3.2.3). To know if the synthesis of glutathione is decreased, it may be an effect of the rate limiting enzyme ϒ-glutamate- cysteine ligase. So serum and peripheral blood mononuclear cells of ED samples were estimated for ϒ-glutamate-cysteine ligase was determined by the method of Hooper et.al., [141].

Table 16: Processing for plasma samples and peripheral blood mononuclear cells:

Reagent	Blank	Control	Test
GCL cocktail (µL)	_____	_____	50
Plates kept in ice to prevent ATP degradation			
Samples (µL)	_____	50	50
Incubate the plates for 15 sec at 37º C			
2 mM Cysteine (µL)	_____	_____	50
Cyclomixed and Incubated on ice for 20 min			
200 mM SSA (µL)	_____	50	50
GCL cocktail (µL)	_____	50	_____
2 mM cysteine (µL)	_____	50	_____
Cyclomixed and Incubated on ice for 20 min. Then the tube was centrifuged for 5 min at 2,500 rpm.			
From this, 20 µl of the supernatant were transferred into black well plate.			
10 mM NDA (µL)	180	180	180
Incubate at the RT for 30 min in dark.			
Read the GCL activity using plate reader at Ex: 485 nm and Em: 538 nm.			
Read the GC activity was read within 10 min			

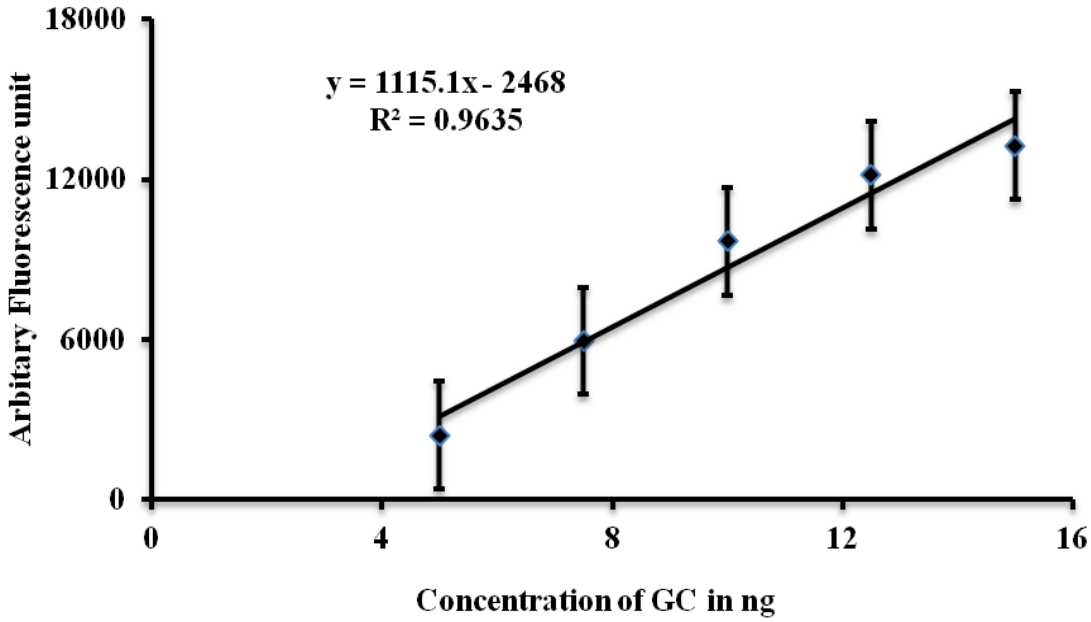


Figure 22: Standard graphs of ϒ- glutamate-cysteine by Spectrophotometer

3.18 Modeling for GCLC

3.18.1 Homology Modelling

In order to model the tertiary structure of Glutamate-Cysteine Ligase Catalytic subunit (GCLC) of human, protein sequence of GCLC was retrieved from the UniProt database (Accession no: P48506). Further, the protein sequence of GCLC BLAST analyzed against PDB to find a suitable template for homology modeling [142]. Based on the E-value, sequence coverage and sequence identity the suitable template was finalized. Based on the retrieved PDB file the protein along with substrates, cofactors and metal ions was modelled using the model-ligand, by the module of Modeller [143, 144]. The best homology model was selected based on the discrete optimized protein energy (DOPE) and molpdf scores.

3.18.2 Molecular Dynamics studies

Molecular dynamics simulation was performed for docked complexes with GROMCS 4.5.5 package for a 30 ns time duration of scale to evaluate the interactions and stability of the cysteine and homo-cysteine substrate with GCLC using Gromos53A6

force-field. PRODRG server was used to generate the topology files for Cso, Cys, Glu and ADP molecules. Further, the protein ligand complexes were placed at the center of a cubic box which was solvated with SPCE explicit water model with a distance of 1.0 Å from the edge of the simulation box. Particle Mesh Ewald (PME) method was used for electro-statistics energy calculations and LINCS algorithm was docked with the covalent bond constraints. The system was neutralized with the addition of 10 Na^+ ions. The complexes were energy minimized using the steepest descent approach, followed by a conjugate gradient approach each for 5000 steps. The system was equilibrated at NVT and NPT ensembles. Finally, 30 ns production run was performed and the obtained trajectories were analyzed.

3.19 : Cell culture experiment

Retinal Pigment epithelial cells :

Cell culture models of retinal pigment epithelial cells provide a system in biological research. It acts as an external barrier, function and regulation of the ocular in vitro. These models have a high advantage of in which conditions and parameters can be easily changed. Any disruption of either the inner or the outer barrier results in the development of edema and can lead to a loss of vision. The physiological function of RPE cells, products the photoreceptor layer which was constantly exposed to reactive oxygen species, including hydrogen peroxide and superoxide anion [145]. These defenses to protect the retina-RPE from oxidative damage, these evidences suggests that oxidised exposure disrupts RPE cell junction and barrier integrity [146]. Investigation of the mechanisms of blood-retinal barrier breakdown, which occurs in diseases such as diabetic retinopathy, retinitis pigmentosa, uveitis and age-related macular degeneration needed the such cell culture models [147]. These models are useful to the development of drug delivery systems targeted to the eye.

The human ARPE-19 cell line was obtained from ATCC (American type cell culture collection) and cultured in DMEM/F12 medium supplemented with 10 % FBS, 2 g/L sodium bicarbonate, 2 mM L-glutamine, 2 mM sodium pyruvate, 100 U/mL pencillin and 100 µg/mL streptomycin. ARPE-19 cells were used within 10 passages.

Human Umbilical Vein Endothelial Cells :

Ethics statement: All the protocols involving the collection and processing of human samples were strictly adhered to the tenets of Helsinki declarations, and approved by the Institutional ethic Board approval during the study was conducted. Reference number –150 –2009–P is the IRB–VRF approval number for this work. Consents were obtained from mothers who volunteered to donate their umbilical cord for research.

Processing of the cord: The method of processing of cord was followed by Baudin, B et.al., [148]. Cords were procured and processed within 3 h. All the reagents were kept in 37°C water bath before use. To 500 ml of 1x phosphate buffered saline (1x PBS), 500 µl of antibiotic solution was added. The cords were washed externally to remove RBCs. A butterfly needle was inserted into one end of the vein and clamped using artery forceps. The cord was internally washed with PBS until the wash appeared to be colourless. Collagenase solution was added through the veins after clamping the cord on both sides and then gently massaged. The cord was then incubated for 20 min at 37°C water bath in a beaker containing PBS. After incubation the cord was gently massaged to dislodge the cells from the vein. The clamp was removed from one end, then 10 ml PBS was allowed to flow through the cord gently thereby collecting the dislodged cells. Cells were collected in 5 ml of EGM medium in a 50 ml falcon tube. Centrifuged for 10 min at 1500 rpm. The supernatant was discarded and the cell pellets were resuspended in 5 ml of EGM medium. The cells were seeded in a fibronectin coated flask and incubated at 37°C in 5 % CO_2. After 2 h, change of medium was done to remove unattached cells and incubated till the cells were grown to confluence.

3.19.1 Maintenance of Cells:

The flasks were observed everyday under phase contrast microscope for morphological changes. Change of medium was given alternate days to the cells. The ARPE-19 cells grown were trypsinized, subcultured and passaged for 10 generations in DMEM-F12 medium with 10 % Fetal bovine serum (FBS).

HUVEC cells grown were trypsinized, subcultured and passaged for 5 generations in Endothelial growth medium (EGM) with 1% FBS.

3. 19.2 Subculture:

The ARPE-19 cells and HUVECs cells were trypsinized using 0.1 % trypsin EDTA for 5 min. The wall of the flask to which the cells were adhered was rinsed with the medium and were transferred into a 15 ml falcon tube and kept for centrifugation at 2500 rpm for 5 min. The supernatant was discarded to the pellet added the medium and mixed gently. The dislodged cells are transferred in to a new flask with a split ratio of 1:3.

3.19.3 Cell Viability:

Cell viablity was done using the trypan blue test. A mixture of 1 volume of dye and 9 volumes of cell suspension was made. From this, 10 µL was loaded to a neubauer chamber. Cell count was carried out in four corners of the slide. The viable cells do not take up the stain whereas dead cells take up the stain.

3.19.4 Cytotoxicity assays:

Cytotoxicity assay was carried out using MTT. The cells were exposed to the experimental conditions and were then assayed by the following methods, after exposure time is over, the medium was carefully removed and replaced with fresh serum free medium. To this MTT (5 mg/ml) is added to 1/6 th volume of the medium. This is incubated for 4 h at 37ºC. After the incubation, the medium was discarded and the formazon crystals formed were solubilised with 100 µL DMSO. After the crystals were dissolved, the plate was read at 540 nm. Results were expressed taking the absorbance of the control cells as 100%.

3.20 Statistical analysis:

All values were expressed as mean ± standard error mean. With SPSS software, (version: 16.0) the raw data were analyzed for statistical significance using student “t” test. Pearson’s correlation test was employed to assess the relationship all the parameters. p value < 0.05 was considered statistically significant.