3. MATERIALS AND METHODS
3.1 Materials

Biological Materials

Human blood samples from healthy normoglycemic volunteers (nonsmokers and non drug addicts) and patients with different grades of diabetes mellitus were collected in heparinised condition. The diabetic patients were mostly registered with Out-Patients Department of N.R.S. Medical College Hospital, Kolkata. Both normal individuals and diabetic patients were from 13-50 years age group.

Chemicals:

Human serum albumin (HSA), Arachidonic acid, Sephadex G-25, Sephadex G-100, Catalase, O-dianisidine, Malondialdehyde, Desferrioxamine, Ferrozine, Deoxyribose, Bromophenol blue, Thiobarbituric acid (TBA), Nitroblue tetrazolium (NBT), Glucose, Acrylamide, Bisacrylamide and TEMED were purchased from Sigma Chemical Company, U.S.A. BioRex-70 and prestained protein marker were purchased from BioRad, India. Trifluoperazine was obtained as a gift from Sun pharmaceuticals, India.

Other chemicals e.g., Hydrogen peroxide, Sodium chloride, Sodium hydrogen phosphate, Disodium hydrogen phosphate, Potassium ferricyanide, Tris-base, Glacial acetic acid, Hydroxylamine hydrochloride, Ethylenediamine tetraacetate (EDTA), Sodium dodecyl sulfate (SDS), Methanol, Coomassie brilliant blue, Hydrochloric acid, Citric acid, Sodium hydroxide etc were of analytical grade and purchased locally.
3.2. METHODS

3.2.1. Preparation and purification of hemoglobin

Oxyhemoglobin was isolated and purified from blood samples of normal volunteers and diabetic patients (Age: 13-50 years). The blood was collected aseptically in 0.02% heparin and was centrifuged at 3000 rpm at room temperature for 10 minutes. The supernatant containing plasma and buffy coat of white blood cells in the upper portion of the sediment were discarded. The erythrocytes thus obtained was washed thrice by centrifugation at 5000 rpm for 5 minutes with 0.15 M NaCl in 50 mM phosphate buffer solution, pH 6.8. Addition of 3 volume of double distilled water to 1 volume of packed cell with slow stirring, led to significant hemolysis of erythrocytes within 15 minutes. The hemolysate was further centrifuged at 15,000 rpm for 30 minutes at 4°C and membrane free hemolysate was carefully collected from upper layer. The concentration of NaCl in hemoglobin solution was then adjusted to 0.15 M NaCl. Hemoglobin thus obtained was then eluted in 0.15 M NaCl in 50mM phosphate buffer, pH 6.5 through a calibrated Sephadex G-100 column (21×1.5 cm) and it gave a single elution peak corresponding to hemoglobin tetramer as shown in Figure-3.1. Figure-3.2 shows the absorption spectrum of purified hemoglobin in the wavelength region 250-700nm. Absorption spectra were recorded in Hitachi U-2000 spectrophotometer using quartz cuvette of path length 1 cm. The isolated hemoglobin shows three absorption peaks at 415nm, 540nm and 577nm respectively, which are characteristic for oxygenated hemoglobin.
Fig.-3.1.: Representative plot showing the elution profile of hemoglobin separated by Sephadex- G100 column chromatography.
Fig.-3.2.: Absorption spectrum of purified hemoglobin (4 μM, heme basis) in 0.15 M NaCl.
The molar extinction coefficients of hemoglobin at the above wavelengths are

\[ \varepsilon_{415\text{nm}} = 125 \text{ mM}^{-1} \text{ cm}^{-1}, \varepsilon_{540\text{nm}} = 12.3 \text{ mM}^{-1} \text{ cm}^{-1}, \varepsilon_{577\text{nm}} = 12.6 \text{ mM}^{-1} \text{ cm}^{-1}. \]

The concentration of hemoglobin was estimated from the molar extinction coefficient at 415 nm.

### 3.2.2. *In vitro* glycation of hemoglobin

Hemoglobin was isolated from the blood sample of normal healthy individual by Sephadex G-100 column chromatography and was incubated in phosphate buffer saline (PBS) containing different concentrations of glucose ranging from 0 to 270 mg/ml (final volume 1 ml containing 5 mg hemoglobin). Hemoglobin and glucose solutions were sterile filtered before incubation. Incubations were done in sterile glass tubes for 5 days at 23°C. After incubation, the amounts of free glucose left in the samples were estimated by glucose oxidase method, where aldehyde group of glucose was oxidised by glucose oxidase to gluconic acid and hydrogen peroxide. \( \text{H}_2\text{O}_2 \), by the action of peroxidase, converted aminophenothiazene, an oxygen acceptor, to a coloured compound, which was measured spectrophotometrically [107], from which the amount of glucose utilized for glycation was determined. Initially, the isolated hemoglobin was 5% glycated. Incubation with glucose caused further glycation.
3.2.3. Estimation of ferrozine detected iron from hemoglobin

Ferrozine detected free iron was estimated according to the method of Panter [108] with slight modification. In a microcentrifuge tube, 250 µl of hemoglobin solution (50µM) and 250 µl of 20% TCA (v/v) were added. The tubes were then centrifuged at 3000 rpm for 10 minutes to pellet the precipitated protein. From clean supernatant, 250 µl was transferred to a new 12 × 75 mm glass test tube containing 250 µl distilled water and 2.5 ml 50 mM acetate buffer, pH 4.5, containing 1.5% hydroxylamine hydrochloride and was mixed thoroughly. Finally, 50 µl ferrozine (0.85% w/v) was added and the color was developed for 30 minutes at 37°C. Absorbance at 560 nm was recorded to estimate free iron content. Similarly, a standard curve for iron was constructed using different amounts of iron from a stock solution containing 5 µg per ml iron. Figure 3.3 is the standard curve of iron detected spectrophotometrically with ferrozine. Using the standard curve free, iron levels (i.e., non-heme iron) were estimated in hemoglobin samples and expressed as µg of iron per gram of hemoglobin.

3.2.4. Isolation of fractions of glycated hemoglobin from hemolysate of diabetic patients

The stroma-free hemolysate was prepared from blood samples of diabetic patients following the method stated in section 3.2.1. The hemolysate was passed through a Sephadex G-100 column (21 × 1.5 cm) equilibrated with 50mM phosphate buffer, pH 6.6 and hemoglobin was isolated as mentioned before. Chromatographically separated hemoglobin was then subjected to ion exchange
Fig.-3.3.: Standard curve of iron detected spectrophotometrically as a complex with the chromophore ferrozine. The data represent mean values ± SD for five independent determinations.
chromatography. The ion exchange column-BioRex-70 (200-400 mesh) resin (27cm \times 1.5 \text{ cm}) was preequilibrated with 50 mM phosphate buffer, pH 6.6. Fractions of glycated hemoglobins were separated by stepwise increase of NaCl concentration in eluted buffer according to the method of McDonald et al [59]. Fractions HbA_{1a1} and HbA_{1a2} were eluted without any added sodium chloride. HbA_{1b} was collected in the fraction containing 0.05 M NaCl in the elution buffer. HbA_{1c} was separated at a salt concentration of 0.1 M and finally, HbA_0 or nonglycated major human adult hemoglobin was eluted at 1.0 M NaCl concentration. The elution profile of the separated fractions is represented in Figure-3.4, in which absorbances of different hemoglobin fractions at 415 nm, 540 nm and 677 nm have been plotted against fraction numbers. HbA_0 fraction was diluted with phosphate buffer immediately after separation to 0.1 M NaCl concentration to avoid subunit dissociation at high concentration of NaCl. The first two major peaks in the plot are for HbA_{1a1} and HbA_{1a2}, the third one for HbA_{1b}, fourth represents HbA_{1c} and the final peak is for HbA_0. The concentration of the hemoglobin was measured from their extinction coefficient at 415 nm which is equal to 125 mM^{-1}cm^{-1} (monomer basis). Both HbA_0 and HbA_{1c} in 50 mM phosphate buffer, pH 6.6 showed a single elution peak corresponding to tetrameric hemoglobin molecular weight 66,800 when applied to a calibrated Sephadex G-100 column. Both HbA_0 and HbA_{1c} showed three absorption peaks around 415 nm, 540 nm and 577 nm (Fig.-3.5a & 3.5b). The methemoglobin fractions of HbA_0 and HbA_{1c} thus isolated were found to be insignificant as evident from very small absorbances at 630 nm, the characteristic absorption
Fig.-3.4.: The elution profile of the separated fractions of glycated hemoglobins on ion exchange chromatography (BioRex70). Fractions of hemoglobin are separated by stepwise increase of NaCl concentration (0-1.0 M) in 50 mM, phosphate buffer, pH 6.6.
Fig.-3.5.: Absorption spectra of purified oxygenated (a) HbA₀ and (b) HbA₁c in 50 mM phosphate buffer, pH 6.6 containing 0.1 M NaCl. The concentration of both hemoglobin samples is 8 μM.
wavelength for methemoglobin form [109]. The extents of oxygenation in \( \text{HbA}_0 \) and \( \text{HbA}_{1c} \) estimated from their respective absorption spectra following Huang and Redfield's equation [110] were found to be nearly 100%.

\[
\text{Oxygen Content (\%)} = \frac{12.73 - 9.92}{3.77 + 3.88} \times 100
\]

where \( A_{560} \) and \( A_{540} \) indicate absorbances at 560 nm and 540 nm, respectively.

3.2.5. Thiobarbituric acid test

Hemoglobin fractions \( \text{HbA}_0 \) and \( \text{HbA}_{1c} \) were tested for carbohydrate contents by using thiobarbituric acid according to the method of Fluckiger and Winterhautler [54]. 2 ml fraction of \( \text{HbA}_{1c} \) or \( \text{HbA}_0 \) was added to 1 ml of 1 M oxalic acid and heated for 4 hours in a boiling water bath. Each test tube was covered with marble ball to avoid evaporation. After cooling, 1.0 ml 40% TCA was added to precipitate protein and centrifuged for 10 minutes at 5000 rpm. 2 ml supernatant was transferred to a separate test tube to which 2 ml thiobarbituric acid (0.025 M) was added. The mixture was then incubated for 1 hour at 37\(^\circ\)C. Spectra from 400 to 500 nm were recorded adjusting the control made similarly using 2 ml 50 mM phosphate buffer, pH 6.6 instead of hemoglobin. Figure 3.6 shows that \( \text{HbA}_{1c} \) exhibited positive reaction for glucose having absorption maximum at 443 nm. On the other hand, \( \text{HbA}_0 \) showed no reaction with thiobarbituric acid.
Fig.-3.6.: Thiobarbituric acid test for confirmation of glycation in hemoglobin fractions (HbA₀ and HbA₁c).
3.2.6. Estimation of iron released from HbA₀ and HbA₁c in the presence of H₂O₂

250 µl of HbA₀ or HbA₁c (50 µM) was taken in different microcentrifuge tubes with varying concentration of H₂O₂ (0-1.25 mM), mixed and incubated for 1 hour at 37°C. After incubation, 250 µl 20% TCA was added to each tube, mixed and centrifuged for protein precipitation. From the protein-free supernatant, 250 µl was taken for estimation of ferrozine detected iron according to the method of Panter as described in Section 3.2.3. Results were expressed as nanogram of iron released per gram of HbA₀ and HbA₁c as calculated from standard solution of iron in iron buffer reagent (Fig.3.3).

3.2.7. H₂O₂-mediated lipid peroxidation by HbA₀ and HbA₁c

Aldehydes are produced when lipid hydroperoxides break down in biological systems. Measurement of these compounds is an index of lipid peroxidation caused by free radical reactions. Malondialdehyde like substances (a mixture of thiobarbituric acid reactive aldehydes) generated from arachidonic acid degradation by HbA₀ and HbA₁c in the presence of hydrogen peroxide are measured by thiobarbituric acid reaction [111].

1 ml sample containing HbA₀ or HbA₁c (40 µM), arachidonic acid (160 µM) and H₂O₂ (1 mM) in 50 mM phosphate buffer, pH 6.6 was incubated at 37°C for 1 hour. The reaction was initiated by adding H₂O₂ and stopped by 20% TCA. Then 0.5 ml each of 1% thiobarbituric acid and 50 mM citrate buffer, pH 3.0 were added. The protein was precipitated by centrifugation and supernatant was incubated at 100°C for 30 minutes. The absorbance was measured at 530 nm and the value was corrected for endogenous TBA reactive
substrates present in arachidonic acid. The results were calculated from a standard obtained with malondialdehyde treated similarly.

3.2.8. \( H_2O_2 \) - mediated deoxyribose degradation by \( HbA_0 \) and \( HbA_{1c} \)

Hydroxyl (OH) radical specifically degrades deoxyribose. Deoxyribose degradation by \( H_2O_2 \) -mediated reaction was determined following the method of Sadrzadeh et al [112] with slight modification. The reaction mixture (1 ml) contained 0.67 mM deoxyribose, 4 μM HbA or HbA1c in 50 mM phosphate buffer, pH 6.6 and \( H_2O_2 \) (0.67 mM). The reaction was started by adding \( H_2O_2 \). The mixture was incubated for 1 hour at 37°C and stopped by adding 0.5 ml TCA (2.8%) and TBA reactivity was developed by adding 0.5 ml TBA (1%) and heated in a boiling water bath for 15 minutes. The resulting chromogen was extracted in 3 ml n-butanol. The product was estimated from emission fluorescence at 553 nm by exciting at 523 nm.

3.2.9. Stability of heme-globin linkage in \( HbA_0 \) and \( HbA_{1c} \)

The bond between heme and globin through proximal histidine of globin chain and fifth co-ordination position of iron is too strong for measurement of its association constant directly. However, heme of ferrihemoglobin (Hb\(^+\)) is rapidly transferred to human serum albumin (HSA) forming methemalbumin (MHA). This heme transfer from Hb\(^+\) to HSA at pH 9.0 is determined spectrophotometrically according to the method of Benesheh [113] and the rate of heme transfer gives an idea of the stability of heme-globin linkage.
Purified HbA₀ and HbA₁c in 50 mM phosphate buffer, pH 6.6 were oxidised to ferric form by adding 1.2 equivalent potassium ferricyanide at room temperature, followed by passing through Sephadex G-25 column (20 ×1.5 cm) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5 to remove ferro and ferricyanide. The concentration of Hb⁺ was determined (ε₅₄₀ = 11.0 ×10⁴ M⁻¹cm⁻¹, heme basis) from absorbance at 540 nm. 1 mM stock of HSA was prepared in 0.5 M Tris-HCl buffer, pH 9.05. 1 mM hemin chloride stock was prepared by dissolving in small amount of 0.1 N NaOH and diluting with Tris buffer, 0.05 M, pH 7.5. When a mixture of equal amounts of hemin chloride and HSA was diluted to twenty folds, it gave a 0.25 mM MHA standard solution. The reaction was started by adding HSA to a temperature equilibrated cuvette (26°C) containing 1.0 ml 0.5 M Tris-HCl buffer, pH 9.05 and Hb⁺ in 0.05 M Tris-HCl buffer, pH 7.5. The final pH was 9.0. The concentrations of Hb⁺A₀ or Hb⁺A₁c and HSA were 40 μM each in final volume. The change in absorbances at 510 nm and 580 nm were recorded at different time intervals. The concentration of MHA formed in the reaction mixture was calculated from a 0.25 mM MHA standard treated in identical condition. In the first phase of reaction, the heme from β chain of hemoglobin is released to form MHA. During this phase, the sum of [Hb⁺] + [MHA] should remain constant and equal to initial Hb⁺ concentration [113]. The percentage of [MHA] formed at different time intervals was calculated according to the formula:

\[
\% [\text{MHA}] = \frac{[\text{MHA}] \times 100}{[\text{Hb}^+] + [\text{MHA}]} = \frac{[\text{MHA}] \times 100}{[\text{Hb}^+_{\text{initial}}]}
\]
3.2.10. Peroxidase activities of HbA₀ and HbA₁c

Peroxidase activities of both types of hemoglobin (HbA₀ and HbA₁c) were assayed according to the method of Everse et al [114] using o-dianisidine as the substrate. The reaction mixture (2 ml) contained 50 mM citrate buffer, pH 5.4, 1.5 μM HbA₀ or HbA₁c, 0.002 % o-dianisidine and the reaction was initiated by adding 17.6 mM H₂O₂. The assay was done at ambient temperature with hemoglobin, buffer and o-dianisidine in the reference cuvette. The increase in absorbance at 450 nm was monitored for 2 minutes.

3.2.11 (a) Auto-oxidation of HbA₀ and HbA₁c

Sterile filtered HbA₀ and HbA₁c (40 μM) in 50 mM phosphate buffer saline, pH 6.6 were taken in sterile tubes and incubated at 4°C for 15 days. The aliquots were taken out aseptically every 24 hour intervals and the absorbances at 577 nm and 630 nm were measured. The increase in methemoglobin level was estimated from the following relation [109]:

\[ \text{[MetHb], pM} = 279A_{577} - 3.0A_{630}. \]

The rate of methemoglobin formation was plotted against time. The rate of disappearance of oxyhemoglobin was estimated using the relation:

\[ \text{[oxy Hb], μM} = 66A_{577} - 80A_{630}. \]

3.2.11 (b) Co-oxidation of HbA₀ or HbA₁c with nitroblue tetrazolium

Nitroblue tetrazolium (NBT) rapidly oxidises oxy form of hemoglobin to its reduced form or methemoglobin. 1 ml solution containing 40 μM HbA₀ or HbA₁c in 50 mM phosphate buffer saline, pH 6.6 and 240 μM NBT in the same buffer was taken in the cuvette, mixed and change in the absorption spectrum
450-700 nm was recorded against time (5 minute interval). NBT was also added to the reference cuvette. Absorbances at 577 nm and 630 nm were noted to estimate the rate of oxidation of HbA₀ and HbA₁c in the presence of NBT following the relation mentioned before.

3.2.12. Thermal denaturation of HbA₀ and HbA₁c

The precipitation test of hemoglobin by thermal denaturation was done according to the method of Olsen [115] with slight modification. For this experiment, HbA₀ and HbA₁c in 50mM phosphate buffer saline, pH 6.6 were further subjected to Sephadex G-100 column equilibrated with 100 mM phosphate buffer, pH 7.4 and single eluted fraction corresponding to tetrameric protein of molecular weight 66,800 was collected. The sample of HbA₀ or HbA₁c (20 μM) in 100 mM phosphate buffer, pH 7.4 was incubated at 62°C in thermostatic water bath. At different time intervals, samples were withdrawn and chilled immediately on ice to stop further denaturation and centrifuged to remove denatured protein precipitates. The absorbances of the supernatants at 523 nm were measured. The percentage of denaturation of hemoglobin was estimated according to the relation [115]:

\[
\text{Percentage of denaturation} = \frac{(A₀ - Aₜ)}{A₀} \times 100\text{ where } A₀ \text{ and } Aₜ \text{ are absorbances at zero time and a particular time, respectively.}
\]
3.2.13. Spectrofluorometric tryptophan quenching titration using acrylamide as a neutral quencher

Surface accessibility of tryptophan in HbA₀ and HbA₁c was estimated from the dynamic quenching of its fluorescence by acrylamide acting as a neutral quencher. The study was performed in Hitachi F-3010 spectrofluorometer using 1 cm pathlength cuvette. The emission maxima of HbA₀ or HbA₁c was at 330 nm, when excited at 285 nm. Quenching of tryptophan fluorescence intensity of HbA₀ or HbA₁c (6.5 µM) in the presence of the added acrylamide was measured from the change of the respective intensity at 332 nm. Excitation of acrylamide at 285 nm does not contribute significantly to the fluorescence emission around 330 nm.

Fraction accessibility of tryptophan was estimated from Lehrer plot of \( \frac{F_0}{\Delta F} \) versus \( \frac{1}{L_t} \) following the equation [116]:

\[
\frac{F_0}{\Delta F} = \frac{1}{f} + \frac{1}{f} \cdot K \cdot \frac{1}{L_t}
\]

where \( \Delta F = F_0 - F \), \( F_0 \) and \( F \) represent fluorescence emission intensities of hemoglobin in the absence and presence of total acrylamide concentration (Lₜ), respectively. \( f \) is the fraction of accessible tryptophan quenched by acrylamide and \( K \) is the bimolecular collisional dynamic quenching constant.

3.2.14. Preparation of trifluoperazine (TFZ) solution and its absorption spectrum

Solution of TFZ was made fresh before each experiment. For this purpose, a small amount of the drug was weighed and dissolved in 0.15 M NaCl. The absorption spectrum (200-400 nm) of the solution was taken in Hitachi U-2000 Spectrophotometer using 1 cm pathlength quartz cuvette. The drug shows two absorption peaks in ultraviolet wavelength region - one at 255 nm and the other at 49
305 nm [Fig.-3.7]. No significant absorption is seen in visible wavelength region. The molar extinction coefficients of the drug are [117]:

\[ \varepsilon_{305\text{nm}} = 3162 \text{ M}^{-1} \text{ cm}^{-1} \]
\[ \varepsilon_{255\text{nm}} = 31,620 \text{ M}^{-1} \text{ cm}^{-1} \]

The concentration of the drug solution was determined spectrophotometrically from the molar extinction coefficient at 305nm. The drug concentration range (25-250 µM) used in this study followed Beer's law. To avoid light induced reactions, the pyrex glass tubes containing the drug solutions were always wrapped with black papers.

3.2.15. Trifluoperazine-hemoglobin binding studies using fluorescence quenching titration

A molecule, when excited in the ground state, undergoes electronic transition from the ground state or lower energy level to the excited state or higher energy level. Following Frank-Condon's principle, the molecule then returns to the ground electronic state by process of emission. This is known as fluorescence. Substances which possess delocalised electrons display significant fluorescence intensities [118]. Fluorescence quenching refers to a process which decreases the fluorescence intensity of a given substance. A variety of processes like excited state reactions, energy transfer, complex formation etc can result in quenching.

In proteins, fluorescence is mostly dominated by the tryptophan residues and the indole nucleus present in these residues is a sensitive and complicated fluorophore. Tyrosine and phenylalanine - other two amino acid residues also
Fig.-3.7.: Absorption spectrum (200-400 nm) of trifluoroperazine (0.1 mM) dissolved in 0.15M NaCl.
cause protein fluorescence, but quantum yield of protein fluorescence intensity due to tryptophan is much higher than these two amino acid residues.

Indole, tryptophan and their derivatives are highly sensitive to solvent polarity and appear to be subject to both general and specific solvent effects. Therefore, the emission spectra of tryptophan residues can reflect the polarity of their surrounding environment. For the purpose of our experiments, all fluorescence measurements were done in Hitachi F-3010 spectrofluorometer using 1 cm pathlength quartz cuvette. Excitation as well as emission bandwidths were kept at 10 nm. Drug from a concentrated stock solution was added to 3 ml 
HbA\textsubscript{0} or HbA\textsubscript{1c} solution (8 μM, monomer basis) by a Tarson micropipette, so that the volume increment in the cuvette was negligible. The emission maxima of both 
HbA\textsubscript{0} or HbA\textsubscript{1c} appeared at 330 nm, when excited at 285 nm. Quenching of fluorescence intensity of both hemoglobin in the presence of the added drug was measured from the change of the respective emission intensity at 330nm. TFZ has its absorption minima at 285 nm and when excited at 285 nm, does not contribute significantly to the fluorescence at the emission wavelength 330 nm. The fluorescence of the maximum concentration of drug (250 μM) used in this study was thirty two fold less than that of 8 μM hemoglobin excited similarly at 285nm.

Binding parameters (binding affinity constant, $K$; degree of co-operativity of binding, $\eta_\text{H}$; binding sites, $p$) of TFZ with HbA\textsubscript{0} or HbA\textsubscript{1c} were estimated from the quenching of fluorescence of a fixed concentration of protein in the presence of the increasingly added TFZ using the relations shown in Results and Discussion section. Binding study was done at different NaCl concentrations to
determine the nature of interaction. Duration of each set of binding experiment was about 15 minutes. TFZ solution after being kept alone in a pyrex glass or quartz cuvette for the above time period showed no change in absorbance at their respective peak absorption wavelength. This indicates that adsorption of the drug to the cuvette used in our experiments is negligible. Fluorescence change of the drug kept similarly for 15 minutes under the same illuminating condition of the spectrofluorometer was also found to be negligible indicating the fact that photodecomposition of the drug, if any, during the above time period does not affect the measurement of binding parameters.

3.2.16. **Spectrophotometric studies of drug-protein interaction (Difference Spectroscopy)**

Absorption spectrophotometric technique (difference spectroscopy) was used to study the interaction of TFZ with HbAo and HbAic. The experiments were done in Hitachi U-2000 spectrophotometer using 3 ml quartz cuvettes of pathlength 1 cm. 3 ml of a particular concentration (8 μM) of HbAo or HbAic in phosphate buffer saline was taken in the sample cuvette and the same volume of buffer was taken in the reference cuvette. Absorbance spectra from 380-500 nm was recorded. The protein absorbance was titrated by adding equal volume of TFZ from a concentrated stock solution to both the sample and reference cuvettes.

3.2.17. **TFZ-induced oxygen release from HbAo and HbAic**

Oxygen release from oxygenated HbAo and HbAic due to interaction with TFZ was measured in a Gilson 5/6 oxygraph machine. The principle of the oxygen electrode is that it consists of a platinum electrode in glass as the cathode
and a silver wire immersed in a chloride solution as the anode. The anode can also be a calomel electrode. When a voltage is imposed across the two electrodes immersed in an oxygen containing solution with platinum electrode negative relative to the reference electrode, oxygen undergoes electrolytic reduction and a current is generated. This current is measured by a galvanometer and recorded by a recorder.

For the purpose of our experiment, the change in partial pressure due to TFZ-induced oxygen release in the hemoglobin solution in a stoppered cell was detected by the membrane covered oxygen electrode fitted with the cell. The output signal was recorded in the oxygraph chart as a function of time. The buffer solution showed no change in the output signal even after 15 minutes of stirring. The amount of free dissolved oxygen in the solvent (0.15 M PBS, pH 6.8) alone was taken to be 250 nmoles per ml at 27°C [119]. Calibration of the oxygraph chart in terms of nmoles of oxygen release was made from the change in the output signal due to the total depletion of free oxygen from 2 ml solvent, when 100 mg sodium metabisulphite was added to it. The temperature during the experiments was maintained at 27°C.

3.2.18(a). Heme loss due to TFZ-hemoglobin interaction: Identification by gel filtration

200 μl of HbA0 or HbA1c (80 μM) was loaded to a Sephadex G-25 column (1×20 cm) pre-equilibrated with 50 mM phosphate buffer saline, pH 6.6 and fractions (1 ml each) were collected in microcentrifuge tubes. The absorbance of the fractions at 415 nm was measured.
In case of treated samples, 800 μM TFZ was added to final volume of 200 μL of HbA₀ and HbA₁c solution and incubated for 10 minutes at an ambient temperature prior to loading of the drug-protein complex over the same column and eluted similarly. The reduction in peak height in case of HbA₁c-TFZ treated sample gave an idea of the possibility of heme loss due to interaction with the drug.

3.2.18(b). Heme loss due to TFZ- hemoglobin interaction: Identification by SDS-PAGE with heme staining

Interaction products between hemoglobin (HbA₀ or HbA₁c) and TFZ were subjected to low temperature (4°C) SDS-PAGE essentially according to the method of Klatt et al [120]. 200 μl HbA₀ or HbA₁c (80 μM) in phosphate buffer, pH 6.6 in the absence or presence of TFZ (800 μM) were incubated for 15 minutes at 25°C. Then 50 μl of 50 μM chilled Laemmli buffer (containing 0.12 M Tris-HCl, pH 6.5, 4% SDS, 20% glycerol and 0.2 % bromophenolblue) was added to each sample. Mercaptoethanol was not added to Laemmli buffer, as it interferes with heme staining. However, absence of this has no effect on migration pattern of hemoglobin. Samples of HbA₀ and HbA₁c were subjected to SDS-PAGE (10%) for eight hours at a constant voltage 120 mV. Gels (17×30×1 mm) and buffers, prepared according to Laemmli [121], were equilibrated at 4°C prior to electrophoresis and the buffer tank was cooled by ice-bath during electrophoresis. Gels were stained for heme with o-dianisidine/H₂O₂ following the method of P. Klatt et al [120] with slight modification. Gels were washed for 10 minutes with 3:7 v/v methanol: sodium acetate (0.25 M, pH 5.0) and subsequently
incubated in the dark for 20 minutes in a freshly prepared solution containing 7 parts of 0.25 M sodium acetate, pH 5.0 and 3 parts 6 mM o-dianisidine. Gels were developed for 1 hour by adding H$_2$O$_2$ to a final concentration of 60 mM, washed for 30 minutes in 3:1:1 v/v H$_2$O: methanol : acetic acid, dried and photographed.

The molecular mass of heme stained region was detected by using prestained of proteins from BioRad.

3.2.19. Circular dichroism experiments

Circular dichroism (CD) results from interaction of a given medium (optically active) with linearly polarized light. One of the aspects of interaction of linearly polarized light with an optically active medium is that the absorption coefficient of this medium for left and right circularly polarized light is different, $\varepsilon_L \neq \varepsilon_R$ i.e., the medium exhibits circular dichroism. All proteins have two fold asymmetry- one due to the asymmetric amino acid residues and the other due to $\alpha$-helix content of the protein. Due to such asymmetry, protein solutions have different absorbances for left and right circularly polarized light. At a given wavelength, the circular dichroism of a particular protein solution is given by the relation $\Delta \varepsilon = \varepsilon_L - \varepsilon_R$ (the differences in molar extinction coefficients for left and right circularly polarized light). Change in the $\alpha$-helical content of the protein causes its conformational change and this is manifested by the change in ellipticity.

Circular dichroic measurements (200-600 nm) of HbA$_0$ and HbA$_{1c}$ and the effect of TFZ (100 $\mu$M and 200 $\mu$M) on the proteins (10 $\mu$M) were done in Jasco-
600 spectropolarimeter. Molar ellipticity $[\theta]$ values were obtained using the relation [123]:

$$[\theta] = \frac{[M_rW][\theta]}{10l.c}$$

where $c$ is the concentration of hemoglobin (HbA$_0$ or HbA$_{ic}$) in gm/ml, $\theta$ (obtained directly from the CD chart) is the observed rotation in degrees (mdeg.), $l$ is the pathlength in cm and $[M_rW]$ is the mean residual molecular weight of the protein ($[M_rW]=110$). The $\alpha$- helical content of HbA$_0$ and HbA$_{ic}$ were determined according to the relation [123]:

$$\text{Fraction of } \alpha\text{-helix (f)} = \frac{[\theta]_{222} + 2340}{-30300}$$

where $[\theta]_{222}$ is the ellipticity at 222 nm.

3.2.20. Microscopic studies on the effect of TFZ on erythrocytes isolated from normal individuals and diabetic patients

3 ml heparinised blood sample from a diabetic patient was collected. There was no hematological abnormality, but the patient was suffering from retinopathy. Erythrocytes isolated from the blood sample were washed three times with 0.15 M NaCl and suspended in 0.15 M NaCl to get an erythrocyte concentration of $10^6$ cells/ml. Erythrocyte suspension ($10^6$ cells/ml of 0.15 M NaCl) was also prepared from the blood sample of a normal healthy individual as a control. Both cell suspensions (control and diabetic) were treated with different TFZ concentrations (0 $\mu$M, 10 $\mu$M, 20 $\mu$M) for 15 minutes at ambient temperature. Equal volume of both untreated and drug-treated cells after same dilution were taken on glass slides, covered with coverslips and observed under
Olympus (AX-70) light microscope. The photographs of the cells were taken with the help of a camera DP-11 attached to the microscope. Micro-image analysis of untreated and drug-treated cells was also done.

3.2.21. TFZ-induced oxygen release from erythrocytes isolated from normal individuals and diabetic patients

Erythrocytes from blood samples of diabetic patient and normal individual were isolated as described before. The packed cells were diluted 100 times with 0.15 M NaCl. The suspension contained hemoglobin concentration = 0.5 gm/dl.

The oxygen release from erythrocyte suspension due to gradual addition of TFZ (100 μM at each time point) was measured in 5/6 Gilson oxygraph machine. The cell concentration was same in each experiment. Calibration of oxygraph chart was made as described in 3.2.16.

3.2.22. TFZ-induced hemolysis of erythrocytes

Erythrocytes from blood samples of normal subjects and diabetic patients were isolated as described before. To 1 ml each of erythrocyte suspension containing 0.5 g/dl hemoglobin, different concentration of TFZ was added and the final volume was made 3 ml with 0.15 M NaCl solution. Samples were incubated at 37°C for 30 minutes and then centrifuged for 5 minutes at 3000 rpm. The absorbances of the supernatants at 530 nm were measured and a plot of A<sub>530nm</sub> against TFZ concentrations was made. It represents the extent of hemolysis of erythrocytes induced by TFZ.
3.2.23. Determination of haptoglobins in plasma isolated from normal individuals and diabetic patients

Haptoglobin levels in blood samples were determined following the method of Ratcliff and Hardwicke [124]. 20 µl normal purified hemoglobin solution was added to 0.5 ml plasma isolated from different diabetic and normal control volunteers. About 5 mg of NaCl was added to the plasma and hemoglobin mixtures and passed through Sephadex G-100 column (30×1.5 cm) pre-equilibrated with 20% NaCl. Eluted profile showed two peaks, the first corresponding to hemoglobin-haptoglobin complex and second free (unreacted) hemoglobin. The concentration of hemoglobin was measured from first peak fraction using the extinction coefficient ($\varepsilon_{415\text{nm}} = 125$ mM$^{-1}$ cm$^{-1}$). Plasma haptoglobin binding capacity was expressed in terms of mg hemoglobin/dl.