

Fructosylation of Human Serum Albumin - Implications in diabetes mellitus

1. Dr. Sufia Naseem

Assistant Professor, Department of Biochemistry J.N.Medical College, AMU Aligarh

2. Dr. Manzoor Ahmad

Assistant Professor Department of Surgery, J.N.Medical College, AMU Aligarh

3. Ummul Baneen*

Assistant Professor, Department of TB & Respiratory Diseases*(Corresponding author),

4. Dr. Shagufta Moin,

Professor,

Department of Biochemistry, J.N.Medical College, AMU Aligarh(Corresponding Author)

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Abstract

Aim:To investigate the alterations in human serum albumin (HAS) due to glycation with varying fructose concentration.

Methods:Human serum albumin (HSA)was glycated using fructose and characterized it using physicochemical studies. The changes and modifications as a result of glycation were studied in a concentration- and time-dependent manner.

Results:Characterization of fructosylated HSA resulted in hyperchromicity of UV spectrum, increase in AGE-formation, quenching of tryptophan fluorescenceandSDS-PAGE. Amadori products formed were estimated by nitroblue tetrazolium. Higher carbonyl content and decreased thiol content was found in fructose modified HSA. Diabetic sera showed increased levels of malondialdehydeand protein carbonyls indicating enhanced oxidative stress. The diabetic seraalso showed higher recognition and binding capability with respect to glycated HSA than to native HSA, reflecting greater specificity to glycated HSA.

Conclusion:Modification of proteins due to glycation leads to formation of epitopes that are regarded as non-self and lead to antibody production. Structural modifications may also reflect as changes in receptor binding properties and alteration in functions.

Keywords:Fructose, Human serum albumin, diabetes, advanced glycation end products, ELISA, SDS PAGE, Epitopes

1.Introduction

During the last few decades, the prevalence of diabetes mellitus has increased at an alarmingly rapid rate. With over 80 million diabetic patients predicted by 2030, India is now regarded as the world's diabetes capital, surpassing the United States in this regard. Not only does the increase in its prevalence have consequences for the person, but it also has consequences for the country in terms of a significant increase in its economic burden. As a result, there is an urgent need to understand the intricate nature of the biomolecules involved in the aetiology of diabetes, as well as their impact on the disease. An important consequence of diabetes is glycation of biomolecules, such as nucleic acids and proteins.¹⁻² Glycation, also called Maillard's reaction, involves a reaction between a reducing sugar and epsilon amino groups of arginine and lysine residues that yieldsadvanced glycation end products (AGEs).³Glycation has been linked to several diseases, including diabetes and its complications,⁴⁻⁶including cataract, atherosclerosis, neurodegenerative diseases, etc.⁷

Glycation is a spontaneous, non-enzymatic reaction that generates Schiff bases that rearrange to form Amadori adducts, such as fructosamine (for glucose). These adducts are relatively stable and can further get oxidized and polymerized to form AGEs.⁸When the reducing sugar is fructose, Schiff base rearrangement is called Heyns rearrangement. Such products have previously been found in ocular lens proteins and liver extracts.⁹Degree of protein modification is proportional to the duration of interaction between the protein and reducing sugars,¹⁰ the type of sugar present,¹¹ and the organ involved.¹²

The AGE-related pathogenesis of complications is broadly classified into two aspects. The first scenario involves interaction between AGEs and their receptor protein(RAGE), which leads to several inflammatory processes that cause tissue damage.^{13,14} Second aspect includes AGE-induced modification of protein either

directly or through cross-linking.¹⁵⁻¹⁷ Protein cross-linking forms detergent-resistant aggregates, which alter protein degradation and other metabolic processes, especially under hyperglycemic conditions.^{18,19} These oxidative cross-links have decreased proteolytic susceptibility that results in their accumulation.²⁰ This leads to platelet aggregation and triggers ROS generation and oxidative stress that decreases glucose uptake, which results in decreased insulin sensitivity in the adipocytes.

The structure of sugar is an important determinant of its glycation ability. Open chain form and furanose structures are predisposed to higher reactivity. Fructose, with its 0.7% open structure, is ten times more reactive than glucose which has 0.002% open chain structure.²¹ Dietary AGE formation from fructose is much more rapid. Although the concentrations of fructose are much less in comparison to glucose levels, escalation in the intake of fructose can be seen nowadays. It has previously been reported that certain organs of diabetic patients have much higher fructose concentration. Fructose exhibits 7.5% higher hemoglobin glycation than glucose.²² Furthermore, crosslinking of proteins is ten times higher in case of fructose than glucose.²³

Glycation-induced protein modification, escalation of oxidative stress, and intracellular accumulation of sorbitol due to an increase in fructose concentration are some of the important contributors towards diabetes pathogenesis. The other aspect contributing to diabetes pathology is protein crosslinking and aggregate formation that leads to altered receptor recognition. Structural alteration also leads to formation of neo-epitopes, which contributes to development of antigenic properties in the modified protein.²⁴ Such non-enzymatic glycation also affects plasma proteins, especially albumin. In healthy individuals, the level of HSA glycation varies from 1-10%; however, this may increase 2-3 folds during hyperglycemia.^{25,26} Thus, glycated albumin can be considered as an indicator of hyperglycemia in diabetic patients.

Since these changes are stable, HSA can also act as a candidate biomarker for diabetes mellitus, instead of HbA_{1c}. It is still used as an alternative biomarker in some cases as it has a longer half-life and is more reactive. It also has the propensity to form AGE aggregates and amyloid-like structures under hyperglycemic states, which makes it important to explore the structural changes and its implications in diabetes.²⁷

The changing lifestyles and dietary habits undoubtedly have an impact on the development of diabetes complications early in life and the progression of the disease throughout the course of the disease, resulting in complications and other pathological consequences. The purpose of this study was to evaluate the changes in HSA that occur as a result of glycation when the fructose content is varied. Changes in HSA structure, glycation-induced glycoxidative changes, changes in the antigenic characteristics of glycated HSA, as well as its identification by diabetes sera, were also investigated. Conclusions The levels of oxidative stress indicators were measured in order to determine the impact of glycoxidation caused by hyperglycemia on the body.

2. Material and methods

2.1. Materials

Human Serum Albumin (HSA), Bovine Serum Albumin (BSA), Agarose, Tween-20, dialysis tubing, Coomassie Brilliant Blue R-250, dinitrophenyl hydrazine (DNPH), para nitrophenyl phosphate (PNPP), anti-human IgG alkaline phosphatase conjugate and para nitrophenyl phosphate (PNPP) were obtained from Sigma, USA. D-fructose, sodium azide, TRIS, copper sulfate, sodium dihydrogen orthophosphate, sodium carbonate, disodium hydrogen orthophosphate, sodium nitrite, potassium chloride, sodium hydroxide, methanol, isopropanol, sodium bicarbonate, sodium chloride, magnesium chloride, ethanol, glacial acetic acid, and ammonium persulfate were purchased from Qualigens Fine Chemicals, India. Round-bottom and flat-bottom ELISA plates were purchased from NUNC, Denmark. Double distilled water was used for all experiments. All chemicals used were of the highest analytical grade.

2.2. Methods

2.2.1. HSA glycation

HSA was glycated *in vitro* according to a previously described method with minor modifications. Each 1 mL reaction mixture contained 1mg HSA in 20mM PBS (pH 7.4). To this mixture varying concentrations of D-Fructose (25, 50, and 75mM) and 0.01% of sodium azide were added. The solutions were incubated for 7, 14, and 21 days at 37 °C. Next, the unbound sugars and low molecular weight reactants were removed via dialysis. Lowry's method was used to determine the protein levels.²⁸ Both HSA and fructose were dissolved in 20 mM PBS (pH 7.4). Separate solutions of HSA and fructose were prepared under identical conditions which served as controls.

2.2.2. UV-Visible spectroscopy

Shimadzu UV-1700 spectrophotometer was used to assess the UV profiles of the native and the glycated samples under wavelengths ranging between 200 and 400 nm.

2.2.3. Fluorescence spectroscopy

The overall effect of phenylalanine, tryptophan, and tyrosine was assessed using excitation wavelength of 280 nm and emission wavelengths of 300 - 400 nm. Tryptophan-specific fluorescence of native and glycated samples was assessed between 300 and 400 nm following excitation at 295 nm.²⁶

Loss of fluorescence (F) was evaluated by the following formula:

$$\% \text{ Loss of F} = \frac{(F_{\text{native HSA}} - F_{\text{modified HSA}})}{F_{\text{native HSA}}}$$

$F_{\text{native HSA}}$

2.2.4. Advanced glycation end product (AGE)-related fluorescence

The formation of AGEs was assessed by measuring the fluorescence using excitation wavelength of 370 nm and emission wavelengths between 400–500 nm.²⁷

2.2.5. SDS-PAGE

Native and glycated samples were characterized using SDS-PAGE. Gels were electrophoresed for 3 to 4 hours at 50V – 80V and Coomassie Brilliant blue R-250 was used to visualize the bands.

2.2.6. Ketoamine estimation by nitroblue tetrazolium test (NBT assay)

Ketoamine levels were quantified by NBT assay as described previously with minor modifications.²⁹ A 96-well microtitre plate was filled with the native and glycated samples in duplicates. Subsequently, each well was filled with 100 μ L NBT reagent (250 μ M NBT dissolved in 0.1 M carbonate buffer, pH 10.35). Then, the plate was incubated for 2 h at 37. Fructosamine content was measured at 550 nm and the molar extinction coefficient (ϵ) of monoformazan (12640 /M/cm) was used for calculation.

2.2.7. Estimation of carbonyl content

Carbonyl content was assessed using a method described previously.³⁰ The absorbance was measured at 370 nm and the ϵ for DNPH (22000/M/cm) was used for calculation.

2.2.8. Determination of free thiol content

Free thiol content was evaluated using the Elman's method with minor modifications.³¹ The absorbance was measured at 412 nm. The ϵ value of 1.36×10^4 /cm/mol was used.

2.2.9. Determination of serum MDA levels

Serum MDA (malondialdehyde) level was estimated in healthy and diabetic patients by assessing the formation of thiobarbituric acid reactive substances (TBARS) as described previously.³²

2.2.10. Recognition of modified DNA

Serum samples were tested for autoantibodies by direct binding ELISA. 96-well polystyrene immunoplates purchased from Nunc, Maxisorp, were used to quantify the antibodies against glycated and native samples. About 100 μ L of antigen (2.5 μ g/mL glycated HSA dissolved in TBS, pH 7.4) was coated on the wells of the plates and the plates were incubated at 4°C overnight and at room temperature for two hours. Each sample was coated in duplicates. Then, the plates were washed thrice with TBS-T. The unoccupied sites were blocked by incubation with 150 μ L of 1.5% non-fat dry milk (dissolved in TBS, pH 7.4) at 4°C for 4 – 5 h. Again, the plates were washed once with TBS-T. Next, each well was filled with 100 μ L of test antibody. The plates were then incubated for 2 h at 37°C, and then, washed with TBS-T for four times. Each well was filled with 100 μ L of anti-IgG alkaline phosphatase conjugate (1:5000 diluted in TBS). The plates were then incubated for 2 h at 37 °C, and again washed using TBS-T for four times followed by washing with distilled water for three times. Each well was then filled with PNPP and the resultant color was evaluated at 410 nm using a microplate reader. Difference between mean absorbance of the test and the control wells ($A_{\text{test}} - A_{\text{control}}$) was calculated.

2.2.11. Competition ELISA

Competition ELISA was used to determine the antibody specificity. Equal amounts of sera from diabetic patients were mixed with varying inhibitor concentrations (0– 20 μ g per mL). Then, the solution was incubated for 2 h at room temperature and at 4 °C overnight. The resultant immune complex was coated on the wells. Further protocol was the same as that employed for direct binding ELISA. Following formula was used to evaluate the percent inhibition:

$$\text{Percent inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \times 100$$

2.3. Statistical analysis

The statistical significance of results was assessed using Student's t-test. $P \leq 0.05$ defined statistical significance.

3. Results

3.1. UV-characterization of glycated HSA

HSA gave maximum absorbance at 280nm. Incubation of HSA with varying concentrations of fructose (25, 50, and 75mM) for 7 days produced hyperchromicity at λ_{\max} compared to native HSA (Fig.1), whereas incubation for 14 and 21 days with the same concentrations of fructose led to hypochromicity (Fig.1). Though, for both cases, no peak shift was observed. Fructose concentration and incubation time had great influence on the UV-absorption spectra of HSA. Results have been summarized in Table 4.

3.2. Intrinsic fluorescence studies

Overall effects of phenylalanine, tyrosine, and tryptophan were assessed by subjecting the fructosylated HSA samples (incubated for 21 days) to excitation wavelength of 280nm and emission wavelengths of 300 - 400nm. After 21 days, the glycated HSA showed emission peak at 310nm, and a concentration-dependent decrease in fluorescence intensity (Fig.2). Owing to its high reproducibility and sensitivity, tryptophan fluorescence was chosen as an index of conformational changes. Tryptophan-specific fluorescence was analyzed for native and fructosylated HSA (incubated for 21 days). Samples were subjected to excitation wavelength of 295nm and emission wavelengths of 300 - 400nm. They showed emission peak at 330nm with no apparent shift. The glycated samples' fluorescence decreased significantly in a concentration-dependent manner (Fig.3). The results could simply be ascribed to modification of tryptophan microenvironment.

3.3. AGE-specific fluorescence

Incubation with fructose for 14 and 21 days led to an increased AGE-specific fluorescence in a time- and concentration-dependent manner (Fig.4). The results indicated that glycation leads to generation of fluorescent AGEs. Increase in AGE-specific fluorescence correlated with a decrease in tryptophan-specific fluorescence, which indicated a transfer of energy between AGEs and tryptophan.

3.4. Ketoamine estimation

The ketoamine moieties were quantified using NBT method (Fig.5). Increase in the fructose concentration and incubation time (till 14 days) led to an increase in ketoamine formation. Further increase in incubation period till 21 days did not produce much effect on the early glycation products. Use of native HSA led to production of minimal level of ketomamine.

3.5. Determination of carbonyl content

Glycated HSA-bound carbonyl groups were quantified using DNPH (Fig.6). The absorbance was recorded at 360 nm against guanidinium chloride as blank. A molar extinction coefficient of 22,000 M⁻¹ cm⁻¹ was used to calculate the concentration in mol/mol protein. The carbonyl contents of HSA incubated with 25, 50, and 75 mM fructose for 7 days were detected to be 13.25 ± 1.4 , 15.6 ± 1.7 , and 16.20 ± 1.6 nmol/mg protein, respectively. Incubation for 14 days led to further increase in carbonyl contents (30 ± 2.03 , 31 ± 3.1 , and 33.2 ± 2.2 nmol/mg protein, respectively). Incubation for 21 days resulted in a significant increase in carbonyl contents (41.6 ± 2.01 , 43.8 ± 2.96 , and 45.4 ± 4.6 nmol/mg protein, respectively). Native HSA showed production of 6.1 ± 2.0 nmol/mg protein-bound carbonyl groups.

3.6. Estimation of free thiols

Free Thiol content of native and glycated HSA was estimated using DTNB (Fig.7). Absorbance was recorded at 412 nm and free thiol group was calculated from molar extinction coefficient value of 13,600 M⁻¹ cm⁻¹ and expressed as mol/mol protein. The thiol contents of HSA incubated with 25, 50, and 75 mM fructose for 7 days were detected to be 2.23 ± 0.15 , 1.45 ± 0.12 and 0.76 ± 0.08 nmol/mg protein, respectively. Incubation for 14 days led to further increase in thiol contents (2.4 ± 0.08 , 1.65 ± 0.2 , and 0.82 ± 0.5 nmol/mg protein, respectively). Incubation for 21 days resulted in a significant increase in thiol contents (2.56 ± 0.36 , 1.72 ± 0.26 and 0.82 ± 0.09 nmol/mg protein, respectively). Native HSA showed 6.8 ± 2.1 nmol/mg protein thiol content.

3.7. SDS-PAGE

Equal amounts of glycated and native samples (10µg/well) were electrophoresed in 10% polyacrylamide gel (Fig.8-11). Native HSA revealed a single band of about 65 kDa. The glycated HSA samples revealed a more intense and wider band, indicating formation of both low and high molecular weight aggregates during glycation.

3.8. Determination of serum oxidative stress markers

The diabetic patients (both with and without complications) exhibited significantly higher serum carbonyl content (6.11 ± 0.45 nmol/mg protein and 4.89 ± 0.61 nmol/mg protein, respectively) than healthy individuals (2.01 ± 0.29 nmol/mg protein) ($P < 0.05$). In addition, the diabetic patients, without any complication, exhibited lower MDA (malondialdehyde) content than those with complications (Fig.12). Likewise, MDA levels of diabetic patients

with complications (6.71 ± 0.59 nmol/mL) were significantly higher than the diabetic patients without complications as well as normal individuals (5.42 ± 0.71 nmol/mL and 2.23 ± 0.51 nmol/mL, respectively).

3.9. Recognition of glycated HSA by diabetic sera autoantibodies

Binding and specificity of serum autoantibodies of diabetic patients to glycated and native HSA was evaluated by direct binding (Fig.13) and inhibition enzyme immunoassay (Fig.14). Native HSA and HSA (20 μ g/mL) incubated for 21 days with 75 mM fructose were used as antigens to evaluate binding of serum autoantibodies in diabetic as well as normal subjects. The diabetic patient sera exhibited higher binding to glycated HSA (0.49 ± 0.078) as compared to native HSA (0.14 ± 0.026). However, greater recognition was obtained in sera of diabetic patients with secondary complications (0.64 ± 0.037). Both groups of sera showed less binding to native HSA (0.14 ± 0.026). Normal human sera showed negligible binding to both glycated and native HSA.

Competition/Inhibition ELISA was used to analyze the HSA specificity of autoantibodies. Sera of 12 diabetic patients were subjected to inhibition studies. The data was plotted and figures for three sera have been shown (Fig.12). The maximum inhibition with native and glycated HSA ranged from 12.5% - 33.2% and 40% - 60%, respectively. Thus, glycated HSA is a more effective inhibitor.

4. Discussion

Glycation does take place *in vivo* and it also occurs in individuals with normal blood glucose levels. Hyperglycemia promotes AGE formation. The degree of AGE accumulation is related to the severity of diabetic complications, including nephropathy, cataract, retinopathy, neuropathy, and vascular complications.^{33,34} Here, HSA was glycated with fructose *in vitro*, characterized by different complementary methods and its possible role in diabetes was investigated. Baynes et al. described that HSA incubation with glucose leads to its glycoxidation in a time-, concentration-, and temperature-dependent manner.³⁵ Non-enzymatic HSA glycation causes refolding of globular proteins, subsequently forming cross β -structure that might lead to aggregate formation, and thereby, change the albumin structure and functions.³⁶⁻³⁸ Compared to native albumin, AGEs are larger in size, carry more negative charges, and have lower isoelectric point. The negativity and size of AGEs increase with increase in incubation time.³⁹ This may also alter the recognition of proteins, which might be associated with the pathological impact of glycation.⁴⁰ In this study, varying concentrations of fructose were mixed with HSA and incubated 37 °C for 7, 14, and 21 days. Fructose was used because, compared to glucose, it incurs a higher rate of glycation. Furthermore, the fructose level of diabetic patients is much higher than that of normal individuals.²³ In UV absorption studies incubation of different concentrations of fructose for 7 days produced hyperchromicity, while incubation for 14 and 21 days led to hypochromicity at 280 nm. There was no apparent shift in λ_{max} . Such hyperchromicity might be due to the unfolding of the aggregates produced that are during glycation, accompanied by encounter of amino acids with a polar environment. It could also be due to glycation-induced protein aggregation that subsequently decreases the UV intensity.

Fluorescence intensity of glycated samples reduced in a concentration-dependent manner. This result was attributed to masking of chromophoric groups due to aggregate formation and/or changes in their microenvironment.⁴¹ We also observed a concentration-dependent reduction in the tryptophan-specific fluorescence without any apparent blue or red shifts after HSA glycation. This result could be attributed to the modification of tryptophan microenvironment and/or its degradation.²⁶ Fructose causes AGE formation upon its interaction with HSA, which causes an increase in AGE-specific fluorescence with λ_{ex} as 380 nm and λ_{em} as 460 nm. NBT method is a standard approach for detection of early glycation products. Glycated samples exhibited a considerable increase in ketoamine generation as compared to native HSA in a time- and concentration-dependent manner. Ketoamines transform into carbonyls via an enediol intermediate that generates a superoxide radical.^{42,43} The interaction between the lysine residues and the reducing sugars could also produce protein carbonyls.^{44,45} Glycated HSA samples exhibited significantly higher carbonyl content than native HSA samples. Free thiol level is an indicator of glycation-induced glycoxidative stress.⁴⁶

SDS-PAGE showed that glycated HSAs revealed a more intense and wider band, which indicates extensive intra- and inter-molecular cross linking after glycation, which is responsible for aggregate formation.³⁷

Protein carbonyls are important biomarkers of AGE formation and protein oxidation.²³ In this study, the diabetic patients exhibited significantly higher carbonyl content, which might be due to enhanced glycoxidation. In addition, diabetic patients with complications exhibited significantly higher MDA levels compared to those without complications. Hyperglycemia is accompanied with an increase in levels of free radicals, which promotes lipid peroxidation, leading to formation of MDA.⁴⁸ MDA, a marker for lipid peroxidation, has been considered as a link between hyperglycemia-induced glycation and subsequent lipid peroxidation.⁴⁹

Here, we assessed the HSA recognition by diabetic sera. Direct binding ELISA revealed higher binding of sera of diabetic patients to glycated samples compared to native samples. The sera of diabetic patients with complications exhibited higher recognition. Hyperglycemic condition promoted *in vivo* AGE formation. As a result, the epitopes on glycated proteins can be recognized as non-self and lead to antibody production. The elevated concentration of these antibodies, coupled with poor clearance, is implicated in the severity of diabetic complications.

4.1. Conclusions

Our results indicated that glycation causes biochemical and conformational changes in HSA. HSA incubation with fructose led to generation of AGEs. Glycation induces enhance glycoxidative stress in diabetes. Structural modification of protein lead to formation of auto-antibodies in diabetes recognized the glycated HSA better than the native form or glycated HSA is a better substrate for autoantibodies in diabetes. Furthermore, glycated HSA of the diabetic patients with secondary complications exhibited higher binding capacity compared to the diabetic patients without secondary complications. Inhibition ELISA confirmed the prevalence of glycated HSA specific autoantibodies in diabetes. Glycation of blood protein plays a crucial role in etiopathogenesis of complications during diabetes as well as in its progression.

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Figure Legends

Fig. 1 UV absorption spectra of native HSA (trace 1) and HSA incubated with 25, 50, and 75 mM fructose (traces 2, 3, and 4, respectively) for (a) 7, (b) 14 and (c) 21 days respectively.

Fig. 2. Fluorescence emission spectra of native HSA (trace 1) and HSA incubated with 25, 50, and 75 mM fructose (traces 2, 3, and 4, respectively) for 21 days. Excitation wavelength was 280 nm.

Fig. 3. Tryptophan fluorescence emission spectra of native HSA (trace 1) and HSA incubated with 25, 50, and 75 mM fructose (traces 2, 3, and 4, respectively) for 21 days. The excitation wavelength was 295 nm.

Fig. 4a. AGE-specific fluorescence spectra of native HSA (black) and HSA incubated with 25, 50, and 75 mM fructose (red, green, and yellow, respectively) for (a) 14.

Figures:

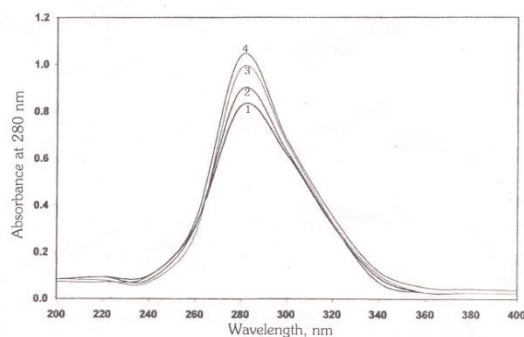


Fig. 1a.

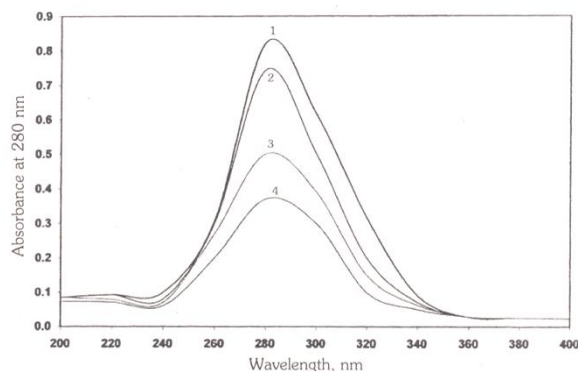


Fig. 1b.

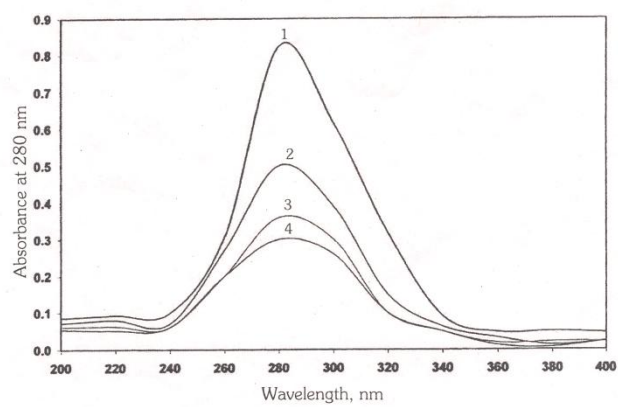


Fig. 1c.

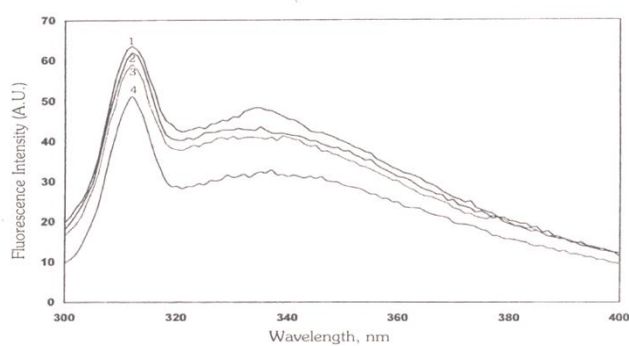


Fig.2.

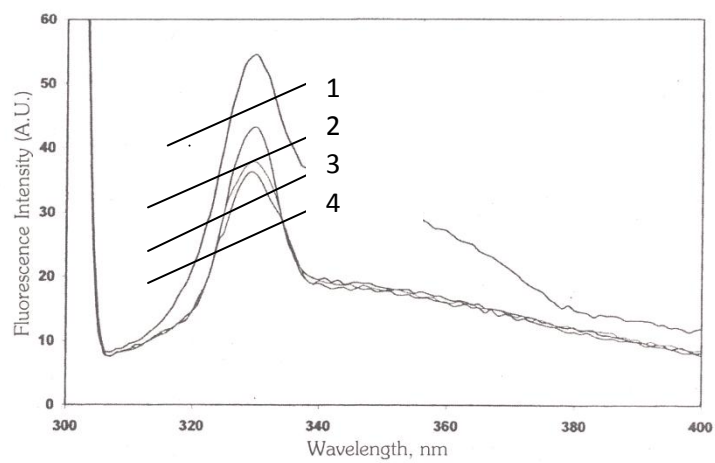


Fig. 3.

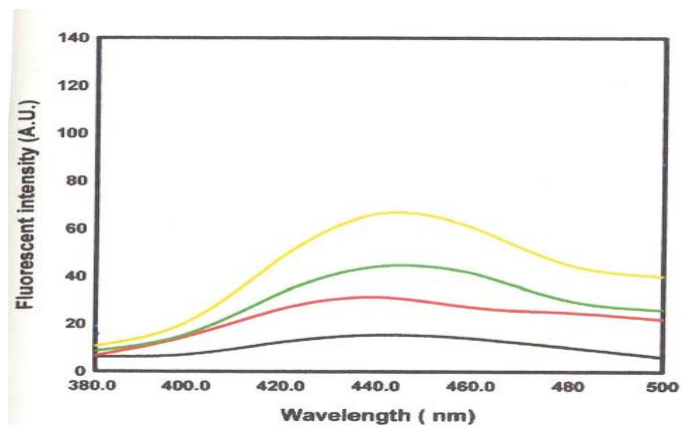


Fig. 4.

