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Human Serum Albumin Structure in Presence of Different Concentrations of Cortisol and Glucose: An In Vitro Modeling Under Normal and Hyperglycemic Conditions

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Abstract

Background: Glucose is an essential element in the supply of body's energy. In diseases such as diabetes, glucose increase is associated with disturbance in metabolism. Cortisol is an important hormone in the regulation of glucose metabolism, and human serum albumin (HSA) is one of the most important glucose and cortisol transmitters in blood. Interaction between albumin and these ligands could affect HSA secondary structure and its stability.
Objectives: The aim of this study was to investigate HSA secondary structure in the presence of different concentrations of glucose and cortisol.
Methods: This was an in vitro (analytical/descriptive) study in which, completely randomized design was used to study the interaction between human serum albumin at 37°C at different glucose concentrations of 0, 80, 180, 240, and 400 mg/dL and cortisol at concentrations of 0, 10, 20, and 40 μg/dL. Intrinsic fluorescence spectroscopy and Circular dichroism (CD) were performed to obtain data.
Results: HSA secondary structure underwent changes in the presence of different concentrations of cortisol and glucose. P values less than 0.01 were considered to be statistically significant. Fluorescence spectroscopy and CD results showed that at normal glucose concentrations, HSA was very flexible, beta-sheet content reduced, and the maximum increase in fluorescence and blue-shift happened. At higher concentrations of glucose, HSA became rigid. Also, HSA in presence of 10 μg/dL cortisol was very flexible; but a cortisol concentration of 40 μg/dL caused stability in HSA structure in presence of different glucose concentrations.
Conclusions: Under normal glucose conditions, very low cortisol concentrations create large changes in HSA secondary structure. At normal glucose concentrations, some of the binding sites of HSA that are all occupied by glucose at higher concentrations become available to cortisol. Cortisol structure is very hydrophobic, which causes large changes in HSA secondary structure and significant increases in quenching and blue shift. In conclusion, binding of compounds such as medicines to HSA sites may be affected by competitive bindings of glucose, depending on its concentration in the blood.

Keywords: Human Serum Albumin, Glucose, Cortisol, Fluorescence Spectroscopy, Circular Dichroism

1. Background

Human serum albumin (HSA) is a non-toxic endogenous protein that can transfer different hydrophobic and hydrophilic drugs in the blood (1). HSA is the most abundant plasma protein (2), has a molecular weight of about 66 kD (3), and possess 585 residues (4). It is a single chain protein with a very high flexibility (1).

In the past decade, most of the studies have focused on the role of HSA as a model of protein fold and ligand binding. HSA has binding sites on the surface with a high affinity to multiple drugs (5). Changes in the structure and stability of proteins may happen when the body's conditions change (such as in diseases). Previous reports indicate that HSA has different and unique structures in the presence of different ligands. At least 6 regions exist in the human serum albumin for non-covalent binding of small molecules and ions (6). In addition to these 6 binding sites in albumin, there is a location with lower affinity to other ligands (7). Binding of ligands to each region of albumin can also change its structure. All these binding sites are used for transferring various ligands by HSA (8, 9).

Normal glucose concentration in the blood is reported to be about 100 mg/dL (2, 7), and that of diabetics is ranged between175 mg/dL and 400 mg/dL (10). About 20% changes in human serum albumin under diabetic condition are induced by non-enzymatic glycosylation (11), and this extent of changes largely happens in the lysine residue 525. Glycation of albumin severely affects the binding of most ligands and even molecules that do not have a common binding site with glucose; the consequence is that the binding tendency of these molecules strongly reduces. It is argued that in diabetes about 80% of albumin is not glycosylated. Therefore, it is important to study conditions under which non-glycated albumin interacts with various molecules (12). Irreversible complications of diabetes are caused by changes in the structure of biomolecules such as albumin, collagen, and hemoglobin; these changes favor the development of complications such as atherosclerosis, nephropathy, and retinopathy (13, 14).

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Cortisol, also called hydrocortisone, is a steroid hormone or glucocorticoid that is secreted by the adrenal glands and its release is controlled by the hypothalamus (15). It is responsible for several stress-related changes in the body, and is known as the stress hormone. The majority of serum cortisol reduces to dihydrocortisol and then to tetrahydrocortisol, which then conjugates to glucuronic acid. The plasma clearance of cortisol is rapid, with a half-life of 66 minutes at normal hormone levels (16). Cortisol is a hydrophobic molecule that is transported in the blood binding to plasma proteins (17). About 90% of total serum cortisol is binding to protein CBG (Corticosteroid Binding Globulin) and albumin, and only 10% of them are free and available for receptors (18).

Illness severity relates more closely to free cortisol levels than total cortisol levels. Cortisol is at the highest level in the morning and at the lowest level at night (19, 20). Values of 7 - 28 μg/dL in the morning and 2 - 18 μg/dL in the afternoon have been reported. Values higher than 18 μg/dL or 28 μg/dL, and lower than 2 μg/dL could be expected in the stimulated and suppressed moods, respectively (21).

2. Objectives

In the present study, it is assumed that cortisol binds to HSA in the presence and absence of glucose, and as a result, alters the secondary structure of the protein. Circular dichroism (CD) and fluorescence intensity methods were used to investigate the secondary structure of HSA at 37°C and pH of 7.5.

3. Methods

3.1. Materials

In this experiment, human serum albumin without fatty acids (a1887) was purchased from Sigma (USA) and used without purification. Hydrocortisone was obtained from Iran Hormone Company and Tris-HCl and glucose were purchased from Merck (Germany).

Tris buffer with pH similar to physiologic pH and concentration of 50 mM was prepared, and was adjusted at a pH of 7.5 by hydrochloric acid. Human serum albumin was prepared at concentration of 0.2 mg/mL in Tris buffer solution. Hydrocortisone powder was solved in Tris buffer solution and solution concentrations of 10, 20, and 40 μg/dL were prepared in albumin Tris buffer solution. Glucose was dissolved in distilled water and different concentrations of glucose (80, 180, and 400 mg/dL) were prepared in albumin Tris solution. Test solutions with different concentrations of glucose and cortisol were incubated for 5 minutes at 37°C.

3.2. Methods

This is an in vitro study in which, the secondary structure of HSA was compared with its structures in presence of glucose, cortisol, and in presence of glucose and cortisol together in four groups. The variables such as alpha, beta, random coil, and F0/F were determined.

This experimental study was carried out at Islamic Azad University, Tehran, in 2015.

3.2.1. Fluorescence Measurements

In this experiment, HSA in presence of different concentrations of 0, 10, 20, and 40 μg/dL of cortisol and 0, 80, 180, and 400 mg/dL of glucose was incubated at 37°C for five minutes. Each sample was excited at 280 nm wavelength and emission was measured between 299 nm and 450 nm.

The possible quenching mechanism can be interpreted by the Stern-Volmer equation (Equation 1):

$$\frac{F0}{F} = 1 + k_q\tau_0 [Q] = 1 + k_{sv} [Q] ; K_{sv} = kq\tau_0 \quad (1)$$

Where F0 and F are the fluorescence intensities before and after the addition of the quencher, respectively, kq is the bimolecular quenching constant, τ_0 is the average lifetime of the biomolecule (10^{-8} s), and [Q] is the concentration of the quencher (22, 23).

The fluorescence spectroscopy device was a Carry -100 Bio; Varian, Australia, and quartz cells with 10 mm diameter were used. The data from fluorescence intensity were analyzed by SPSS software. This instrument was calibrated prior to each run.

3.2.2. Circular Dichroism Measurements

The conditions of structural changes against wavelength were illustrated through elliptic curves. As reported previously, helices have negative bands at 222 and 208 nm and positive band at 190 nm. Beta-sheets show a negative band at 218 nm and positive band at 196 nm. To evaluate changes in the random coil, 212 nm bands were compared with each other (24, 25).

CD changes were studied in far –UV region (190 - 250 nm) with a 0.1 cm diameter cell by an Aviv 215 apparatus (USA) and 0.2 nm interval distance wavelength between each recording, and each test was conducted in triplicate. The CD spectra were analyzed using CDDN software. This instrument was calibrated prior to each run.

3.2.3. Statistical Analysis

This is an in vitro (analytical/descriptive) study, employing completely randomized design. All values were means of three replicates. Data processing and analysis were performed using SPSS software (version 16, Chicago,

IL, USA). P values of less than 0.01 were considered to be statistically significant. Normal distribution of the data was checked using the Kolmogorov-Smirnov test. In normally distributed data, one-way ANOVA was used to compare the variables between the groups. In this study, 16 trials (4 levels of cortisol concentrations versus 4 levels of glucose concentrations) in 3 replicates were carried out; the correlations between F0/F, alpha, beta, and random coil were evaluated using the Pearson's correlation coefficients.

4. Results

The structure of HSA in the absence and presence of glucose at 80, 180, 240 and 400 mg/dl has been shown in Figure 1. These concentrations were used to investigate their effects on albumin separately, corresponding to the normal condition, threshold of diabetic condition, diabetic condition, and critical diabetic condition, respectively. According to Figure 1B, at its minimum concentration, glucose caused highest changes in HSA fluorescence intensity. In the corresponding CD spectra, this is observed as an increase in HSA helices content (Figure 1A). Also in this the the structure of HSA has been shown in the presence of different amounts of cortisol. Different concentrations of cortisol correspond with specific states in the body; 10 μg/dL concentration was used as representative of low levels of cortisol, while 20 μg/dL and 40 μg/dL concentrations were indicative of normal and high levels, respectively. Fluorescence intensity decreased linearly with increasing the amounts of cortisol, and the structure of HSA at minimum concentration of cortisol contained the highest amount of helices (50.8%), indicating a significant difference in presence of other cortisol concentrations (Figure 1B).

Figure 2 shows the concurrent effects of normal glucose condition (80 mg/dL) and different amounts of cortisol on HSA. At cortisol concentration of 10 μg/dL, HSA contained the highest amount of helices. This result is similar to the findings in absence of glucose and presence of different concentrations of cortisol (Figure 2), but the fluorescence intensity changes show irregularities with increasing cortisol concentration (Figure 2B).

In Figure 3, HSA is placed in the threshold glucose diabetes condition (180 mg/dL), in the presence of different concentrations of cortisol. Figure 3A shows an increase in the beta structures of albumin at 10 and 20 μg/dL of cortisol concentrations. Also in Figure 3B, it is observed that fluorescence intensity decreased linearly with increasing concentrations of cortisol. This result is similar to the results of Figure 1B, where there was no glucose in the environment.

Figure 4 depicts the effect of different concentrations of cortisol at critical glucose concentration (400 mg/dL). According to Figure 4A, random coil changes in albumin are not significant in the presence of cortisol compared with the absence of cortisol. The results of fluorescence in Figure 4B show that the HSA structural changes in the absence and presence of 10 μg/dL and 20 μg/dL cortisol concentrations are not significant and only high concentration of cortisol (40 μg/dL) markedly reduces the albumin fluorescence intensity.

In Figure 5, albumin secondary structure is compared in different glucose and cortisol concentrations. As can be seen, in the range of normal concentration of glucose, different concentrations of cortisol are ineffective in random coil structure (Figure 5B); but in this range of glucose, different concentrations of cortisol make statistically significant differences on alpha helices (Figure 5A) with P value of less than 0.01. Different concentrations of glucose at 10 μg/dL cortisol induce a significant effect on the alpha helices structures of HSA; but at 40 μg/dL cortisol, different concentrations of glucose have no effect on HSA structure.

Figure 6 shows a strong negative correlation (-0.894) between alpha helices and beta sheets and also a weak positive correlation (0.101) between random coil and beta sheets.

As observed in Tables 1 and 2, P values less than 0.01 were considered to be statistically significant.

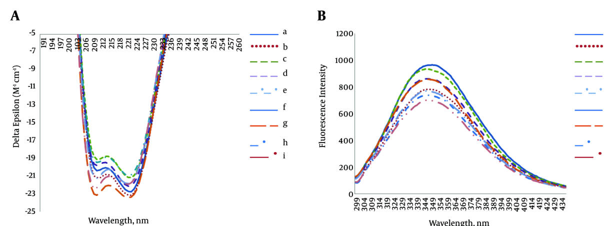
5. Discussion

In a previous report, after incubation of HSA at different glucose concentrations for one week, it was observed that small amounts of glucose cause the largest change in HSA structure, based on HSA intensity fluorescence results, and it was concluded that HSA is "shocked" under normal glucose condition (26, 27). In the present study, although HSA was investigated without long-term incubation, the mentioned structural shock was also observed in the presence of low concentrations of glucose and cortisol.

In the first trial, HSA was put in different concentrations of glucose and showed severe changes in fluorescence intensity under normal glucose condition compared to the threshold of diabetic condition (Figure 1B). Under the same glucose condition, but against different concentrations of cortisol, HSA faced again with a severe quenching in the low (10 μg/dL) cortisol level and the increased amounts of cortisol caused further enhancement in the fluorescence intensity (Figure 2B).

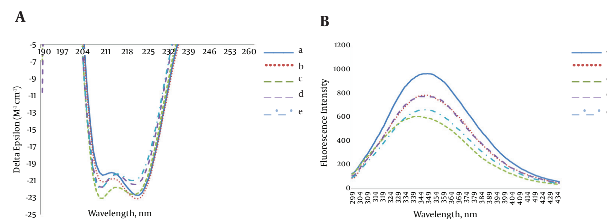
Perhaps the reason for this reduction is related to the empty binding sites that would be consequently occupied by glucose at its higher concentrations; these sites may interact with cortisol and can cause tryptophan and tyrosine to transfer to a less polar environment. Thus, a "blue shift"

Figure 1. A, CD Spectra; B, Intrinsic Fluorescence Emission Spectra



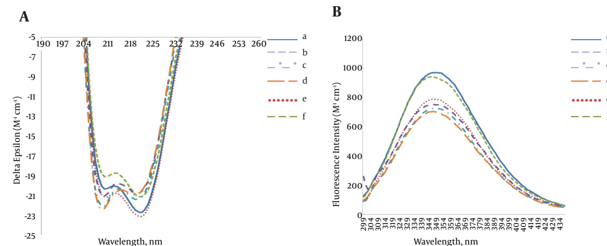
HSA secondary structures analysis a, in the absence; or presence of glucose at b, 80; c, 180; d, 240; and e, 400 mg/dL concentration and f, in the absence or presence of cortisol at g, 10; h, 20; and I, 40 μg/dL.

Figure 2. A, CD Spectra; B, Intrinsic Fluorescence Emission Spectra



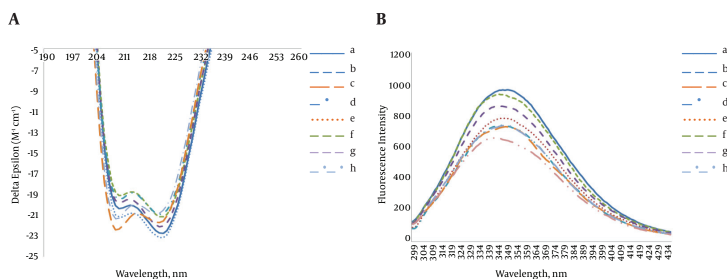
HSA secondary structures a, in the absence or presence of cortisol at b, 10; c, 20; and d, 40 μg/dL and in the presence of glucose at e, 80 mg/dL.

Figure 3. A, CD Spectra; B, Intrinsic Fluorescence Emission Spectra



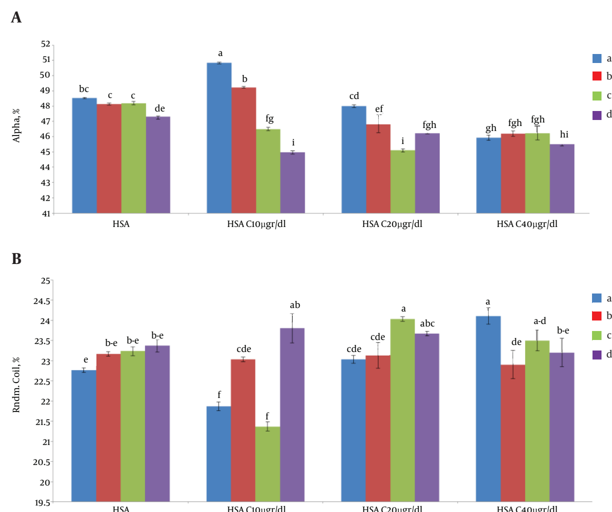
a, HSA secondary structures in the absence; b, or presence of cortisol at 10; c, 20; d, 40 μg/dL and in the absence or presence of glucose at e, 80; f, 180 mg/dL.

Figure 4. A, CD Spectra; B, Intrinsic Fluorescence Emission Spectra



A, HSA secondary structures in the absence or presence of cortisol at b, 10; c, 20; d, and 40 μg/dL and in the absence or presence of glucose at e, 80; f, 180; g, 240 and h, 400 mg/dL.

Figure 5. A, Alpha Helices; B, Random Coils



HSA secondary structures percentage in the absence or presence of cortisol and a, in the absence or presence of glucose at b, 80; c, 180; d, 400 mg/dL.

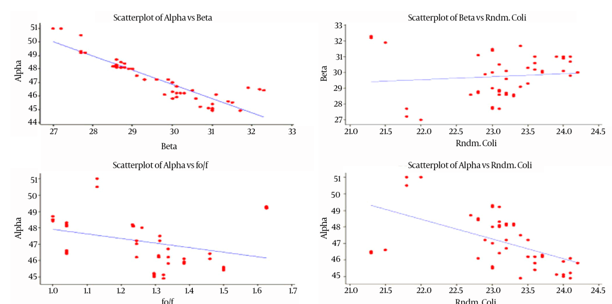


Figure 6. The Correlations Between Alpha Helices, Beta, Random Coils and f0/f Evaluated Using the Pearson's Correlation

Table 1. Three Replicates Analyzed for Each Treatment

	df	Sum of Squares			
		Alpha, %	Beta, %	Random Coil, %	f0/f
Glucose	3	39.63[a]	26.9[a]	2.3[a]	0.44[a]
Cortisol	3	37.27[a]	20.1[a]	6.9[a]	0.45[a]
Glucose × Cortisol	9	39.82[a]	36.5[a]	13.6[a]	0.42[a]
Error	32	1.47[a]	1.6[a]	1.1[a]	0.00[a]
Total	47	118.19[a]	85.2[a]	23.9[a]	1.32[a]

Abbreviations: df, degree of freedom; sov, Sum of squares.
[a] Significance at $P \leq 0.01$.

occurs and maximum wavelength is transmitted toward shorter wavelength.

In this region of fluorescence emission, the fluorescence emission intensity of phenylalanine is much lower in comparison with that of tryptophan and tyrosine, and we assume that during HSA structural changes, these two

Table 2. Three Replicates Analyzed for Each Treatment

	df	Mean Squares			
		Alpha, %	Beta, %	Random Coil, %	f0/f
Glucose	3	13.209[a]	8.981[a]	0.772[a]	0.147[a]
Cortisol	3	12.423[a]	6.708[a]	2.306[a]	0.150[a]
Glucose × Cortisol	9	4.424[a]	4.054[a]	1.512[a]	0.047[a]
Error	32	0.046[a]	0.050[a]	0.034[a]	0.00000017[a]
Total	47				

Abbreviation: df, degree of freedom.
[a] Significance at $P \leq 0.01$.

residues are more likely to transfer into the protein interior or locate at surface clefts, which are out of water reach (28).

This result is entirely consistent with the CD experiment results, which show a marked difference in the random coil structures at a concentration of 10 μg/dL cortisol when compared to other cortisol concentrations in the absence of glucose and presence of normal glucose concentrations. In the presence of 20 μg/dL cortisol, the amounts of random coil structures is the same as those of zero and normal concentrations of glucose, while major changes happen at diabetic threshold concentrations of glucose, and the structure remains constant afterward (Figure 5).

When glucose concentrations are in range of diabetic threshold concentration and HSA comes into contact with cortisol (Figure 3), its quenching behavior is completely linear and follows cortisol concentration. CD experiment results confirm a linear decrease in helical structure content of HSA alongside with the increase in cortisol concentration, which is similar to the condition when HSA is exposed to cortisol in the absence of glucose (Figure 5A).

The fact that HSA structure presents a linear decrease in helices content may be due to glucose interactions with common binding sites of cortisol and glucose when the diabetic threshold concentration is present. Cortisol may not have access to the sites with which its interaction alters the secondary structure of HSA. It may also be suggested that in this concentration of glucose, HSA is more sensitive and it has more flexibility toward ligands.

In HSA, the possible binding sites of the ligands have been studied by competitive binding with other ligands having known binding sites. Even temperature changes can affect this competition (29).

In critical concentrations of glucose, HSA remains unchanged (Figure 5B) in a random coil structure in the presence of all concentrations of cortisol. It can be argued that in this concentration of glucose, the secondary structure of albumin switches, meaning that glucose interacts with sites, leading to an increase in HSA rigidity and a decrease in HSA movement.

In critical concentrations of glucose (Figure 4B), quenching of albumin is similar to the quenching in 10 μg/dL and 20 μg/dL cortisol. Only the 40 μg/dL concentration of cortisol causes further quenching and shifts the maximum wavelength toward shorter wavelengths. This concentration of cortisol drives a higher number of tryptophan and tyrosine residues to non-polar regions or clefts. CD results also suggest further changes in the structure of albumin at 40 μg/dL concentration of cortisol. This finding corresponds to the suggestion that HSA structure changes so much in high concentrations of glucose that other ligands have no more effect on it.

According to Figure 5A, among the selected concentrations of cortisol, the concentration of 40 μg/dL induces the same behaviors in alpha helices at all glucose concentrations. Furthermore, changes in glucose concentration are ineffective in changes of fluorescence quenching of HSA, and 10 μg/dL cortisol induces a significant difference in alpha helices amount in different glucose concentrations. 40 μg/dL cortisol is a specific concentration that may prevent structural changes of HSA, possibly due to the fact that HSA and cortisol interaction could result in HSA being "locked" in its conformation. This event happens also in glucose concentrations under the critical diabetic condition where the "switched" albumin molecule has no more sensitivity toward cortisol concentrations in random coil.

A previous study has established that Fluoxetine (FLX) and cortisol affect the structure of HSA, and FLX competes with cortisol over binding sites on the protein and thus affects the transport of cortisol in the blood. The results of the present study confirmed the effect of different concentrations of cortisol on albumin structure; and as mentioned earlier, it is also possible that common binding sites exist for cortisol and glucose, as they exist for FLX and cortisol (30).

The largest decrease in HSA fluorescence emission intensity, as observed in our study, could happen in low levels of hormones; a condition that suggests natural condition of the body, where these molecules would interact with critical binding sites that would preferably bind with glu-

cose.

Ligands can change biomolecules secondary structure but other factors such as temperature and pH can have a similar effect, too. pH values between 6.5 and 5 cause changes in the secondary structure of BSA (31).

The binding between tolbutamide and glycated HSA followed a two-site model as a set of high affinity sites and a set of lower affinity interactions, thus glycation may change the interactions of drugs such as tolbutamide with HSA in diabetes (32).

In this study, it has been demonstrated that different concentrations of glucose may change the interactions of ligands such as cortisol to non-glycated HSA in diabetes.

5.1. Conclusions

According to this study, the reason for extreme decrease in fluorescence intensity of albumin in presence of low levels of some hormones or drugs is the interaction of these molecules with sensitive sites, which interact preferably with other molecules such as glucose in the body.

Some ligands concentrations induce either flexible or rigid and "locked" structure in HSA. In this study, a normal concentration of glucose locked HSA secondary structure, therefore different concentrations of cortisol had no effect on its structure, and the fluorescent intensity decreased linearly with a gentle slope. On the other hand, 80 mg/dl glucose concentration caused albumin to become quite flexible and sensitive to the different concentrations of cortisol. 20 μg/dL cortisol concentration also locked albumin structure, causing glucose different concentrations to not have any effect on HSA. Perhaps determination of the concentration of molecules that cause flexibility or rigidity in the biomolecules such as HSA can be used to treat diseases more effectively.

Amyloid β (Aβ) is bound to albumin and transported in blood, so albumin has an important role in Alzheimer's disease (A). Albumin complexes with beta-amyloid are reduced in AD patients. Since the diabetic threshold concentration of glucose and 20 μg/dL cortisol cause induction of higher amounts of beta structures in HSA, a similar condition in the body may affect the complexes between albumin and amyloid β; this phenomenon could be of importance in AD (33). A weak, but positive, correlation (0.101) between random coil and beta sheets must be checked with regard to its relation with amyloid β.

In this study, a strong negative correlation (-0.894) between alpha helices and beta sheets was obtained that proves the structural changes happening at the secondary structure level.

Numerous studies have been conducted on non-enzymatic glycated HSA, but less than 20% HSA is non-enzymatic glycated. Thus, it seems crucial to study the interaction of this 80% of HSA with ligands.

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Footnote

Conflict of Interests: The authors declare that there is no conflict of interest in this study.

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