Original Article

Effect of Glucose Concentrations on the HSA-Hydrochlorothiazide Interaction – a study using Circular Dichroism and Molecular Docking

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ABSTRACT

Diabetes Mellitus is a chronic disease characterized by hyperglycemia and is accompanied by disturbances in the metabolism of carbohydrates, lipids, and proteins. It requires a lot of attention, as its complications l ead to serious health damage and even death. Hydrochlorothiazide (HCTZ) is a diuretic that is well-tolerated in antihypertensive treatments, it reduces blood volume and, therefore, decreases peripheral vascular resistance. However, there are some comments in the literature on the possibility that high blood concentrations of blood glucose (GLU) induce tolerance and cause adverse metabolic effects, which might generate clinical complications. This study aimed to present results by using circular dichroism (f ar and near UV) to study the interaction between human serum albumin (HSA) and hydrochlorothiazide in the absence and presence of glucose at 37ºC. Methods- Experimental data were correlated for theoretical analysis through molecular docking simulations. Th e ability of hydrochlorothiazide to bind with HSA was verified in normoglycemic (containing glucose 80 mg/dl) and hyperglycemic (containing glucose 320 mg/dl) solutions. Far-UV analysis showed that HCTZ caused little perturbation on the secondary structure of albumin for the three HSA: HCTZ ratios (1:12, 1:24, and 1:48). We observed a strong disturbance in the secondary structure of albumin was for GLU in hyperglycemic concentration, with a maximum reduction of 12.66% at 222 nm. An increase in the blood GLU level causes a functional perturbation in the binding of HCTZ with HSA. HCTZHSA binding is spontaneous and causes weak perturbation on the secondary and tertiary structure of albumin, however, at high GLU concentration, the perturbation increases which makes the albumin structure unstable.

Key words: Diabetes Mellitus, Glucose, Hydrochlorothiazide, Human Serum Albumin, CD-Spectroscopy, Molecular Docking

In the study performed by Soares. Et al we saw the interaction of HSA with hydrochlorothiazide (HCTZ) in both the absence and presence of glucose using UV-Vis absorption and Spectro fluorescence spectroscopy at 37 ºC. The study also demonstrated competitive binding using warfarin and digitoxin, markers for Sudlow I and Sudlow III sites, respectively. As this drug was used in the treatment of hypertensive diabetic patients, we were interested in verifying if blood glucose (GLU) concentration could interfere with this drug-protein interaction. For this, HSA solutions containing GLU 80 mg/dl and 320 mg/dl were used named normoglycemic and hyperglycemic solutions, respectively. Diabetes mellitus (DM) is a chronic disease characterized by high blood sugar levels, resulting from reduced or non-insulin production and/or reduced tissue sensitivity [2]. Hyperglycemia is accompanied by disturbances in the metabolism of carbohydrates, lipids, and proteins. It is a disease that requires a lot of attention, as its complications can cause serious health damage and even death, especially among young people [3] [4]. By its diuretic effect, HCTZ reduces blood volume and, therefore, decreases peripheral vascular resistance, being in general a well-tolerated drug in antihypertensive treatments [5] [6] [7].

However, there are some comments in the literature on the possibility that high blood concentrations of GLU induce tolerance and cause adverse metabolic effects, which might generate clinical complications [8] [9] [10] [11]. G However, some studies disagree with these opinions [12], suggesting long-term use and high doses (>50 mg/day) as factors that may be behind the adverse effects of HCTZ [13] [14]. The comparison of our results of the experiments using spectrofluorescence and UV-Vis spectroscopy showed that the microenvironment disturbance around aromatic amino acid residues of HSA by its interaction with HCTZ was considerably greater in hyperglycemic solution than in normoglycemic solution [15]. The Fluorescence quenching experiments showed that this drug binds to albumin forming a complex in the absence that might generate a conformational change in albumin and a reduction in the number of sites available for drug binding in the IIA subdomain.

As the biodistribution and effects of drugs depend on their ability to bind to plasma proteins, especially albumin, which is the main drug transporter, it becomes relevant to enlarge the study on disturbances caused by elevated blood glucose in the HSA structure and in the HCTZ-HSA interaction. Circular dichroism (CD) spectroscopy is a widespread technique for checking perturbation on the secondary (far-UV) and tertiary (near-UV) structures of macromolecules upon ligand binding [15]. It is known that HSA exhibits two negative bands in the far-UV: one at 208 nm (π-π* transition) and the other at 222 nm (n-π* transition), which are characteristic of the protein's structural units [16]. Complementing spectroscopic analysis, which allows obtaining experimental data on the binding affinity of a ligand with HSA and theoretical analysis using molecular docking explains the binding ability at the molecular level, which helps in understanding the experimental data [17] [18]. Aiming to complement the results obtained in the previous work [1], we studied the interaction of HSA-HCTZ in the presence and absence of GLU through the circular dichroism technique (far and near UV) also at 37 ºC. Current and previous data were correlated to theoretical analysis through molecular docking simulations, including the evaluation of the main GLU conformation (open or cyclic) in the protein binding pocket.

MATERIAL AND METHODS

Materials and Equipment - HCTZ (6-chloro-3-3, 4dihydro-2H-1,2,4-benzothiadiazole-7-sulfonamide1,1dioxide), D-glucose, HSA, warfarin, digitoxin as well as phosphate buffer solution (PBS) were purchased from Sigma-Aldrich Chemical Company, St. Louis, USA. There was no further purification. Methanol (spectroscopic grade) was obtained from Vetec, Química Fina Ltda, Rio de Janeiro, Brazil. A Millipore Milli-Q system (Merck KGaA, Darmstadt, Germany) was used to obtain purified water.Circular dichroism (CD) spectra were measured on a Jasco J-815 spectrophotometer coupled to a Jasco PFD425S15F thermostatic cuvette door with 0.1 ºC accuracy, with 3 channels, one for steady-state fluorescence, another for the CD and the third for the UV-Vis (Jasco Easton, MD, USA) [19].

Circular Dichroism (CD) Measurements - The circular dichroism (CD) analysis was performed using 3.0 ml of HSA solution (10-6 M, in PBS, pH=7.4) in the absence and presence of HCTZ (1:12, 1:24, and 1:48 HSA-HCTZ solution, in PBS). The effect of the GLU concentration on HSA-HCTZ CD spectra were evaluated in solutions containing 80 mg/dl and 320 mg/dl GLU. All CD spectra were recorded to both far-UV (200-250 nm, for secondary structure) and near-UV (260-320 nm, for tertiary structure) [20, 21]. The intensity of the signals in the CD spectra was expressed as molar residue ellipticity (MRE, in deg cm2 dmol-1), defined according to the following equation [21].

$$MRE = \frac{\theta}{10.n.l.C_p}, \quad (3)$$

Where θ, *n*, *l*, and *Cp* are the observed ellipticity (mdeg), a number of amino acid residues (585 to HSA) [22], optical cuvette length (1.0 cm), and molar HSA concentration (10^{-6} M), respectively. In order to calculate the quantitative loss of the helical structure of the protein due to HCTZ binding, the MRE values at 208 and 222 nm were obtained according to the equations [23].

$$\alpha - helix\ \% = \left[\frac{-MRE_{208} - 4000}{(33000 - 4000)}\right] \times 100, \quad (4)$$

$$\alpha - helix\ \% = \left[\frac{-MRE_{222} - 2340}{(30300)}\right] \times 100, \quad (5)$$

Where MRE_{208} and MRE_{222} are the significant ellipticity molar residues (deg.cm^2/dmol) at 208 nm and 222 nm, respectively.

Molecular Docking Simulations - The crystallographic structure of HSA was obtained from Protein Data Bank (PDB) with access code 1N5U [22l]. The chemical structure of HCTZ and GLU (open and cyclic forms) were built and minimized in terms of energy using the Functional Density Theory (DFT) by Becke-3-Lee Yang Parr (B3LYP) with the standard base set 6-31G*, available in the Spartan'14 software (Wavefunction, Inc.) [24].

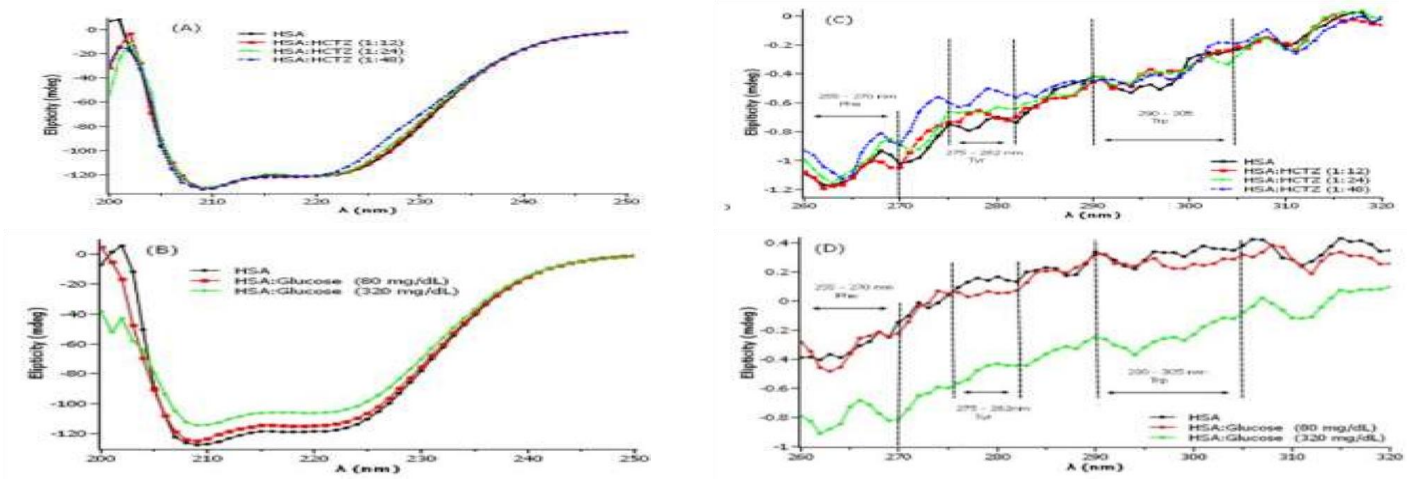

Figure 1 - Far-UV CD spectra for HSA-HCTZ (A) and HSA-glucose (B) at 37 ºC, respectively. Near-UV CD spectra for HSA-HCTZ (C) and HSA-glucose (D) at 37 ºC, respectively. [HSA]= 1.00 × 10-6 M and [glucose] = 80 and 320 mg/dl

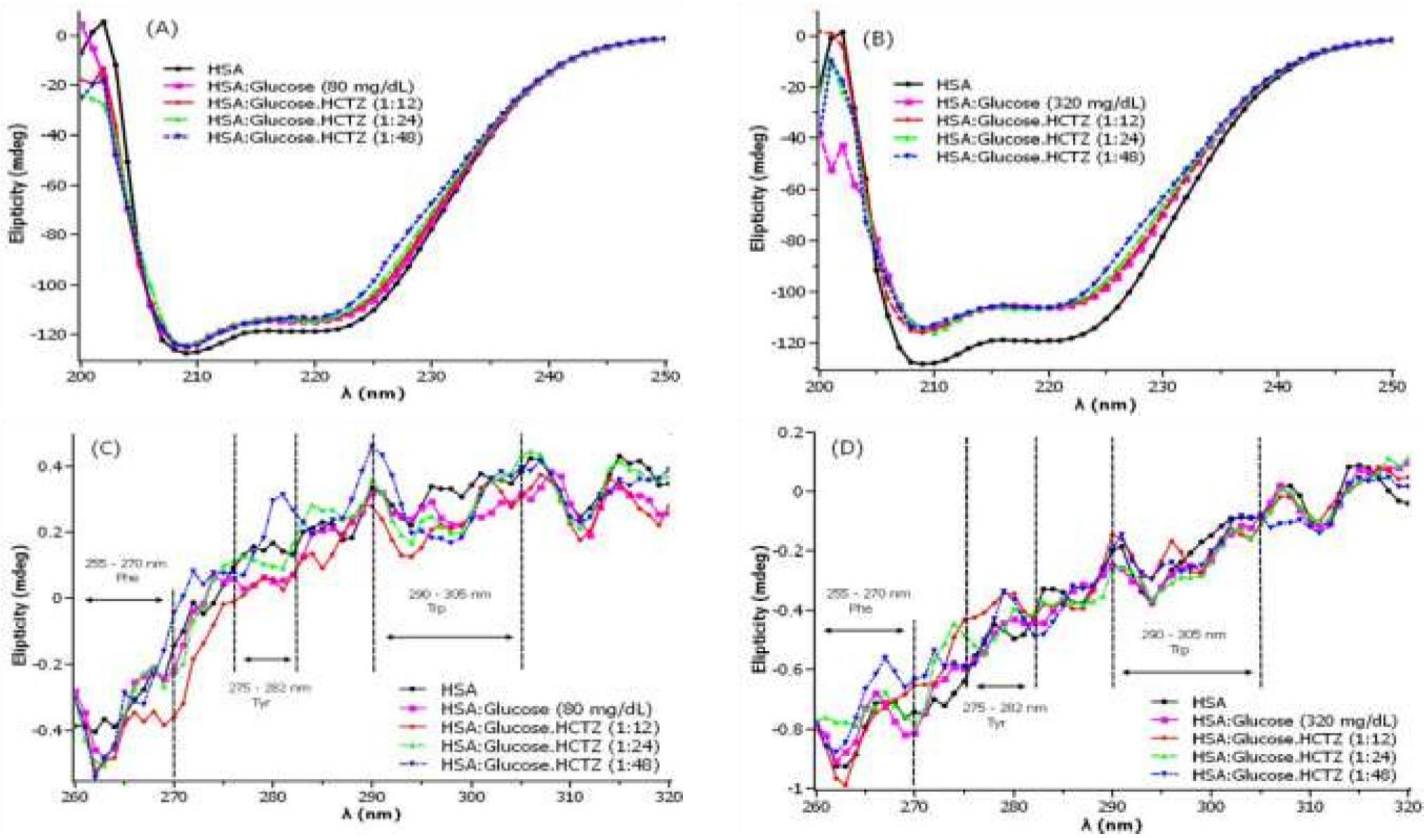

Figure 2 - Far-UV CD spectra for HSA-HCTZ in the presence of glucose, 80 mg/dl (A) and 320 mg/dl (B) at 37 ºC. Near-UV CD spectra for HSA-HCTZ in the presence of glucose, 80 mg/dl (C) and 320 mg/dl (D) at 37 ºC. [HSA] = 1.00 × 10-6 M and [glucose] = 80 and 320 mg/dl.

Molecular docking studies were performed using GOLD 5.7 software (Cambridge Crystallographic Data Centre - CCDC) [25]. Hydrogen atoms were added to the albumin structure according to the data inferred by the program on the ionization and tautomeric states. It is known that HSA presents three main binding pockets for different ligands (sites I, II, and III, which are identified through Trp214, Tyr-411, and Tyr-161 residues [23] [26] [27]. In order to identify the main amino acid residues involved in the interaction HSA-HCTZ, as well as to evaluate the main possible binding site, a spherical 10 Å radius around Trp-214, Tyr-411, and Tyr-161 residues was defined, and molecular docking simulations were carried out [28].

Furthermore, the theoretical evaluation of the GLU effect (in the open and cyclic forms) on HSA-HCTZ binding was simulated, as well as the docking calculations were carried out to HCTZ and GLU (in both forms) at the same time under the same spherical radius described above. A more positive docking score value indicates better interaction, due to the negative sum of a series of energy terms involved in the protein binding interaction process. The number of genetic operations (crossing, migration, and mutation) in each docking run was defined as 100,000. The scoring function used was “ChemPLP”, which is the standard function of the GOLD 5.7 software. The figure of the best docking pose for each case was generated with PyMOL DeLano Scientific LLC software [29].

RESULTS AND DISCUSSION

Three different cases were analyzed by CD measurements: (I) evaluation of the albumin structure upon HCTZ binding, (II) evaluation of the albumin structure upon GLU binding, and (III) GLU effect on HSA-HCTZ CD spectra. Figure 1 A shows that in the far-UV, HCTZ caused little perturbation on the secondary structure of albumin for the three investigated HSA: HCTZ ratios (1:12, 1:24, and 1:48). The content of α-helix (%) at 208 and 222 nm (Table 1) confirms that HCTZ caused little perturbation in the secondary structure of albumin, since, for the 1:48 ratio, the maximum reductions of 3.47% and 3.79% at 208 and 222 nm, respectively, were found [30].

In the absence of HCTZ, GLU at normoglycemic concentration also caused a weak disturbance, with a maximum reduction of 3.45% at 222 nm. But a relatively strong disturbance in the secondary structure of albumin was observed for GLU in hyperglycemic concentration, with a maximum reduction of 12.66% at 222 nm (Figure 1B and Table 2). Thus, high concentrations of GLU in diabetic patients can significantly disrupt the secondary structure of albumin, possibly destabilizing the interaction forces (e.g., hydrogen bonding) responsible for protein stability [31].

Table 1 - α-helix % for HSA without and in the presence of HCTZ in PBS solution at 37 ºC

Ratio HSAHCTZ	HSAHCTZ (208 nm)	HSAHCTZ (222 nm)	Reduction % (208 nm)	Reduction % (222 nm)
1:0	54.50	51.49	-	-
1:12	54.04	50.40	0.84	2.11
1:24	52.92	50.27	2.90	2.37
1:48	52.61	49.54	3.47	3.79

In addition, the near-UV CD spectra for HSA- HCTZ reveal weak signals in the range 290-305 nm, which associates with the amino acid residue Trp-214 (Figure 1C). This indicates that the binding of HCTZ to the subdomain IIA, which is located at the primary site for this drug in HSA [1], does not significantly perturb the protein tertiary structure, as demonstrated by the slight increase in CD signals. These results suggest that there is an increase in hydrophilicity in the Trp-214 environment, the same trend observed in the results of steady-state fluorescence quenching [32]. Verifying the effect of GLU in pure HSA solution in Figure 1D, we observed that the CD signals in the Trp-214 region show a significant decrease, mainly in the hyperglycemic condition, indicating an increase in the tertiary structure of the protein by an increase in hydrophobicity. This may be due to a collapse transition which can directly impact the capacity of ligands binding to HSA [33]. These results are relevant since they are suggesting that high blood GLU levels cause conformational changes in the albumin structure, which can decrease the ability to bind not only to HCTZ but also to other drugs [34].

Table 2 - α-helix % for HSA without and in the presence of glucose in PBS solution at 37 ºC.

[glucose]	HSA-glucose (208 nm)	HSA-glucose (222 nm)	Reduction % (208 nm)	Reduction % (222 nm)
0	55.22	52.12	-	-
80 mg/dl	52.68	50.32	4.60	3.45
320 mg/dl	47.26	45.52	14.42	12.66

Table 3 - α-helix % for HSA-HCTZ in the presence of glucose (80 and 320 mg/dl) in PBS solution at 37 ºC

	80 mg/dl glucose			320 mg/dl glucose		
Ratio HSA-HCTZ	208 nm	222 nm	Reduction % (222 nm)	208 nm	(222 nm)	Reductio n % (222 nm)
1:0	60.5 0	58.6 9	-	61.4 6	58.9 1	-
1:12	58.7 3	56.0 6	4.48	54.0 6	51.5 2	12.54
1:24	58.9 1	55.6 6	5.16	51.9 0	51.0 7	13.30
1:48	59.4 0	54.8 8	6.49	53.2 5	50.7 9	13.78

The same trend was also observed in the region of the spectrum associated with Phe–255 (at 270 nm) and Tyr–275 (at 282 nm) residues. The same kind of analysis was conducted for HSA-HCTZ in the solutions containing GLU 80 and 320 mg/dl. Figures 2A and 2B indicate moderate and strong perturbations in the secondary structure of HSA for normoglycemic and hyperglycemic conditions, respectively [35]. The α-helix content at 222 nm (Table 4) clearly shows a higher tendency of perturbation on the secondary structure upon HCTZ addition in the presence than in the absence of GLU (for HSA: HCTZ = 1:4, maximum reduction of 6.49% and 13.78% at 222 nm, in 80 and 320 mg/dl, respectively). These results are related to spectrofluorimetry results that suggested a conformational change in the HSA structure caused by GLU in hyperglycemic concentration as responsible for the marked decrease in the binding capacity of HCTZ to HAS [1].

Table 4 - α-helix % for HSA-HCTZ in the presence of glucose (80 and 320 mg/dl) in PBS solution at 37 ºC.

	80 mg/dl glucose			320 mg/dl glucose		
Ratio HSA-HCTZ	208 nm	222 nm	Reduction % (222 nm)	208 nm	222 nm	Reduction % (222 nm)
1:0	60.5 0	58.6 9	-	61.4 6	58.9 1	-
1:12	58.7 3	56.0 6	4.48	54.0 6	51.5 2	12.54
1:24	58.9 1	55.6 6	5.16	51.9 0	51.0 7	13.30
1:48	59.4 0	54.8 8	6.49	53.2 5	50.7 9	13.78

For the near-UV CD spectra of HSA-HCTZ in the presence of GLU, unusual behavior can be seen (Figures 2C and 2D). In the hyperglycemic condition, the tertiary contacts decreased in Phe and Tyr regions, due to the decreased αhelical content in the GLU concentration range under study, which may be responsible for the structure loss [32]. But an opposite effect is found in the Trp-214 region (290-305 nm). Overall, the presence of GLU destabilized the protein structure, and this destabilizing effect of GLU becomes more evident with the increase in the drug concentration. **Theoretical Evaluation of the Binding Ability** To identify the possible main binding sites (sites I, II, or III, located in subdomain IIA, IIIA, and IB, respectively) [36], as well as the amino acid residues involved in the HSAHCTZ interaction, and the impact of GLU addition (in open and cyclic forms) in the binding ability, molecular docking calculations were performed with GOLD 5.7 software.

From literature, it is known that in a plasma medium, Dglucose is found essentially as a mixture of two anomers (roughly one-third of α-D-glucopyranose and about twothirds as β-D glucopyranose), with practically no furanose forms [37] and the interconversion between open-chain aldehyde and ring forms is fast and affected by the medium conditions, being energetically favorable in the cyclic than the open form (e.g. for GLU, about 99.98% is cyclic in water) [38]. Thus, all molecular docking calculations for GLU were carried out only assuming β-D-glucose in both cyclic and open forms. Table 5 shows the docking score values (dimensionless) for the best docking pose at the three main binding sites of the HSA structure available for interaction HSA-HCTZ, HSA-GLU, and HSA-GLU.HCTZ.

Table 5 - Docking score values (dimensionless) for the interaction of HSA with HCTZ and glucose at sites I-III.

Sample	Site I	Site II	Site III
HSA-HCTZ	58.3	51.1	40.8
HSA-Open-GLU	56.6	43.5	43.6
HSA-Cyclic-GLU	47.6	41.8	39.6
HSA-Open-GLU.HCTZ	64.6	41.2	50.4
HSA-Cyclic-GLU.HCTZ	49.9	39.0	41.8

The site I had the highest docking score value, the results of molecular docking suggested the subdomain IIA as the main binding site for HSA-HCTZ, HSA-Open-GLU, and HSA-Cyclic-GLU, confirming results from spectrofluorimetric [1]. Although GLU in its open form is in a smaller proportion than its cyclic form, the results of molecular docking suggested a higher docking score for the open than for the cyclic form (56.6 and 47.6, respectively), probably due to the greater flexibility of the open-GLU to adapt to the protein-binding pocket. In addition, if HCTZ and GLU simultaneously bind to the same protein pocket, the results of molecular docking also suggested site I as the main region for this interaction. Furthermore, according to the theoretical analysis, the interaction HSA-OpenGLU.HCTZ is more favorable than HSA-Open-GLU and HSA-HCTZ (docking score values: 64.6, 56.6, and 58.3, respectively). On the other hand, cyclic-GLU showed the opposite trend, decreasing the binding capacity of HCTZ to the same binding pocket. Experimental data also indicated a negative effect of GLU on the HSA-HCTZ interaction [1], which may probably be associated with the fact that GLU is in its pyranose form. According to the molecular docking analysis (Figure 3 and Table 3), hydrogen bonding and van der Waals interactions are the main intermolecular forces involved in HSA-HCTZ, HSA-GLU, and HSA-GLU.HCTZ interactions (for both open and cyclic forms of GLU).

As an example, the hydrogen atom of the -NH3+ group of Lys194, Lys-198, and Lys-443 residues is a potential donor for hydrogen bonding with the sulfonamide group of HCTZ at distances of 2.10, 3.50, and 3.70 Å, respectively. On the other hand, the oxygen atom of Gln-220, Glu-449, and Asp450 residues is a potential acceptor for hydrogen bonding with the NH-portion of the HCTZ structure at distances of 2.10, 1.60, and 1.60 Å, respectively. In addition, the amino acid residue Arg-221 also interacts with the sulfonamide group of HCTZ via hydrogen bonding at a distance of 1.80 Å. Finally, van der Waals interactions between HCTZ and amino acid residues Trp-214 and Val-342 at distances of 3.10 and 2.80 Å, respectively, have also been observed.

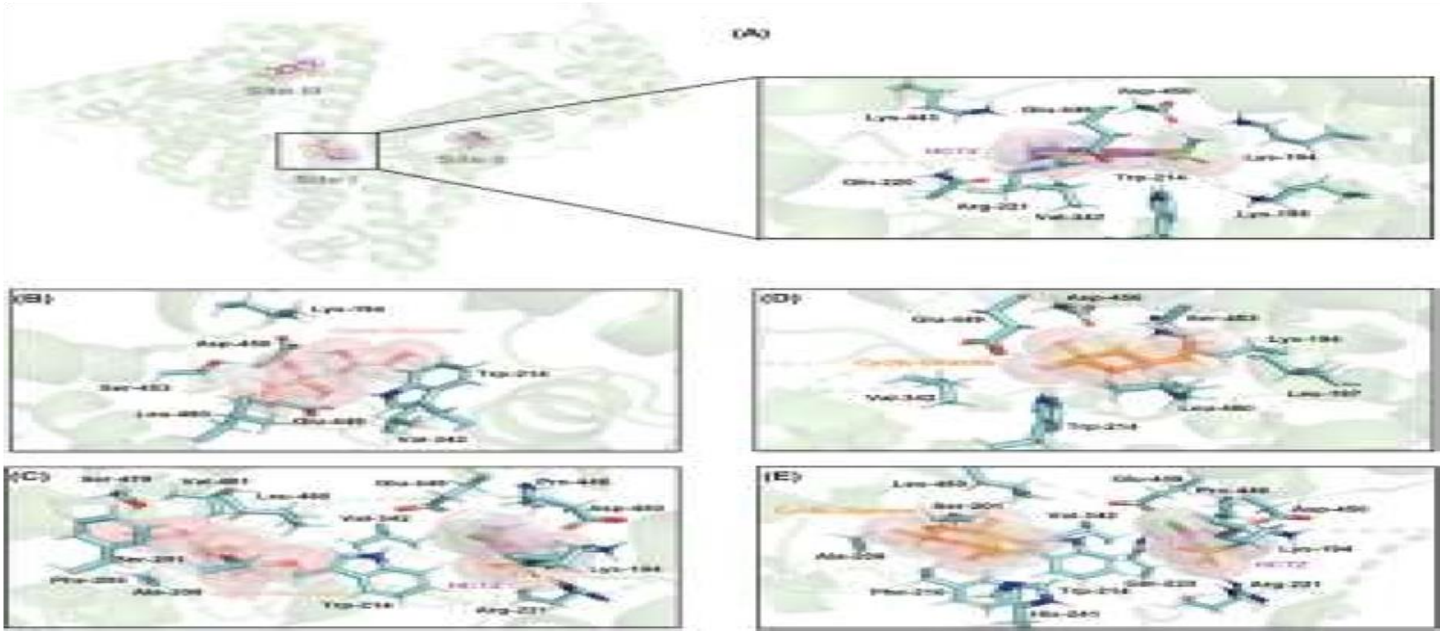

Figure 3 - (A) Best docking poses for the interaction of HSA-HCTZ at sites I, II, and III. The zoom representation shows the main amino acid residues involved in the interaction HSA-HCTZ at a site I. Best docking pose for the interaction between HSA-glucose (B) and HSA-GLU.HCTZ (C), respectively for glucose in the open form at a site I. Best docking pose for the interaction between HSA-glucose (D) and HSA-GLU.HCTZ (E), respectively for glucose in the cyclic form at the site I. Amino acid residues, HCTZ, and glucose in the open and cyclic forms are in cyan, purple, beige and orange colors, respectively. Hydrogen, oxygen, nitrogen, chloro, and sulfur are in white, red, dark blue, green, and yellow, respectively.

CONCLUSION

Circular dichroism confirms the decrease in the binding ability of HCTZ to albumin with an increase in GLU concentration due to the perturbation of the albumin structure. The binding HCTZ-HSA is spontaneous and causes weak perturbation on the secondary and tertiary structure of albumin, however, at high GLU concentration, it was observed an increase of perturbation on these structures increases, conducting the albumin structure to an instability condition and difficulty the bind of HCTZ to the protein binding pocket. There is just one primary binding site for HCTZ in subdomain IIA (the site I), and in this binding pocket of HSA, hydrogen bonding and van der Waals interactions as the key forces for the association. Overall, the experimental and theoretical results suggest that the increase in the blood GLU level can cause functional perturbation in the ability of HCTZ to bind to albumin. It suggests that the adverse effects of HCTZ in hyperglycemic patients may be related to increased levels of free drug in the bloodstream by the decrease of its binding to albumin. In this way, effective treatment for diabetic patients with HCTZ is linked to adequate control of the glycemic index.

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How to cite this article: Marilia Amável Gomes Soares, Otávio Augusto Chaves, Antônio Augusto Fidalgo Neto, Dilson Silva, Dari Cesarin-Sobrinho, Célia Martins Cortez. Effect of Glucose Concentrations on the HSA-Hydrochlorothiazide Interaction – a study using Circular Dichroism and Molecular Docking. Indian J Pharm Drug Studies. 2022: 1(2) 56-62.

Funding: None *Conflict of Interest: None Stated*