6.1. INTRODUCTION

Ranitidine hydrochloride (RTN), chemical name is \( N-(2-[(5-[(\text{dimethylamino})\text{methyl}]\text{furan-2-yl}])\text{methylthio}]\text{ethyl})-\text{N}'\text{-methyl}-2\text{n} \text{itroethene}1,1\text{-diamine} \). It is a histamine \( \text{H}_2\text{-receptor antagonist} \) that has a furan ring structure and is widely used for the effective treatment of gastric and duodenal ulcers. It is also widely used in paediatrics\(^1\), where it is administered orally or intravenously\(^2\). Oral preparations are licensed for treatment of peptic ulcer in children above 3 years, but the injection is not\(^3\). It competitively inhibits the action of histamine on the \( \text{H}_2 \) receptors of parietal cells. Competitive inhibition results in reduced basal and nocturnal gastric acid secretion\(^4\). Ranitidine also decreases the amount of gastric acid released in response to stimuli, such as food, caffeine, insulin, betazole, or pentagastrin. Other actions of ranitidine include an increase in gastric bacterial flora, such as nitrate-reducing organisms. The chemical structure of ranitidine hydrochloride is

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{H}_3\text{C} & \quad \text{NH} & \quad \text{O}_2\text{N} \\
& \quad \text{S} & \quad \text{NH} & \quad \text{NH} \\
& \quad \text{H}_3\text{C} \\
\end{align*}
\]

\( \cdot \text{HCl} \)

The detailed information of HSA is given in Chapter IV (p. 120). To the best of our knowledge there is no work with regard to the binding of this drug by human serum albumin in the literature. Since the study of binding phenomena will provide the basic information on the pharmacological actions,
bio-information, bio-distribution, etc., there is a need for understanding the binding mechanism of this compound. Therefore, it was thought worthwhile to investigate the interactions of HSA with RTN, and such a study is given in this chapter.

6.2. EXPERIMENTAL

6.2.1. Reagents & instruments

Ranitidine hydrochloride and fatty acid–free (<0.005%) and globulin free HSA was purchased from Sigma Chemical Company, St. Louis, USA. The preparation of solutions, instruments used and other relevant information are given in Chapter IV (p.121 & 123). Atomic force microscope (AFM) measurements were carried out using a Nano–Surf easy scan atomic force microscope, TAP-190-Al-G, Switzerland, in air mode, using amplitude 211mV & Time/Line: 703ms. The Raman spectra were measured on a NXR FT–Raman spectrometer, Nicolet USA using an Nd:YVO₄ laser excitation with a wavelength of 1064 nm, the laser power was 500mW.

6.3. RESULTS AND DISCUSSION

6.3.1. Fluorescence quenching mechanism

The fluorescence quenching mechanism was studied as given in Chapter IV (p.125). The fluorescence spectra of HSA in presence of different amounts of RTN were recorded in the range of 250-500 nm upon excitation at 280 nm (Fig.VI (i) (p. 191)). Fig.VI (i) (p. 191) shows the fluorescence
Figure VI (i)
Fluorescence spectra of HSA in presence of RTN; HSA concentration was 5µM (a) RTN concentration was at 5µM (b) to 45µM (j); ($\lambda_{ex} = 296$ nm; $\lambda_{em} = 340$ nm)

Figure VI (ii)
Modified Stern–Volmer plots at three different temperatures

(a) 288 K 
(b) 298 K 
(c) 308 K
emission spectra of HSA in the absence and presence of RTN. RTN causes a concentration dependent quenching of the intrinsic fluorescence of HSA with changing the emission maximum and shape of the peaks. These results indicated that there were interactions between RTN and HSA, further confirmed by synchronous, 3D fluorescence techniques. The quenching was accompanied with an evident red shift (from 340–355 nm), which signified that the binding of RTN was associated with changes in the micro environment of HSA. The Stern–Volmer equation used is same as given in Chapter IV (p.125).

The Stern–Volmer curves of $F_0/F$ versus $[Q]$ at different temperatures were shown in Fig.VI (ii) (p. 191) and the calculated $K_{SV}$ and $k_q$ were presented in Table VI (i) (p. 193). The plots showed that within the investigated concentration, the results exhibited a good linear relationship. Table VI (i) (p. 193) shows that $K_{SV}$ values increased with increase in temperature which revealed the presence of dynamic quenching mechanism in the interaction of RTN with HSA. The fluorescence data was further examined using modified Stern–Volmer equation, and is given in Chapter IV (p.128). The plot of $F_0 / (F_0-F)$ versus $1 /[Q]$ yielded $1/f$ as the intercept and $1/ f K_a$ as the slope. Thus the ratio of the intercept and slope gave the $K_a$ value and the corresponding results at the three investigated temperatures are shown in Table VI (i) (p. 193). The increasing trend of $K_a$ with increasing temperature is consistent with a dynamic quenching mechanism.
**Table VI (i)**

Stern–Volmer quenching constants for the interaction of RTN with HSA at three different temperatures

<table>
<thead>
<tr>
<th>Temp (T)</th>
<th>$K_{SV}$ values $K$</th>
<th>$k_q$ values $K$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L mol$^{-1} \times 10^4$</td>
<td>L mol$^{-1} \times 10^{12}$</td>
<td></td>
</tr>
<tr>
<td>± SD</td>
<td>± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>288</td>
<td>6.15 ± 0.036</td>
<td>6.15 ± 0.036</td>
<td>0.997</td>
</tr>
<tr>
<td>298</td>
<td>7.93 ± 0.023</td>
<td>7.93 ± 0.023</td>
<td>0.995</td>
</tr>
<tr>
<td>308</td>
<td>7.97 ± 0.007</td>
<td>7.97 ± 0.007</td>
<td>0.998</td>
</tr>
</tbody>
</table>

**Table VI (ii)**

Modified Stern-Volmer association constants $K_a$ and relative thermodynamic parameters of the RTN–HSA system

<table>
<thead>
<tr>
<th>$T$ (K)</th>
<th>Binding constant $K_a \times 10^{-4}$ L mol$^{-1}$ ± SD</th>
<th>$R^2$</th>
<th>$\Delta H^0$ (kJ mol$^{-1}$)</th>
<th>$\Delta S^0$ (J K$^{-1}$ mol$^{-1}$)</th>
<th>$\Delta G^0$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>288</td>
<td>2.05 ± 0.020</td>
<td>0.999</td>
<td>$-23.85 \pm 0.068$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>298</td>
<td>4.16 ± 0.010</td>
<td>0.997</td>
<td>$44.15 \pm 0.04$</td>
<td>$236.13 \pm 0.02$</td>
<td>$-26.21 \pm 0.040$</td>
</tr>
<tr>
<td>308</td>
<td>6.80 ± 0.011</td>
<td>0.998</td>
<td>$-28.57 \pm 0.055$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.3.2. Analysis of binding constants and binding sites

The binding constant \(K_a\) and the number of binding sites \(n\), were calculated as given in Chapter IV (p.131). By the plot of \(\log{(F_0 - F)/F}\) versus \(\log{[Q]}\), the number of binding sites \(m\) and binding constant \(K_b\) can be obtained at 298 K as 1.169 and \(3.914 \times 10^5\) \(L\) \(mol^{-1}\), respectively. From the data of \(m\), we may infer that there was one independent class of binding sites on HSA for RTN. Hence, RTN most likely binds to the hydrophobic pocket located in subdomain IIA; that is to say, Trp–214 is near or within the binding site.

6.3.3. Thermodynamic analysis

The equations used to calculate thermodynamic parameters, are given in Chapter IV (p.129). The thermodynamic parameters are summarized in Table VI (ii) (p. 193). The \(\log{K_a}\) versus \(1/T\) plot as shown in Fig.VI (iii) (p. 196). Accordingly, as shown in bar diagram (Fig VI (iv) (p. 196)), the negative values of \(\Delta G^0\) reveal that the binding process is spontaneous and the positive \(\Delta H^0\) and \(\Delta S^0\) values mean that hydrophobic forces play a major role in the binding between RTN and HSA

6.3.4. Displacement experiments with site markers

The displacement of specific probes from HSA by drugs can measure the specificity and relative strength of binding of drugs to the two sites. When
two ligands (drug and probe) bind to HSA simultaneously, two types of interaction can occur:

\[
\begin{align*}
\text{probe} + \text{HSA} & \rightarrow \text{probe - HSA} \quad \text{probe - HSA} + \text{drug} \\
\text{probe} + \text{HSA} & \rightarrow \text{probe - HSA} \quad \text{probe - HSA} + \text{drug}
\end{align*}
\]

The displacement experiments were performed by keeping the concentration of HSA and the site probe constant (each of 5µM). The fluorescence intensity of HSA-probe was recorded in presence of increasing amounts of drug and the corresponding results are shown in Table VI (iii) (p. 198). It was observed that the binding constant of RTN–HSA decreased markedly in presence of warfarin indicating the competition between warfarin and RTN for site I of subdomain IIA. No significant effects were observed in presence of ibuprofen and digitoxin. This revealed that these two site markers did not compete with RTN for same binding site on HSA. Hence, the site I located in subdomain IIA near Trp-214 is proposed to be the main binding site for RTN in HSA.

6.3.5. Distance measurements using resonance energy transfer

The binding distance between RTN and HSA was calculated using same equations as given in Chapter IV (p.133, 135). The extent of spectral overlaps between fluorescence spectrum of HSA and absorption spectrum of RTN Fig VI (v) (p. 199), determines the extent of energy transfer. In the present case using the \( k^2 = 2/3 \), \( N = 1.336 \) and \( \Phi = 0.15 \) value to calculate, \( J = \)
Figure VI (iii)

log $K_a$ versus $1/T$ plot for the binding of RTN–HSA system

Figure VI (iv)

Bar diagram showing thermodynamic parameters of the interaction of HSA with RTN at 298 K

$\Delta S^0$ (J K$^{-1}$ mol$^{-1}$) 236.13

$\Delta H^0$ (kJ mol$^{-1}$) 44.15

$\Delta G^0$ (kJ mol$^{-1}$) -26.21
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1.940 \times 10^{-14} \text{ cm}^3 \text{ L mol}^{-1}, E = 0.204, R_0 = 2.85 \text{ nm} \text{ and } r = 3.40 \text{ nm}. The average distances between a donor fluorophore and acceptor fluorophore on the 2–8 nm scale, this criterion is satisfied in the above case and hence quenching of tryptophan fluorescence of HSA in the presence of the probe is attributed to energy transfer.9

6.3.6. Effect common ions

The effect of common ions viz., Ca^{2+}, Cu^{2+}, K^+, Mg^{2+} and Ni^{2+} on the binding constant of RTN–HSA system was investigated at 298 K by recording the fluorescence intensity in the range of 285–500 nm upon excitation at 280 nm. In plasma, there are some common ions, which can affect the reactions of the drugs and serum albumins. The fluorescence intensity was changed after the addition of some common ions. Therefore, effects of interference of some common ions on the binding constants of RTN–HSA system were investigated. The values of the apparent binding constant $K_a$ as acquired are summarized in Table VI (iv) (p. 198).

The fluorescence intensity was changed after the addition of some common ions viz., Ca^{2+}, Cu^{2+}, K^+, Mg^{2+} and Ni^{2+} to RTN–HSA system. The binding constant between RTN and HSA increased in the presence of Cu^{2+}, K^+ and Ni^{2+} ions implying stronger binding of RTN to HSA. The higher binding constant observed in presence of these Cu^{2+}, K^+ and Ni^{2+} ions might be resulted from the interaction of the ion with RTN to form a complex, which further interacted with the protein. This prolonged the storage time of RTN in
### Table VI (iii)

The comparison of binding constants of RTN–HSA before and after the addition of site probes (warfarin, ibuprofen and digitoxin)

<table>
<thead>
<tr>
<th>Systems</th>
<th>Binding constant (L mol$^{-1}$) × 10$^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA + RTN</td>
<td>4.16</td>
</tr>
<tr>
<td>HSA + RTN + Warfarin</td>
<td>2.35</td>
</tr>
<tr>
<td>HSA + RTN + Ibuprofen</td>
<td>4.01</td>
</tr>
<tr>
<td>HSA + RTN + Digitoxin</td>
<td>3.98</td>
</tr>
</tbody>
</table>

### Table VI (iv)

Effect of common ions on binding constant of HSA–RTN

<table>
<thead>
<tr>
<th>Systems (cations)</th>
<th>Binding constant (L mol$^{-1}$) × 10$^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA + RTN</td>
<td>4.16</td>
</tr>
<tr>
<td>HSA + Ca$^{2+}$ + RTN</td>
<td>3.92</td>
</tr>
<tr>
<td>HSA + Cu$^{2+}$ + RTN</td>
<td>4.27</td>
</tr>
<tr>
<td>HSA + K$^{1+}$ + RTN</td>
<td>5.33</td>
</tr>
<tr>
<td>HSA + Mg$^{2+}$ + RTN</td>
<td>3.46</td>
</tr>
<tr>
<td>HSA + Ni$^{2+}$ + RTN</td>
<td>5.79</td>
</tr>
</tbody>
</table>
Figure VI (v)

The overlap of fluorescence spectrum of HSA ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 340$ nm
$C_{HSA} = 5\, \mu$M) (a) and absorbance spectrum of RTN ($C_{RTN} = 5\, \mu$M) (b),
([HSA]:[RTN] = (1:1))
blood plasma and enhanced the maximum effectiveness of RTN. The decrease in the binding constant of Ca\(^{2+}\) and Mg\(^{2+}\) ions may be due to the formation of metal ion–protein complexes. The formation of such complexes is likely to affect conformation of protein, which may influence RTN binding kinetics and even inhibit RTN-HSA binding.

6.3.7. Conformational analysis

6.3.7.1. UV – vis absorption measurements

The absorption spectra of HSA in the presence and absence of RTN were recorded and presented in Fig.VI (vi) (p. 202). It can be seen from Fig.VI (vi) (p. 202), with the addition of RTN the absorbance intensity increases, which indicates that there is an interaction between RTN and HSA. This result reconfirms that the probable fluorescence quenching mechanism of HSA by RTN is a dynamic quenching process.

6.3.7.2. Circular dichroism

The procedure for CD measurements was same as given in Chapter IV (p.141). The CD spectra of HSA with various concentrations of RTN are shown in Fig.VI (vii) (p. 202). It can be seen from this figure that HSA exhibits two negative bands at 208 and 222 nm in the ultraviolet region, characteristic of typical \(\alpha\)-helix structure of protein\(^{11}\). The reasonable explanation is that the negative peaks of 208 and 222 nm both contribute to the \(n\rightarrow\pi^*\) transfer for the peptide bond of \(\alpha\)-helix\(^{12}\). With increasing addition
of RTN, the band intensity of curves b–d decreased regularly Fig.VI (vii) (p. 202) indicating that the binding of RTN to HSA induced the conformational changes in the protein, which is consistent with the fluorescence results.

The α–helicity of the HSA was calculated, it is decreased from 81% in free HSA to 36.70% in RTN–HSA. Further, the CD spectra of protein in the presence and absence of RTN are observed to be similar in shape, indicating that the structure of the HSA is also predominantly α-helical even after binding with drug\textsuperscript{13}. In the presence of HSA, no appreciable perturbation of secondary and tertiary structures in the protein is observed (Fig.VI (vii) (p. 202)). Thus, RTN, the aromatic and peptide regions do not show any appreciable change in the CD Spectra.

6.3.7.3. Life time measurements studies

The life time measurements were made as given in Chapter IV (p.152). The Fig.VI (viii a and b) (p. 203) shows the fluorescence decay of human serum albumin in the presence and absence of RTN. The fluorescence decay of HSA in the presence of different concentrations of the quencher was found to be biexponential (Fig.VI (viii a and b) (p. 203)). The fluorescence lifetimes ($\tau_1$, $\tau_2$) and their corresponding pre-exponential factors ($\alpha_1$, $\alpha_2$) as well as the average lifetimes ($\tau$), intensity fractions ($f_1$, $f_2$) and chi-square ($\chi^2$) are listed in Table VI (v) (p. 205). The value of $K_S$ and $K_D$ were calculated using the equations as given in Chapter IV (p. 152).
**Figure VI (vi)**
Absorption spectra of RTN, HSA and HSA-RTN system; (×) Indicates 5μM of RTN only. HSA concentration was at 5μM (a), RTN concentration for RTN–HSA system was at 5μM (b) to 45 μM (j).

**Figure VI (vii)**
The CD spectra of the RTN–HSA system obtained at 298 K.
Figure VI (viii)

Time–resolved fluorescence spectra of HSA (a), and the RTN + HSA (b) system
The value of $K_D$ was found to be $7.183 \times 10^3 \text{ M}^{-1}$ from the plot of $\tau_o/\tau$ versus [Q]. The value of $K_S$ found to be $6.312 \times 10^3 \text{ M}^{-1}$. The dynamic quenching constant is greater than that of the static quenching constant which suggests that the quenching mechanism is predominantly dynamic than that of static $^{14}$.

6.3.7.4. Synchronous fluorescence spectra

The detailed information of Synchronous fluorescence spectra is given in Chapter IV (p.144). The conformational changes of HSA caused by the RTN binding were evaluated by measuring the synchronous fluorescence intensity of HSA before and during the addition of the compound. In the presence of RTN, the intensity of fluorescence of Trp and Tyr residues decreased without any remarkable shift in the wavelength (Fig.VI (ix a and b) (p. 206)). This indicated that the binding between RTN and the HSA did not result a change in the polarity of microenvironment of Trp and Tyr residues, but a change in the internal packing of the protein $^{15}$.

6.3.7.5. 3D fluorescence spectroscopy

3D measurements were made as given in Chapter IV (p. 147). The 3D fluorescence spectra of HSA and the HSA – RTN system are shown in Fig.VI (x a and b) (p. 207 and 208) and tabulated in Table VI (vi) (p. 205).

Peak “a” was the Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$) it appeared to match with the “chine” pattern in Fig.VI (x a and b) (p. 207 and 208). At the same time, there were two “humps” in the three dimensional fluorescence
### Table VI (v)

Lifetimes of fluorescence decay of HSA in phosphate buffer of pH 7.4 at different concentrations of RTN

<table>
<thead>
<tr>
<th>[RTN] (µM)</th>
<th>Analysis</th>
<th>$\tau_1$(ns)</th>
<th>$\tau_2$(ns)</th>
<th>$\tau_{av}$(ns)</th>
<th>$f_1$</th>
<th>$f_2$</th>
<th>$\alpha_1$</th>
<th>$\alpha_2$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Biexp.</td>
<td>7.03</td>
<td>2.47</td>
<td>4.75</td>
<td>0.81</td>
<td>0.18</td>
<td>12.4</td>
<td>7.24</td>
<td>1.09</td>
</tr>
<tr>
<td>40</td>
<td>Biexp.</td>
<td>6.84</td>
<td>2.39</td>
<td>4.61</td>
<td>0.84</td>
<td>0.15</td>
<td>11.9</td>
<td>7.83</td>
<td>1.00</td>
</tr>
<tr>
<td>80</td>
<td>Biexp.</td>
<td>6.77</td>
<td>2.18</td>
<td>4.47</td>
<td>0.86</td>
<td>0.14</td>
<td>13.0</td>
<td>7.04</td>
<td>1.10</td>
</tr>
<tr>
<td>120</td>
<td>Biexp.</td>
<td>6.60</td>
<td>2.04</td>
<td>4.32</td>
<td>0.82</td>
<td>0.11</td>
<td>13.7</td>
<td>7.38</td>
<td>1.00</td>
</tr>
<tr>
<td>160</td>
<td>Biexp.</td>
<td>6.56</td>
<td>1.99</td>
<td>4.27</td>
<td>0.84</td>
<td>0.15</td>
<td>12.9</td>
<td>7.65</td>
<td>1.03</td>
</tr>
<tr>
<td>200</td>
<td>Biexp.</td>
<td>6.44</td>
<td>1.60</td>
<td>4.02</td>
<td>0.82</td>
<td>0.17</td>
<td>12.1</td>
<td>7.17</td>
<td>1.11</td>
</tr>
</tbody>
</table>

### Table VI (vi)

Three–dimensional fluorescence spectral characteristics of HSA and HSA–RTN system

<table>
<thead>
<tr>
<th>System</th>
<th>Peak position (Ex/Em)</th>
<th>Peak a</th>
<th>Peak b</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>Peak position (Ex/Em)</td>
<td>260/260–340/340</td>
<td>280/340</td>
</tr>
<tr>
<td></td>
<td>Relative intensity (F)</td>
<td>8.685–3015</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>Relative intensity (F)</td>
<td>1.87–938.8</td>
<td>381.4</td>
</tr>
</tbody>
</table>
Figure VI (ix)

Synchronous fluorescence spectra of HSA–RTN (T = 298 K, pH 7.40), (A) $\Delta \lambda = 15$ nm (B) $\Delta \lambda = 60$ nm. (a) [HSA] = 5 µM; (b) [RTN] = 5 µM to (j) 45 µM
Figure VI (x)

Three-dimensional fluorescence spectra of HSA without RTN

(a) Buffer + HSA

(b) Emission (nm)

(c) Excitation (nm)
Figure VI (x)

Three-dimensional fluorescence spectra of HSA with RTN
spectra for HSA and RTN–HSA, in which the peaks were, marked peak “b” and peak “c”. Peak “c” was the second ordered scattering peak ($\lambda_{em} = 2\lambda_{ex}$). In Fig.VI (x a and b) (p. 207 and 208), the fluorescence intensity of peak “a” increased with the addition of RTN, possible reason might be due to the formation of complex formation between RTN–HSA. So the diameter of the macromolecule increased and resulted in the enhanced scattering effect. 

Peak “b” mainly reflected the spectral characteristics of Trp and Try residues. The fluorescence intensity of peak “b” decreased markedly and the maximum emission wavelength of the peak was changed following the addition of RTN. Analyzing from the intensity changes of peak “a” and peak “b” revealed that the binding of RTN to HSA induced some conformational and micro–environmental changes in HSA.

6.3.7.6. The interaction between RTN and HSA based on AFM

Samples were prepared by deposition free HSA 5 µM and mixed (RTN 5µM + HSA 5µM) of the samples on freshly cleaved mica plates and were dried overnight and the AFM measurements were then performed. AFM has been proved to be a very reliable tool for biological applications and for the investigation of protein structure and protein behavior. The size and surface morphology of HSA and its RTN mixture were analyzed by AFM. All the 2D and 3D height images are given in Fig.VI (xi) (p. 210) respectively. As shown in Fig.VI (xA and A1) (p. 210), the HSA was adsorbed evenly on the mica surfaces. The mean height of the individual HSA molecules was
Figure VI (xi)

(A) AFM topography image of free HSA; (B) the AFM topography images of the HSA–RTN samples were adsorbed onto mica with taping mode in air, and the scan size of the image is 2 µm × 2 µm. The corresponding 3D images are given by (A1) and (B1), scan area are given in brackets.
235.5 nm (image size is 2.49 µm). These dimensions are consistent with those from previous AFM studies\textsuperscript{20}. After the addition of RTN, the HSA molecule became swollen. In addition, the mean height of the HSA reached 109.2 nm (image size is 3.5 µm) Fig.VI (xi B and B1) (p. 210)). The aggregation of the HSA molecules is also shown in Fig.VI (xi B and B1) (p. 210). After interacting with the RTN, the microenvironment around the HSA became more hydrophobic. These results again support the formation of protein probe complex with morphology totally different from the free probe or the free protein. Protein–protein hydrophobic interaction is also an important factor that leads to protein aggregation. To minimize the number of unfavorable factors for the formation of a stable structure, the HSA molecule reduced its surface area contact with water by molecular aggregation. In addition, these results revealed that a hydrophobic interaction between HSA and RTN may occur.

6.3.7.7. Analysis of Raman spectrum

The Raman spectra were recorded in wavelength ranges of 500–1800 cm\textsuperscript{-1} with a resolution of 8cm\textsuperscript{-1}. Under the temperature of 298K, record the Raman spectra of 10 µM HSA and the same concentration of HSA–RTN system. Raman spectroscopy, a powerful tool was used to analyze the structural and conformational of proteins\textsuperscript{21}. The polypeptide backbone and amino acid residues which were marked specific Raman are well described in the literature\textsuperscript{22}. In order to detect the changes of structure and the
The microenvironment of side chains in protein, the Raman spectra of free HSA and the HSA–RTN system were analyzed. In the Raman spectra, amide I band appears\(^{23}\) at 1610–1700 cm\(^{-1}\). Amide I bands in the spectral region between 1650 and 1600 cm\(^{-1}\) can be attributed to \(\alpha\)-helix which is the main content of conformation for protein\(^{24}\). In amide I bands of HSA, the assignments of the bands for other small contents structure were as follows: 1630 cm\(^{-1}\) to short segment, 1684 cm\(^{-1}\) to \(\beta\)-turn\(^{25-27}\).

The serum albumin molecule consists of 17 disulphide bridges and one free cysteine residue. In Raman spectra, the region of 550–500 cm\(^{-1}\) can be due to bands of S–S bridges stretching vibration\(^{28}\). The bands occurred around 500–510 cm\(^{-1}\) are due to the gauche–gauche–gauche (g–g–g) conformation, and the peaks around 515–525 cm\(^{-1}\) and 535–545 cm\(^{-1}\) are attributed to gauche–gauche–trans (g–g–t) or trans-gauche-gauche (t–g–g) conformation and trans- gauche-trans (t–g–t) conformation, respectively\(^{29}\).

After addition of RTN, an increase in the intensity of the bands at 522 cm\(^{-1}\) and 542 cm\(^{-1}\) was observed. This result indicates that most of the disulfide bridges change their local conformation from ggg to ggt and tgt after interaction with RTN. The spectral region of 800–860 cm\(^{-1}\) cm contains bands at about 830 cm\(^{-1}\) and 850 cm\(^{-1}\) (called the Fermi-resonance Tyr doublet), which are potentially useful for monitoring the micro-environment of tyrosyl residues.
6.3.7.8. Molecular modelling

The importance of Molecular docking using Surflex–Dock is given in Chapter IV (p. 123). The application of molecular modeling was employed to further understand the possible conformation of RTN-HSA complex. HSA has three structurally homologous domains (I-III), each domain has two subdomains A and B, possessing common structural elements, with six $\alpha$-helices in subdomain A and four $\alpha$–helices in subdomain B\textsuperscript{30}. It is important to note that Trp214 is in subdomain IIA. The interaction of warfarin with HSA was widely investigated as a model to study ligand/HSA interaction, Sudlow and co-workers\textsuperscript{31} reported that site I of serum albumin showed affinity for warfarin and site II showed affinity for ibuprofen etc. The binding of digitoxin to HSA was found to be independent of sites I and site II \textsuperscript{32, 33}. In order to identify the binding site on HSA for RTN, competitive binding studies were performed with warfarin, ibuprofen and digitoxin as site markers.

RTN was capable of forming some key non-covalent interactions with amino residuals around it, docking results for HSA from surflex-dock were listed in Fig.VI (xii a) (p. 215). As shown in Fig.VI (xii a) (p. 215), nitrogen of dimethyl amine group make H–bond with NH$_3$ of Lys195 (2.33 Å) that of oxygen atom of furan moiety make H-bond with NH$_3$ of Lys199 (2.00 Å). Secondary amine hydrogen near to sulphur make H-bond with Glu292 (2.09 Å) and the two oxygen atoms of nitro group makes two H–bonds with hydrogen of His288 (2.36 Å) and Ser192 (2.16 Å).
It was important to note that RTN was surrounded by residues Ser192, Lys195, Lys199, Leu203, Phe211, Trp214, Arg218, Arg222, His242, His288, Ala291 and Glu292 of subdomain IIA of HSA; which indicated that the binding location was Sudlow’s site I in subdomain IIA. Fig.VI (xii b) (p. 215) reveals that the distance between RTN and Trp214 is ~7 Å. These results illustrated that hydrophobic interactions and hydrogen bonds might contribute to the stability of the RTN–HSA complex. The docking results were in good agreement with the above experimental results.

6.4. IMPORTANCE OF CHAPTER

In this chapter, we used different approaches to explore the interaction between RTN and HSA under physiological conditions. The experimental results clearly show that RTN quenches the fluorescence of HSA by a dynamic quenching mechanism. The values of thermodynamic parameters demonstrate that the binding process is spontaneous and hydrophobic forces play a major role in the interaction study. According to Förster’s non-radioactive resonance energy transfer theory, energy transfer from HSA to RTN occurs with high probability. Our study is expected to provide important insight into the interactions of the physiologically important protein HSA with RTN, facilitating further investigation on the pharmacological behavior of RTN.
Figure VI (xii)

(a) Docking conformation of the RTN at the HSA site I in subdomain IIA; (b) The difference between essential amino acid Trp214 and RTN observed at ~7 Å. The color ramp for DI ranges from red (lowest distance area) to gray (highest distance area) between Trp214 and RTN.
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