Molecular docking and multi-spectroscopic approaches to unravel the mechanism of the interaction between thiocolchicoside and bovine serum albumin

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Abstract. A popular muscle relaxant for the treatment of severe, painful muscular spasms is thiocolchicoside (TCS). Although the precise mechanism underlying its ability to relax muscles is unknown, it demonstrates a specific affinity for the inhibitory gamma-aminobutyric acid and glycinergic receptors. This study used a variety of spectroscopic methods and molecular docking to examine the interaction of TCS with bovine serum albumin (BSA). UV absorption and fluorescence spectroscopic titration analysis supported the conclusion that TCS suppressed BSA’s fluorescence through a blend of static and dynamic mechanisms. The thermodynamical constraints revealed that the interaction between BSA and TCS is spontaneous and that van der Waals and hydrogen bonding forces play key roles in stabilising the complex. TCS binds to the site III on BSA, as demonstrated by competitive binding assays utilising site-specific markers and molecular docking studies. By binding TCS, BSA exhibits minor microenvironmental modifications near the tryptophan amino acid residue, according to a structural study employing synchronous fluorescence.

1 Introduction

Colchicine's natural derivative thiocolchicoside, a semi-synthetic version of the naturally found colchicoside obtained from Gloriosa superba (Liliaceae) seeds, is derived from colchicine. In Africa and Southeast Asia, this medicinal plant has long been used as a traditional medicine to treat a variety of illnesses. Gloriosa superba’s tuberous roots are frequently used to treat inflammation, ulcers, skin conditions, and snakebites. Rheumatic and muscular symptoms are relieved by its seeds[1]. Because it is a centrally acting muscle relaxant, it is utilised in medicine. It also has analgesic and anti-inflammatory effects[2]. While the substance has long been used in European nations, the first thiocolchicoside-containing formulation was first licenced in India in 2008[3].

Thiocolchicoside has analgesic, muscle-relaxing, and local anaesthetic properties because it is an agonist of the central nervous system's g- amino butyric
acid (GABA) receptors [4]. Thiocolchicoside is therefore recommended for the treatment of orthopaedic, traumatic, and rheumatologic illnesses [5]. However, the European Medicines Agency's Committee on Human Medicinal Products has advised that the authorised uses for thiocolchicoside-containing medications used for oral or injectable usage should be constrained in light of new findings of the formation of aneuploidy [6]. It is now only advised to use thiocolchicoside and other formulations containing the medicine as an additional treatment for painful muscle contractures (permanent tightening of the muscle tissue) brought on by spinal disorders in adults and adolescents 16 years of age or older. Thiocolchicoside dosage should also be limited when administered orally or intravenously. Thiocolchicoside is offered in oral, parenteral, and topical forms in India. [7].

![Structure of Thiocolchicoside](image)

**Fig. 1.** Structure of Thiocolchicoside

One of the key elements affecting the free drug concentrations in the bloodstream is the binding of drug and protein. Serum albumin protein is widely distributed in the circulatory system of mammals. It is in charge of a varied array of bodily processes, plus the storage & transportation of several endogenous and exogenous substances, regulation of the bodily pH, and upkeep of oncotic pressure. In a few cases, a drug's ability to bind to serum albumin may improve its ability to reach its target or vice versa. And also, it will be helpful in deciding the minimum doses required. BSA is frequently used as a model of serum proteins for in vitro research on protein-drug interactions, because of its low cost, structurally close to human serum albumin (HSA), and ease of use. The single-chain, big globular protein known as bovine serum albumin has 583 amino acid residues. It has three anatomically similar domains (I, II, and III) split into nine loops (L1 to L9) by 17 disulfide links. Every domain is made up of two subdomains (A and B). At certain binding sites, BSA functions as a transporter and distributor of a variety of endogenous and exogenous chemicals, including fatty acids, amino acids, hormones, metals, and medicines. BSA and HSA's sequences are roughly 76% homologous. The key distinction between the two proteins is the presence of one tryptophan (Trp 214) on sub-domain IIA in HSA vs the existence of two tryptophan residues (Trp 134 and Trp 212) on sub-domains IA and IIA, respectively, in BSA [8-10]. Therefore, in this study, several mathematical models, particularly Stern-Volmer's, Hill's, and thermodynamic equations, were used on the fluorescence quenching data to suggest the interaction process and the binding parameters. Additionally, molecular docking was used to examine the structural details of BSA-thiocolchicoside interaction, plus the preferred orientation, the favourite binding location, and the binding energy.
2 Experimental Section

2.1 Materials

BSA was acquired commercially from Sigma Aldrich, Bangalore, India. The site
markers warfarin, ibuprofen, hemin and ligand Thiocolchicoside were supplied by
Tokyo Chemical Industry Co., Ltd. (TCI). Stock solutions of BSA are produced and
diluted as necessary in 10 mM sodium phosphate buffer (pH 7.4). In distilled water,
stock solutions of ibuprofen, warfarin, and thiocolchicoside were made at a
concentration of 5 mM. The tests also used various chemicals, all of which were
analytical-grade.

2.2 Measurements of absorption spectra

On Shimadzu double-beam spectrophotometer (Model UV-1900i), UV absorption
spectra of BSA in the presence and absence of thiocolchicoside were measured by
means of quartz cuvettes with a 1 cm pathlength. All measurements were conducted
in the 190–800 nm wavelength range. BSA's working concentration was set at 5µM,
while of thiocolchicoside ranged from 0 to 70 µM.

2.3 Measurements and mathematical operations in fluorescence
spectroscopy

The Shimadzu RF-6000 Spectrofluorimetric Device with a temperature control
system was utilised to conduct this test. The fluorescence emission spectra were
obtained in the wavelength range (300–500 nm) with the excitation wavelength
established at 295 nm. The slit width was fixed to 5 nm. In fluorescence-based
quenching tests, BSA (5µM) was titrated with different TCS concentrations (0-
100µM) at three distinct temperatures (298K, 311K, and 318K).

2.4 Competitive study of site probes

Using warfarin, ibuprofen, and hemin as probes for sites I, II, and III correspondingly[11],
the competitive displacement tests were done so as to clarify
the binding site of thiocolchicoside on BSA. At a temperature of 310 K, each site
marker in a range of concentrations (0–10µM) was titrated with an equivalent molar
concentration solution of BSA and thiocolchicoside (5µM).

2.5 Molecular Docking

Using the well-known docking programme Auto Dock 4.2[12], molecular modelling
was done to determine molecular interaction between BSA and thiocolchicoside. It
was possible to get the crystal structure of BSA from the RCSB Protein Data Bank
[PDB: 1AO6][13]. To prevent any obstacles, all of the water molecules were
eliminated[14]. The 3D structure of thiocolchicoside [CID: 9915886] was saved in
SDF format and translated to pdb format using Chimera 1.13 from
pubchem.ncbi.nlm.nih.gov [15]. Using Accelrys Discovery Studio 4.5, post-
modelling analysis was carried out with all other docking parameters left at their
default values.
3 Results and discussion

3.1 Thiocolchicoside's impact on the BSA absorption spectrum

The mechanism of interaction between a protein and a ligand can be identified with the aid of a careful investigation of the absorption spectra[16]. Collisional quenching impacts the fluorophore's excited state, where the absorption spectra remain unchanged. On the other hand, the creation of ground-state complexes typically modifies the fluorophore's absorption spectra[17]. Here, BSA showed a significant peak in absorption at 272 nm, which corresponded to the absorption properties of the tryptophan (Trp) and tyrosine (Tyr) residues. In the presence of thiocolchicoside (1–55µ M), an increase in absorbance was noted in a concentration-dependent way. Additionally, thiocolchicoside appeared to shift the highest absorption peak of BSA toward the blue, from 272 to 258 nm. Additionally, one additional peak at 370 nm was formed by the interaction of BSA with thiocolchicoside (Fig.2). Such an observation suggests that a compound between BSA and thiocolchicoside has formed [18]. It also implies that the interaction between BSA and thiocolchicoside caused a microenvironmental change just next to the chromophore.

Fig. 2. Absorption spectra of BSA-TCS complex

3.2 Emission spectra of the interaction of TCS with BSA

To identify the quenching mode in drug-protein interactions, fluorescence quenching studies are a useful technique[19]. The protein's native fluorescence is fundamentally incredibly sensitive to even minute modifications to the protein's structure or surroundings [20]. These alterations are frequently brought on by ligand binding. In the current investigation, different quantities of TCS were added, and the fluorescence intensity of BSA measured at 334 nm decreased as well as depicted blue shift. The extent of quenching reliant on concentration (Fig. 3(a)). Quenching can generally be caused by a variety of methods, such as static, dynamic, or mixed quenching[21]. Dynamic quenching is instigated when quencher molecules collide with a fluorophore that is in its excited state, whereas static quenching is produced when ground-state complexes are formed [22]. Combination or mixed quenching is the term used when both processes take place together. By analysing the connection
between quenching rate constant and temperature, these mechanisms can be separated from one another. The quenching constant for dynamic quenching increases with rising temperature while in static, increasing the temperature causes the quencher/fluorophore ground-state complex to become less stable, which in turn causes the quenching rate constant to drop\[23\] The data was analysed using Stern-Volmer Eq.

\[
\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{sv} [Q] \tag{1}
\]

In the above equation \( F_0 \) is the fluorescence without quencher (TCS) and \( F \) with quencher, \([Q]\) is the concentration of TCS; \( K_{sv} \) is the Stern Volmer constant; \( K_q \) is the biomolecule quenching rate constant; and \( \tau_0 \) is the average lifetime of the fluorophore in excited state, which is typically \( 10^{-10} \) s for a biomolecule \[24\]. The effects of fluorescence quenching at 298, 311, and 318 K were examined. Fig.3 (b)) depicts the Stern-Volmer plot for BSA-TCS at 3 diverse temperatures. Results exposed a straight relationship among \( K_{sv} \) values and temperature (Table 1), suggesting that the dynamic interaction between BSA and TCS is what primarily regulates the fluorescence quenching process.

\[\text{Fig. 3. (a) BSA-TCS system's Fluorescence Quenching}\]
Although they were all greater than the highest value for collisional quenching in aqueous solution ($10^{10}$ L mol$^{-1}$ s$^{-1}$), the bimolecular quenching-rate constant values derived using $k_q = K_{sv}/\tau_0$ (Table 1) are suggestive of the role played by the static mechanism in the quenching process. As a consequence, a mixture of dynamic and static mechanisms is likely to be responsible for the suppression of fluorescence intensity of BSA by TCS[25].

In order to further analyse the fluorescence data and gain more knowledge, the following modified Stern-Volmer equation (Eq. (2)) [18-20]:

$$\frac{F_0}{F_0 - F} = \frac{1}{fQ/K_a} + 1/f$$

(2)

In this equation, $f_a$ denotes the proportion of fluorophores that are accessible, and $K_a$ is the effective quenching constant for those fluorophores (Fig.4(a))

Table 1. The Sterne-Volmer quenching constants ($K_{sv}$) and bimolecular quenching rate constants ($K_q$) for TCS – BSA system

<table>
<thead>
<tr>
<th>Complex</th>
<th>T(K)</th>
<th>$K_{sv}$ (X10$^4$ M$^{-1}$)</th>
<th>$K_q$ (X10$^{12}$ M$^{-1}$ S$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-TCS</td>
<td>298</td>
<td>5.61</td>
<td>5.61</td>
<td>0.9342</td>
</tr>
<tr>
<td></td>
<td>311</td>
<td>6.71</td>
<td>6.71</td>
<td>0.9799</td>
</tr>
<tr>
<td></td>
<td>318</td>
<td>6.42</td>
<td>6.42</td>
<td>0.9852</td>
</tr>
</tbody>
</table>

3.3 BSA-TCS complex binding constant and stoichiometry
Using the double logarithmic Scatchard Eq. (3) binding constant (Kb) and stoichiometry of the interaction (n) were calculated.

\[
\frac{\log(F_0 - F)}{F} = \log K_b + n \log [Q]
\]  

(3)

The intercept and slope of the log \((F_0 - F)/F\) against log\([Q]\) plot was used to determine Kb & n (Table 2) values at 298, 311 and 318 K, respectively (Fig. 4). A strong binding exists between TCS and BSA, according to the binding constant[26].

![Fig. 4. (a) Modified Stern Volmer Plot’s b) Double logarithmic Plot for BSA-TCS systems at 298, 311, and 318 K](image)

Table 2. The binding constants (Kb) and number of binding sites ("n") for TCS-BSA complex

<table>
<thead>
<tr>
<th>Complex</th>
<th>T(K)</th>
<th>Kb (X10^7 M(^{-1}))</th>
<th>n</th>
<th>R(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-TCS</td>
<td>298</td>
<td>2.14</td>
<td>1.6</td>
<td>0.9844</td>
</tr>
<tr>
<td></td>
<td>311</td>
<td>0.12</td>
<td>1.28</td>
<td>0.9852</td>
</tr>
<tr>
<td></td>
<td>318</td>
<td>1.095</td>
<td>1.51</td>
<td>0.9870</td>
</tr>
</tbody>
</table>

3.4 Thermodynamic depiction

In order to further investigate the fluorescence data, a van't Hoff plot was used, as revealed in Fig.5, to shed light on the forces that influence how BSA and TCS are bound. The binding process between ligands and macromolecules is mostly governed by non-covalent forces, including as hydrophobic interactions, electrostatic forces, hydrogen bonds, and van der Waal's forces. The values of heat enthalpy (ΔH), Gibb's free energy change (ΔG), and entropy change (ΔS) were also computed from the following equations:

\[
\ln K_a = -\Delta H /RT + \Delta S /R
\]  

(4)

\[
\Delta H = \Delta G - T\Delta S
\]  

(5)

This method yielded the thermodynamic values shown in Table 2. The primary forces that bind BSA and TCS together are hydrophobic interactions, due to the positive values of \(\Delta H\) and \(\Delta S\)[27]. Additionally, Eq. (5) was used to calculate \(\Delta G\). The results of the TCS and BSA interaction were spontaneous as evidenced by the fact that the values of \(\Delta G\) got at 298, 311 and 318 K were all negative (Table 3).
Table 3. Thermodynamic parameters and the Stern-Volmer association constant (Ka) for TCS-BSA

<table>
<thead>
<tr>
<th>T(K)</th>
<th>Ka (X10^4 M^-1)</th>
<th>ΔH (kJmol^-1)</th>
<th>ΔS(J mol^-1 K^-1)</th>
<th>ΔG(kJmol^-1)</th>
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</thead>
<tbody>
<tr>
<td>298</td>
<td>1.49</td>
<td>49.47</td>
<td>246.44</td>
<td>-23.97</td>
</tr>
<tr>
<td>311</td>
<td>4.47</td>
<td></td>
<td></td>
<td>-27.17</td>
</tr>
<tr>
<td>318</td>
<td>4.90</td>
<td></td>
<td></td>
<td>-28.90</td>
</tr>
</tbody>
</table>

3.5 Identification of the main TCS binding site on BSA

Various markers/probes were employed in dislocation experimentations to support the binding location of TCS on BSA. Three primary drug-binding sites, commonly referred to as sites I, II, and III, are present in the structure of BSA. The competitive binding experiments were conducted to further recognize the TCS-BSA binding site. In this experiment, TCS and BSA were put at the same concentration and titrated with fluorescent probes. [10, 41]. The following approach can be used to calculate the probes' percentage of TCS displacement (I)-I(%) = (F2/F1X 100 %).
where \( F1 \) and \( F2 \) represent, respectively, the peak fluorescence emission intensities of BSA complexed with TCS when various probes are absent and present. Fig. 6 depicts the relationship between displacement percentage(I) values and various molar ratios of a probe to BSA. It was found that TCS binds to site-III in subdomain IB of BSA, as evidenced by the fact that percentage of TCS dislodgment from BSA by hemin is noticeably more compared to the dislodgment achieved in the company of warfarin and ibuprofen.[33–35].

3.6 Molecular docking

As an active technique for further validating the outcomes of the experiments, in silico molecular docking was used to study the binding of TCS to BSA. It is understood that the bulk of ligands attach to subdomains IIA and IIIA in hydrophobic regions. The TCS binding site on BSA was discovered by molecular docking to be close to site III in subdomain IB, as illustrated in Fig. . The optimum conformation's Gibbs free energy value was -7.8 kcal/mol. The results of the studies using the site probe displacement were supported by the docking results. The amino acid residues around TCS that are directly engaged in the interaction include Lys\textsubscript{114}, Leu\textsubscript{115}, Lys\textsubscript{116}, Pro\textsubscript{117}, Leu\textsubscript{122}, Lys\textsubscript{136}, Tyr\textsubscript{137}, Glu\textsubscript{140}, Ile\textsubscript{141}, Arg\textsubscript{144}, Ile\textsubscript{160}, Glu\textsubscript{182}, Met\textsubscript{184}, Arg\textsubscript{185}, Val\textsubscript{188}, Leu\textsubscript{189} and Thr\textsubscript{518}. 

![Molecular docking diagram](image-url)
In the current article, we used various spectroscopic techniques and a molecular docking methodology to examine the binding characteristics of TCS with BSA. Studies have also revealed that TCS spontaneously binds to BSA 1:1.5 stoichiometry by hydrophobic forces, causing a little alteration in BSA’s conformation. Furthermore, the moderate affinity of BSA for TCS was supported by the high value of binding constant. Thermodynamic information indicates that the interaction between TCS and BSA is an enthalpy-driven, spontaneous process based on hydrophobic interactions. The TCS binding site on BSA was discovered by molecular docking to be close to site III in subdomain IB which was experimentally also supported by markers results.

5 References
1. S. Jana and G. S. Shekhawat, Fitoterapia 82, 293 (2011)
6. Spain, European Medicines Agency Recommends Restricting Use of Thiocolchicoside by Mouth or Injection Medicine Only to Be Used at Low Doses for Additional Short-Term Relief of Painful Muscle Contractures (2013)