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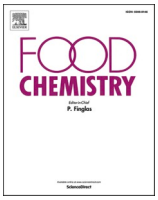

# Change in thermal stability and molecular structure characteristics of whey protein beta-lactoglobulin upon the interaction with levamisole hydrochloride

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ABSTRACT

The interaction between beta-lactoglobulin (BLG) and anthelmintic compounds including levamisole (LEV) is a matter of great concern as it not only poses potential health and environmental risks but also has significant implications for food processing and production. The mechanisms of LEV-BLG interaction were investigated through spectral and molecular modeling approaches. Fluorescence and UV–Visible investigations indicated the formation of a spontaneous and stable LEV-BLG complex. Structural changes of BLG were revealed by circular dichroism and Fourier transform infrared studies. The thermal stability of BLG increased in the presence of LEV. Molecular docking studies indicated the best mode of LEV-BLG interaction and molecular dynamics simulation confirmed the stability of the LEV-BLG complex. In conclusion, our study sheds light on the potential of BLG to interact with deleterious substances such as anthelmintic agents, thus highlighting the necessity of further research in this field to assure food safety and prevent any health hazards.

## 1. Introduction

Imidazothiazole and its derivatives are anthelmintic drugs commonly employed in veterinary medicine to control parasite infestations (Campillo, Eiden, Boussinesq, Pion, Faillie, & Chesnais, 2021; Jedziniak, Szprengier-Juszkiewicz, & Olejnik, 2009). Levamisole (LEV), belonging to a class of synthetic imidazothiazole derivatives, is a potent and broad-spectrum anthelmintic drug extensively used in food-producing animals to treat gastrointestinal and pulmonary nematode infections (Sajid, Iqbal, Muhammad, & Iqbal, 2006). Another significant characteristic of LEV is related to its immuno-modulating effects (Corum, Durna Corum, Atik, Altan, Er, & Uney, 2019). However, in recent years, many concerns have been raised regarding the use of LEV; this is since chronic human exposure to this drug is linked to some serious health problems such as cutaneous necrotizing vasculitis (Farhat, Muirhead, Chaffins, & Douglass, 2010), multifocal inflammatory leukoencephalopathy (Xu et al., 2009), and agranulocytosis (Zhu, LeGatt, & Turner, 2009). According to this background, it is essential to identify the ways in which human beings might be exposed to such drugs and their metabolites. Levamisole is generally administered as a monohydrochloride salt (Fig. 1), via injection or in an oral manner, to livestock. Subsequently, inappropriate administration or misuse of LEV in food-producing animals may result in the production of milk containing LEV residues, which eventually leads to human contamination. To safeguard individuals from exposure to these harmful substances introduced through food, strict adherence to specified withdrawal periods is crucial. This ensures that residue levels in food fall below safe thresholds, protecting consumer health and well-being. Animal breeders play a pivotal role in this process, as they bear the responsibility of diligently monitoring the withdrawal time for medicinal products before slaughtering animals or selling milk. Through their meticulous oversight, animal breeders ensure that animal-derived products intended for human consumption from treated animals are free from excessive residue levels. This crucial step guarantees the safety and integrity of the food supply chain, providing consumers with peace of mind. In relation to this issue, a recent study investigating the existence of levamisole in tissues of treated livestock uncovered that, within a few days, the concentration of levamisole decreases to levels considered safe; and the exact timeframe varies depending on factors such as the species, type of tissue, and method of treatment (Ciucă, Rusănescu, & Safta, 2022). It is

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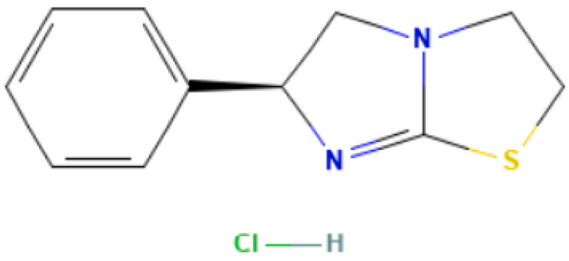


**Fig. 1.** Chemical structure of Levamisole Hydrochloride.

imperative to note that the interaction between drugs and proteins plays a pivotal role in governing the drug's pharmacokinetic profile, including its transport and distribution within the body (Ghosh, Mondal, & Mukherjee, 2015). Hence, a meticulous study regarding the interaction between life-threatening drugs including LEV and proteins, particularly those involved in transportation, is critical in order to comprehend the drugs' behavior in the body in terms of pharmacokinetics and pharmacodynamics.

Beta-lactoglobulin (BLG), accounting for more than 50% of whey content and about 10% of the total ruminant milk protein, is an 18.3-kDa small protein containing 162 residues as a monomer (Zheng, Liu, Zhu, Zheng, & Liu, 2016). When the pH is neutral, BLG typically exists in the form of a stable dimer that splits into individual molecules (monomer) at pH values <3.5 or greater than 7.5 owing to the significant electrostatic force of repulsion between the two components of the dimer (Mercadante et al., 2012). As a member of an expanding collection of small extracellular proteins called the lipocalin-protein superfamily, its secondary structure is characterized by one alpha-helix and nine antiparallel beta-strands labeled from A to I. The alpha-helix positioned at the exterior of the beta-barrel together with eight beta-strands of A to H combine to form a flattened conical barrel called the calyx, which serves as a substrate-binding site (Berino et al., 2019; Shafaei et al., 2017). Generally, BLG has two high-affinity binding sites. The first one (main site) is located inside the calyx, while the second one is placed on the dimer interface, in a cleft between alpha-helix and beta-barrel, involving Trp19, Tyr20, Tyr42, Gln44, Gln59, Gln68, Leu156, Glu157, Glu158 and His161 residues (Dumitraşcu, Ursache, Aprodu, & Stănciuc, 2019). In this regard, previous research has demonstrated that BLG possesses the ability to bind and subsequently transport small-molecule drugs, including cyclosporine A (Mohseni-Shahri, Moeinpour, Malaekeh-Nikouei, & Nassirli, 2017), which serves as an immunosuppressant agent, anthracycline antineoplastic drugs like doxorubicin (Agudelo, Beauregard, Bérubé, & Tajmir-Riahi, 2012), and widely used antibiotics such as ciprofloxacin and kanamycin (Mehraban et al., 2017). The BLG protein also exhibits extraordinary withstand against proteases of the stomach and the acidic environment, implying that it can retain its structure during its passage through the stomach and release carrying ligands further in the intestine, which is the primary site of the absorption (Mehraban et al., 2017). In light of the detrimental impact of LEV on human health as previously outlined, and by considering the capability of BLG in facilitating the binding and delivering of numerous ligands, it is crucial to conduct thorough research on the LEV-BLG interaction, thereby necessitating high-quality studies. Therefore, the current investigation endeavors to furnish a comprehensive understanding of the potential mechanism of the LEV-BLG interaction within physiological conditions. In order to achieve this objective, fluorescence and UV–Visible spectroscopy were employed to determine binding parameters and thermodynamic parameters associated with possible binding interaction between the BLG and LEV. Additionally, circular dichroism and Fourier transform infrared spectroscopies were applied to find whether this ligand binding triggered any alternations in the structure of BLG. Moreover, molecular docking along with MD simulation methodologies were utilized to further explore the LEV-BLG interaction and also support the validity of outcomes obtained from the aforementioned experimental studies. As a result, the outcome of this study is significant in terms of providing insights into the potential of BLG to form a complex with LEV and transport it.

## 2. Materials and methods

### 2.1. Reagents and preparation of solutions

LEV (Product Number: 31742; ≥ 98 purity) and BLG (beta-lactoglobulin from bovine milk; Product Number: L0130) were both obtained from Sigma-Aldrich (St. Louis, USA). All other chemical compounds were of analytical grade and triple-distilled water free from any fluorescent contaminants was used throughout the experiment. The buffer employed to maintain the pH and ionic strength of all solutions was Phosphate Buffer Solution (PBS, 50 mM, pH = 7.4). BLG (0.1 mg/ml) and LEV stock solutions were freshly prepared in PBS just 15 min before each experiment, and if needed the resulting solutions were stored at 0–4 °C.

### 2.2. Steady-state fluorescence titration

Shimadzu fluorimeter (Model: RF-5301, Japan) along with dual-path length quartz cells and a water circulator (±0.1 °C) were employed for the scanning of all the fluorescence spectra by setting the bandwidths for excitation and emission at 3 and 5 nm, respectively. For fluorescence measurements, BLG concentration (0.1 mg/ml) was maintained constant, while that of LEV continued to increase from 0 to 0.045 mM. All the fluorescence spectra were obtained at 296, 306, and 316 K through the wavelength of 295–450 nm after being excited at 290 nm.

### 2.3. Absorbance spectroscopy measurements

UV–Visible spectroscopy was conducted with the help of an ultraviolet–visible spectrophotometer (Model Ultrospec 4000 Pharmacia Biotech, England) equipped with quartz cuvettes of 1 cm optical path length. All the absorption spectra were scanned within the wavelengths of 200–450 nm at room temperature. The concentration of BLG was fixed at 0.1 mg/ml and that of LEV varied from 0 to 0.075 mM. Phosphate buffer was used as a blank.

### 2.4. Thermal stability analysis

By employing a Pharmacia 4000 UV–Visible spectrophotometer that was equipped with an external thermostat, the influence of LEV binding (in concentrations ranging from 0 to 0.03 mM) on the thermal stability of BLG (with a concentration of 0.1 mg/mL) was studied by observing the absorbance spectra at 280 nm. All the samples were initially vacuumed and then subjected to heating, starting from 318 K and ending at 372 K with a 1 K/min rate (54 min of thermal treatment).

### 2.5. Fourier transform infrared (FT-IR) spectra analysis

The FT-IR spectra of all solutions were scanned on a PerkinElmer Spectrum Two spectrometer. This was done with the help of a Universal Attenuated Total Reflectance (UATR) accessory. All measurements were performed as 256 scans with a resolution of 4 $cm^{-1}$ in the range from 2000 to 1450 $cm^{-1}$. The background effects were mitigated during each measurement by capturing and digitally subtracting the absorbance values attributed to the buffer and LEV solutions from the absorbance of the native BLG and LEV-BLG complex solutions, respectively.

### 2.6. Circular dichroism (CD) spectroscopy

Investigation into the far-UV CD spectra of BLG (concentrated at 0.1 mg/mL) was performed by employing an AVIV 215 spectropolarimeter. The experiments were conducted within the wavelength range of 190–250 nm, and a quartz cuvette with a path length of 0.1 cm was utilized. All the measurements were carried out under both the absence and presence of LEV (concentrated at 0–0.03 mM) at 300 K. Next, the secondary structure content of BLG was quantified by recruiting the CDNN software, with the results being presented as the mean residue ellipticity in degrees $cm^2$ $dmol^{-1}$, as a function of the given wavelength.

### 2.7. Computational docking studies

The optimal mode of interaction for the LEV-BLG complex was recognized through an in silico docking study utilizing the Auto Dock software and the Lamarckian genetic algorithm approach. Initially, the crystallographic structure of the BLG receptor was acquired from the RCSB Protein Data Bank (PDB ID: 3NPO) with a resolution of 2.20 Å. Subsequently, the optimization of the receptor was performed by executing the following procedures: (1) incorporating polar hydrogen atoms, (2) assigning Kollman united-atom partial charges, and (3) removing water molecules. The three-dimensional structure of LEV (identified with CID 27944) was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in the form of an SDF file, which was then transformed into PDB format by employing Chem3D 15.0.0.106. The energy minimization of the ligand (LEV) was performed utilizing the UCSF Chimera software. In order to assess all possible binding sites, a docking grid was designed with dimensions of 126, 92, and 116 along the X, Y, and Z axes, respectively, and a grid spacing of 0.615 Å. The experiment was carried out with a specified number of 300 runs and the docking model featuring the lowest binding free energy was deemed appropriate for additional evaluation. The selected docked structure was finally visualized using the UCSF Chimera and Discovery studio 3.5 software.

### 2.8. Molecular dynamic (MD) simulations

The MD simulations of both the native BLG and the LEV-BLG complex were conducted utilizing the biomolecular simulation software GROMACS version 4.6.5, with the Amber99sb-ildnff force field serving as the selected method for calculating intermolecular forces. In two separate simulation runs, BLG and LEV-BLG complexes were soaked into a dodecahedron box of sufficient TIP3P waters. To begin with, the whole systems undergo neutralization through the addition of the proper number (8) of sodium ions in the simulation box. Then, energy minimization of both systems was carried out with the help of the steepest descent method to eliminate any initial stress and provide the system's relaxation. Subsequently, applying both the NVT and NPT ensembles, each system was subjected to a simulation of 100 picoseconds, maintained at a temperature of 300 K and an atmospheric pressure of 1, with the objective of achieving the equilibration phase. Finally, the simulation production was performed for 100 ns with 1 fs timestep.

### 2.9. Statistical analysis

The statistical evaluation of the experimental findings was conducted through the employment of the SPSS software version 16 (SPSS Inc., Chicago, ILL). Results were presented in terms of the mean values with corresponding standard deviations. The Tukey's test was performed by one-way analysis of variance (ANOVA), and $P$ values $< 0.05$ were considered statistically significant.

## 3. Results and discussion

### 3.1. Spectrofluorometric evaluations

#### 3.1.1. Effect of LEV on the fluorescence emission by BLG

Fluorescence spectroscopy as a very simple and sensitive methodology has long been used for the efficient and reliable investigation of the interactions between the small compound and biological macromolecules, particularly proteins (Yadav, Kumar Yadav, Dixit, Agarwal, & Kumar Awasthi, 2019). This technique could provide important information concerning the ligand–protein binding interaction, including binding constants, thermodynamic parameters, intermolecular distances, energy transfer efficiency, binding mode, and mechanism (Millan et al., 2017). The intrinsic fluorescence emission of proteins is owing to the existence of fluorescent residues, including Tyrosine (Tyr), Phenylalanine (Phe), and Tryptophan (Trp) (Alsaif, Wani, Bakheit, & Zargar, 2020). The amino acid sequence of BLG encompasses two Trp residues and four Tyr residues, which serve as the source of its intrinsic fluorescence emission. Among these, the chief contribution originates from Trp residues (Alsaif et al., 2020). The fluorescence emissions of BLG alone and in the presence of varying LEV concentrations at 296, 306, and 316 K are illustrated in Supplementary Data, Figs. S1–S3. Following the excitation at 290 nm, BLG showed a strong fluorescence band around 346 nm. The fluorescence signal of the BLG maximum decreased with increasing temperature (Fig. S4). This is because when the temperature increases then the fluorophores are exposed to an aqueous environment in which they have lower fluorescence, ultimately leading to the decrease of the fluorescence signal of the protein (Lakowicz, 1983). Further, as a result of the increasing concentration of LEV in the protein solution, the fluorescence signals of BLG exhibited a progressive decline in the maximum emission wavelength (346 nm). It was found that the highest concentration of LEV (0.045 mM) quenched the BLG fluorescence signals by %14.96, %17.94, and %21.38 at 296, 306, and 316 k, respectively. When considering the reduction in the fluorescence emission of BLG protein across each investigated temperature, it becomes apparent that the sole factor leading to the quenching of protein fluorescence emission is the binding of varying concentrations of LEV to the protein, rather than temperature change. These findings confirm the quenching of BLG by LEV, which is indicative of the LEV-BLG complex formation. In a study, based on various spectroscopic techniques and molecular modeling methodologies, the molecular interaction between BLG and two widely used antibiotics (ciprofloxacin and kanamycin) in physiological situations was examined. The findings of this research indicated that the increase in drug concentration leads to a decline in the fluorescence intensity of BLG (Mehraban et al., 2017). Similar quenching results are also reported in previous studies for several drugs (Agudelo et al., 2012; Mohseni-Shahri et al., 2017; Paul, Ghosh, & Mukherjee, 2014; Shahraki & Shiri, 2018).

#### 3.1.2. Determination of fluorescence quenching mechanism

Fluorescence quenching can be defined as a phenomenon in which the fluorescence intensity of a fluorophore, a molecule that exhibits the ability to fluoresce, is decreased due to molecular interactions. This decrease in fluorescence quantum yield is triggered by several different mechanisms including molecular rearrangements, non-radical energy transfer, excited state reactions, ground-state complexation, and collisional quenching (Habibian-Dehkordi, Farhadian, Ghasemi, & Evini, 2022; Zhao, Guo, Sun, Lan, & Li, 2017). The phenomenon of fluorescence quenching in proteins can be generally classified into two categories: static and dynamic. Static quenching occurs as a result of the formation of a non-fluorescent complex between the fluorophore and the quencher at the ground state. On the other hand, dynamic quenching is brought about by collisions that occur in the excited state. These two quenching mechanisms could be differentiated from each other on the basis of their quenching constant behavior with respect to the temperature increase. Briefly, in the case of static quenching, temperature

raising leads to a decrease in complex stability and thereby lowers quenching constants. However, in the dynamic quenching case, raising the temperature results in faster diffusion and more vigorous collision, thus increasing the quenching constant (Mousavi & Fatemi, 2019). The determination of the mode of the quenching mechanism was established through the evaluation of the data generated from fluorescence spectroscopy at temperatures of 296, 306, and 316 K. The following widely recognized Stern-Volmer equation (Eq. (1)) was employed for this purpose (Eslami-Farsani, Farhadian, Shareghi, & Asgharzadeh, 2022):

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau_0 [Q] \quad (1)$$

In this relationship, $F_0$ and F are the fluorescence of BLG with and without LEV; $K_{SV}$ demonstrates the Stern-Volmer quenching constant; $K_q$ shows the quencher rate coefficient of the macromolecule; [Q] is the LEV concentration, and $\tau_0$ defines the average integral fluorescence lifetime of the fluorophores without LEV (here $\tau_0$ value is approximately equal to 1.28 ns). Fig. S5 in Supplementary Data demonstrates the Stern–Volmer plots of $F_0$/F vs. [Q] for the interaction between LEV and BLG, and Table 1 summarizes the related $K_{SV}$ and $K_q$ values. A gradual decline in the values of $K_{SV}$ was found with the temperature increasing from 296 to 316 K, suggesting LEV-BLG complexation at the ground state. Besides, corresponding $K_q$ values were found to surpass the established upper limit for dynamic quenching of biomolecules, which is $2 \times 10^{10}$ $Lmol^{-1}s^{-1}$, thus illustrating the existence of a static sort of quenching mechanism in the interaction between LEV and BLG (Habibian-Dehkordi et al., 2022). The quenching parameters were not significantly different among the studied temperatures ($P > 0.05$). Within this context, the investigation of the binding interaction between cyclosporine A and BLG by multi-spectroscopy and molecular dynamics simulation approaches has shown that this protein efficiently bind to this drug. The results of this study showed that the fluorescence of BLG regularly decreased with the increasing concentration of cyclosporine A, indicating that the drug interact with BLG and quench its intrinsic fluorescence. Similar to our investigation, the type of fluorescence quenching in this study was static quenching, which means the formation of a ground-state protein drug complex. Due to the localization of Trp19, which is deemed to underlie the intrinsic fluorescence of BLG protein, within the internal cavity of the beta-barrel, its accessibility for collision with various ligands is restricted. As a result, the predominant mechanism of quenching observed during BLG protein binding with various ligands is static (Mohseni-Shahri et al., 2017).

### 3.1.3. Evaluation of binding constant and the number of binding sites

In pharmacokinetics, the relationship between the bioavailability of drugs and their binding affinity to carrier proteins is direct and well-established. In fact, the stronger the binding affinity between a drug and its carrier proteins, the greater the likelihood that the drug will be effectively absorbed, distributed, metabolized, and excreted by the body, thus leading to an increase in the overall efficacy of the drug. Given the static nature of the fluorescence quenching of BLG and the supposition that the BLG structure comprises separate and similar binding sites for LEV, the binding constant ($K_b$) and the number of binding sites (n) for the LEV-BLG interaction were quantified with the help of the following version of the Stern-Volmer equation (Eq. (2)) (Vahedi, Farhadian, Shareghi, Asgharzadeh, & Evini, 2022):

$$log\frac{F_0 - F}{F} = logK_b + nlog[Q] \quad (2)$$

where, $K_b$ shows the binding constant and n signifies the number of binding sites for LEV on each molecule of protein, respectively. The logarithmic linear plot of log[($F_0$-F)/F] against log[Q] (depicted in Supplementary Data, Fig. S6) was employed to determine the $K_b$ and n values, which are shown in Table 2. The order of the binding constants was found to be on the magnitude of $10^3$ for all temperatures examined, illustrating moderate binding affinity of LEV toward BLG when compared to protein–ligand complexes with higher binding constants (Rahman et al., 2019; Zhang, Wang, Yan, & Xiang, 2011). The value of the binding constant was significantly ($P < 0.01$) higher at 316 K temperature as compared with 296 K. In this particular context, the study by Patel et al. (Patel, Sepay, & Mahapatra, 2019) that delved into the interaction between tartrazine (an additive food dye) and BLG has revealed that the binding constants were on the order of $10^4$ L/mol. This observation indicates that BLG protein is capable of forging notably stronger chemical bonds with other molecules. According to Table 2, the value of n was nearly equal to 1, indicating a 1:1 complex formation between the BLG and LEV (presence of a binding site within the BLG structure for LEV). Also, the n values showed an increasing trend with the increase in temperature and there was a significant difference ($P < 0.05$) among all temperatures. In this regard, the investigation carried out by Ghalandari et al. (Ghalandari et al., 2015), reported that oxaliplatin, an anticancer drug, could bind to BLG and subsequently quench the fluorescence emission through a combination of static and dynamic quenching with a predominant contribution of static mode. The number of binding sites in this study for oxaliplatin on each molecule of BLG was reported to be around 1, which is consistent with the results of our study.

### 3.1.4. Thermodynamics of LEV-BLG binding

The stabilization of protein–ligand complexes is primarily attributed to several key intermolecular forces, which mainly include hydrogen bonds, van der Waals forces, electrostatic attractions and repulsions, and hydrophobic interactions (Habibian-Dehkordi et al., 2022). According to the literature, the primary binding interactions involved in complexation processes can be determined through the assessment of the signs and magnitudes of corresponding thermodynamic parameters. Consequently, in order to comprehend the dominant forces involved in the binding of LEV to BLG, the associated thermodynamic parameters were evaluated utilizing the following equations (Eq. 3–4):

$$lnK = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

In the above equation, K and R indicate the binding constant and the universal gas constant (8.314 J/mol.K), respectively. The values of ΔH and ΔS were assessed by using the slope and intercept of the log K versus 1/T plot depicted in Supplementary Data, Fig. S7. Table 2 shows the corresponding thermodynamic parameters for LEV-BLG interaction. Our findings indicate that ΔG is negative, thus showing that the

**Table 1**
The quenching parameter of the LEV-BLG interaction. Values are expressed as mean and standard deviation.

| T(K) | $K_{sv}$ ($\times 10^3$ L.mol$^{-1}$) | $K_q$ ($\times 10^{12}$ L.mol$^{-1}$.S$^{-1}$) | R2 |
|---|---|---|---|
| 296 | 2.501 ± 0.272 | 1.953 | 0.99 |
| 306 | 2.228 ± 0.229 | 1.740 | 0.97 |
| 316 | 2.054 ± 0.111 | 1.604 | 0.98 |

**Table 2**
Thermodynamic and binding values for the LEV-BLG interaction at pH 7.4. Values are expressed as a mean of the group (n = 3) ± SD.

| T (K) | $K_b$ ($\times 10^3$ mol$^{-1}$) | N | ΔG (kJ/mol) | ΔH (kJ/mol) | ΔS (J mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|---|---|
| 296 | 0.649 ± 0.130[a] | 0.86[a] | −15.907 ± 0.091 | | |
| 306 | 1.140 ± 0.1 | 0.93[b] | −17.901 ± 0.230 | 45.659 ± 12.489 | 208.121 ± 41.608 |
| 316 | 1.858 ± 0.524[c] | 0.99[c] | −19.710 ± 0.704 | | |

[a, b, c] indicate significant differences ($P < 0.05$) among the groups.

complexation process is spontaneous. Ross and Subramanian (Ross & Subramanian, 1981) have reported the relationship between the kind of interaction that plays a crucial role in protein–ligand complexation processes and thermodynamic characteristics. When both enthalpy change and entropy change are positive, then hydrophobic interactions are the primary driving force; if the enthalpy change is negative and the entropy change is positive, electrostatic forces serve as the main driving force; van der Waals forces and/or hydrogen bonding are the main when the changes in both enthalpy and entropy are negative. Our results showed positive ΔH and ΔS values, implying that the stability of the complex formed between BLG and LEV is predominantly attributed to hydrophobic interactions. In one study published in 2018, the binding interaction of isoxsuprine hydrochloride and levothyroxine to BLG from the perspective of comparison was investigated. The results of this study indicated that the interaction of the aforementioned drugs with the protein was a spontaneous process. Additionally, it was found that the formation and stabilization of the levothyroxine-BLG complex were primarily influenced by hydrogen bonds and van der Waals interactions, whereas hydrophobic interactions were the dominant factor in the formation of the complex between BLG and isoxsuprine hydrochloride (Shahraki et al., 2018). Such results collectively demonstrate that BLG is endowed with a remarkable capability for forming a wide array of interactions (including hydrogen bonds, van der Waals forces, and hydrophobic interactions), which enable it to bind with diverse molecules and ultimately transport them.

### 3.2. Uv–vis spectral measurements

UV–Vis spectrum scanning measurement as a basic and straightforward approach was conducted to verify the development of a complex between LEV and BLG (Lyu & Wang, 2021). The recorded UV–Vis spectrum for BLG in the presence of varying concentrations of LEV is illustrated in Supplementary Data, Fig. S8. As shown, the spectrum of BLG has two special absorption peaks near 210 and 280 nm. The stronger absorption band positioned at around 210 nm originates primarily from the peptide bond, while the weaker (at 280 nm) comes from the Trp, Tyr, and Phe $\pi \rightarrow \pi^*$ transition. Following the gradual addition of LEV to the BLG solution, an apparent decrease in absorption intensity of BLG at both 210 and 280 nm bands (hypochromic effect) was observed, which could be explained by the development of the LEV-BLG complexes through static quenching.

### 3.3. FT-IR spectroscopy evaluations

As a simple and powerful method, FT-IR spectroscopy is extensively exploited to provide information regarding the influence of small-molecule drugs on the secondary structure of proteins. According to available kinds of literature on this topic, the protein's spectra in the infrared region show a bunch of amide bands, reflecting different peptide chain vibrations. The vibrations associated with the amide I and II bands, in particular, are tightly tied to the protein's conformation, providing valuable insights regarding its secondary structural arrangements. The peak position of amide I is typically in the wavelength ranging from 1700 to 600 $cm^{-1}$ and includes contributions from the C═O stretching vibration of the amide group. The amide II band, on the other hand, corresponds to the stretching vibration of C—N as well as the deformation vibration of N—H and appears in the range between 1600 and 1500 $cm^{-1}$. As mentioned, a significant association exists between both bands and the secondary structure of proteins; although, amide I exhibits greater sensitivity to structural alterations than amide II. In this work, the IR spectra of BLG were examined to look at the structural changes that take place when it forms a complex with LEV (Fig. 2). Upon interaction with LEV the peak intensity of both amide bands reduced. These results demonstrate that LEV interacted with the polypeptide backbone of the BLG and caused some conformational alternations in its structure (Wang et al., 2020).

### 3.4. Circular dichroism studies of BLG

The application of Far-UV spectroscopies is acknowledged as a reliable and robust technique for examining alternations in the secondary structural composition of proteins resulting from binding with small molecular ligands. Therefore, in order to gain a detailed insight into the global structural changes of BLG, the CD spectra of BLG before and after the treatment with LEV were assayed and corresponding data were presented in Fig. 3 and Table 3. Our results showed that the structure of

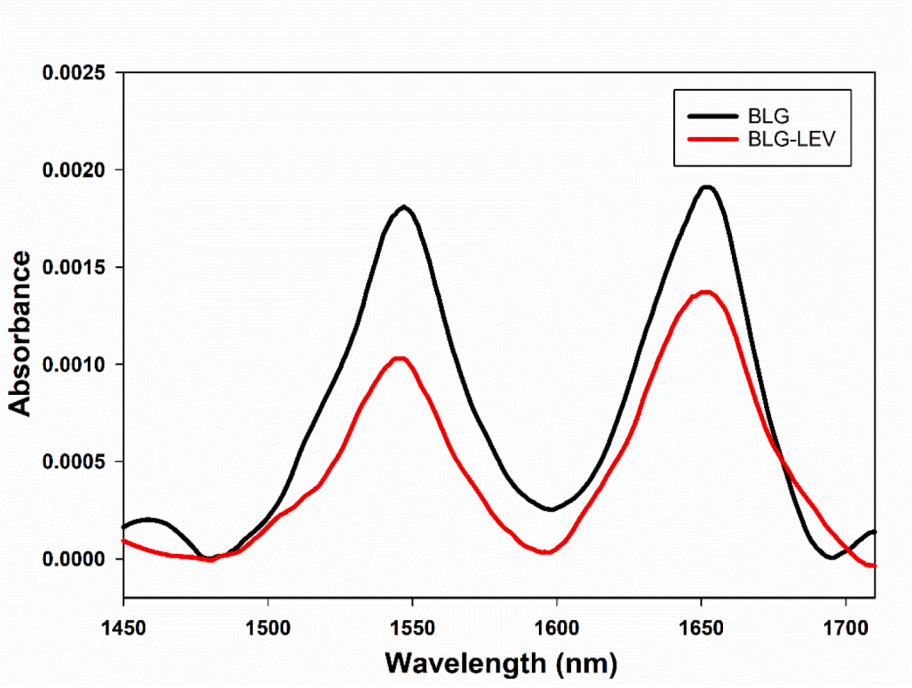


**Fig. 2.** FT-IR difference spectra for native BLG and LEV-BLG systems.

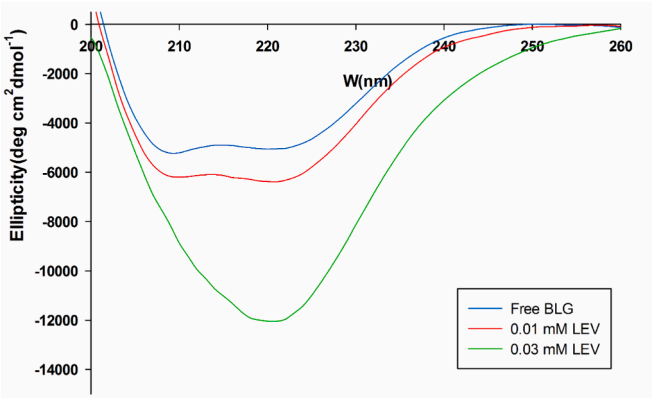


**Fig. 3.** Circular dichroism spectra for BLG without and in the presence of various LEV concentrations.

**Table 3**
The CD results for the interaction between BLG and LEV, calculated by CDNN software.

| [LEV] (mM) | alpha-helix% | beta-sheet% | beta-turn% | random coil% |
|---|---|---|---|---|
| 0.00 | 13.8 | 32.3 | 18.9 | 34.9 |
| 0.01 | 15.7 | 29.9 | 19.4 | 34.7 |
| 0.03 | 25.7 | 22 | 19.25 | 33.1 |

BLG is approximately made up of 32.3% beta-sheet, 34.9% random coils, 18.9% beta-turn, and 13.8% alpha-helix, which aligns with previous findings and studies (Mehraban et al., 2017). Upon binding with LEV, the alpha-helix content of BLG showed an increase. On the contrary, after complexation with LEV, the beta-sheet content of BLG decreased. Our results, which are in line with MD simulation findings, indicated that the development of a complex between BLG and LEV leads to a change in the secondary structure of BLG. In this vein, previous studies have shown that BLG shows different structural behavior when binding to different ligands. For instance, in the study that examined the binding interaction between kanamycin and ciprofloxacin with BLG, it was observed that while the binding of Kanamycin did not induce any significant structural alterations, the binding of ciprofloxacin led to a partial conformational change and unfolding of BLG through the decreased of beta-sheet and an increase in alpha-helix content of the protein (Mehraban et al., 2017). Also, the study carried out by Habibian-Dehkordi et al. (2022), which investigated the interaction of BLG with amoxicillin in a neutral environment, showed that the conformational structure of the BLG was altered upon the complexation with amoxicillin.

### 3.5. Thermal stabilization of BLG by LEV

According to previous studies, molecular interaction of proteins with small-molecule ligands to leads to an alternation in protein structure and eventually affects its thermal stability, which could be considered indirect evidence of ligand–protein complexation. Accordingly, through the calculation of transition temperature (Tm), we examined how LEV affects the thermal stability of BLG. Tm represents a special temperature that a protein experiences thermal denaturation and reaches an irreversible point (Zhang et al., 2021). Fig. 4 shows the Tm values of BLG protein before and after the interaction with increasing concentrations of LEV. Our findings show that a progressive rise of LEV concentration in the protein solution leads to an increase in the Tm profile of BLG. This observation suggests that the presence of LEV and its binding to the BLG substantially protects the protein from heat stress.

### 3.6. Docking insights into the LEV-BLG molecular complex

The LEV-BLG interaction was subjected to further examination through the utilization of molecular docking technology. Computational docking is beneficial in identifying the most favorable binding location of ligands within the structure of biological macromolecules and detecting the residues involved in the interaction. The LEV's preferred position in the BLG structure is depicted in Supplementary Data, Fig. S9. This figure provides a conspicuous demonstration of LEV's successful incorporation into the BLG structure. From Fig. S10 in Supplementary Data, it could be seen that LEV is surrounded by several amino acid residues, including Trp19, Tyr20, Tyr42, Glu157, Gln59, Glu158, Glu44, Gln159, Leu156, His161, Ser21, Val43 in LEV-BLG complex. The present

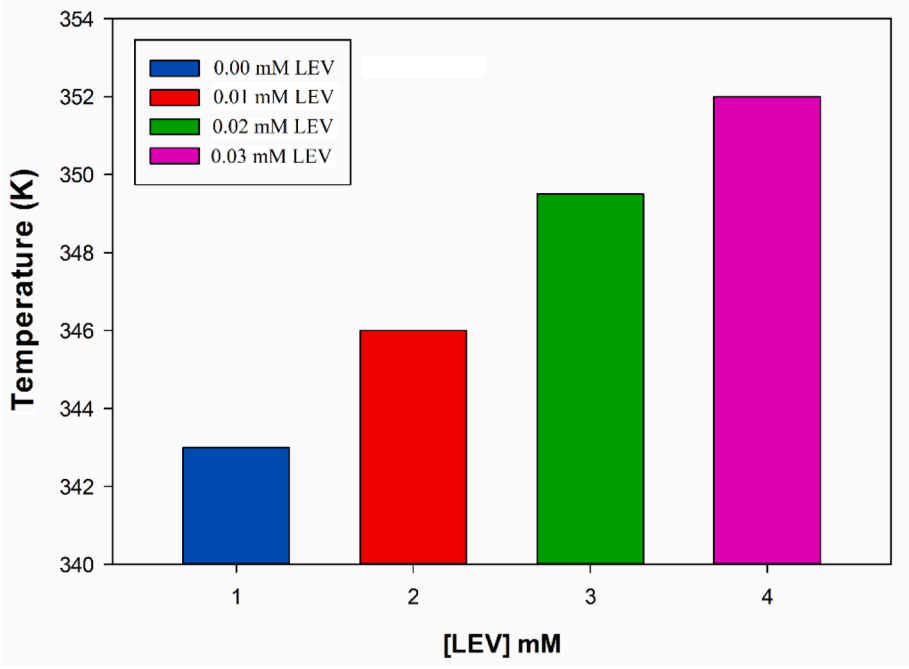


**Fig. 4.** The Tm characteristics of BLG before and after the interaction with varying LEV concentrations.

findings are in consonance with the outcomes of other investigations that have employed docking studies to examine the BLG's interactions with diverse drugs. For instance, during the interaction between levothyroxine and BLG Val43, Gln59, His161, Try20, Tyr42, Ser21, Glu158, Leu156, and Glu157 residues were placed close to the ligand (Shahraki et al., 2018). Also, investigation of the interaction of norfloxacin with BLG showed the involvement of Trp19 in the process of complexation between BLG and norfloxacin (Paul et al., 2014). A detailed inspection of the binding site revealed that the binding location of the LEV was close to the Trp 19 residue (dominant fluorophore of BLG), thus providing structural evidence for the efficient quenching of BLG fluorescence in the presence of LEV (see section 3.1.1). The calculated binding free energy as determined by the docking process was −21.08 kJ/mol (Supplementary Data, Table S1). Remarkably, this value falls within a comparable range to the ΔG that was obtained through the employment of fluorescence investigations. The dissimilarity observed between the values of ΔG as calculated by thermodynamic and docking studies can be attributed to the fact that the underlying principles and methodologies employed by the two approaches are fundamentally distinct; because the impacts of unbounded molecules like water and buffers are all ignored in docking investigations (Shaghaghi, Rashtbari, Vejdani, Dehghan, Jouyban, & Yekta, 2020).

### 3.7. MD simulation results

As a robust and meticulous theoretical approach, MD simulation has the potential to offer atomistic-level comprehension into the dynamic behavior of proteins when interacting with different molecules (Das et al., 2020). Therefore, the application of this method was employed to generate a visual representation, facilitating the understanding of the structural attributes, stability, conformational compactness, and flexibility of BLG, prior to and during the presence of LEV. To achieve these goals, in this study, some dynamic structural properties, including root mean square deviations (RMSD), root mean square fluctuation (RMSF), and radius of gyration (Rg) were monitored and the related findings were reported as mean and standard deviation. The outcomes of MD trajectories are thoroughly discussed in the following.

Initially, the RMSD profile of BLG was acquired and examined for both unbound and LEV-bound states to ascertain the stability of BLG and establish the validity of the MD simulation. As we know, the smaller values for RMSD offer smaller fluctuations of structure and consequently more stability of the studied system. In Fig. S11 of Supplementary Data, the RMSD value of the LEV-BLG complex is depicted over the course of 100 ns simulations, relative to the native BLG. The plot exhibits the fluctuation in RMSD values over time for the two systems, enabling a comparative analysis of the dynamic behavior of the LEV-BLG system in comparison to its unbound counterpart. After the simulation run, the temporal evolution of the RMSD values of both systems revealed an initial increase, followed by the attainment of an equilibrium configuration after approximately 30 ns of simulation. Based on this observation, the final 70 ns of the time-dependent MD trajectories were selected for further analysis, given that they capture the dynamic behavior of the systems when they reach a stable state. The current findings are in accordance with the results of other investigations that have utilized MD simulation to investigate the molecular interactions between BLG and diverse ligands. Within this context, a previously conducted investigation offered valuable insights regarding the interaction of cyclosporine A with BLG through MD simulations. The study determined that for both the unbound BLG protein and the BLG-cyclosporine A complex, the RMSD values reached equilibrium and oscillated around the average value after about 20 ns simulation time (Mohseni-Shahri et al., 2017). Table 4 provides the RMSD profile of BLG prior to and during the interaction with LEV. The data revealed that over the last 70 ns of the simulation period, the average value of the RMSD for the LEV-BLG system was lower than that of the native BLG. This finding indicates that the binding of LEV has led to an increase in the stability of the BLG protein.

**Table 4**
The mean values and standard deviation of RMSD, RMSF, and Rg for BLG and BLG-LEV systems based on the data of the last 70 ns (equilibrium state).

| System | RMSD | RMSF | Rg |
|---|---|---|---|
| BLG | 0.282 ± 0.025 | 0.083 ± 0.034 | 1.404 ± 0.006 |
| LEV-BLG | 0.263 ± 0.015 | 0.105 ± 0.059 | 1.398 ± 0.005 |

The subsequent analysis involved the investigation of the RMSF profiles for both the BLG and LEV-BLG systems, with the aim of tracking the mobility of individual amino acid residues in relation to their average positions, as well as assessing the overall flexibility of the protein structures under study (Habibian-Dehkordi et al., 2022). The RMSF value is the indicator of the system's flexibility; the larger value of RMSF reflects higher structural flexibility and vice versa. In Fig. S12 from Supplementary Data, the RMSF values are depicted as a function of residue number for both the native and complex forms of BLG. Using the RMSF values for unliganded BLG as a baseline, it was observed that the presence of LEV led to an increase in fluctuations across most regions. Upon a detailed analysis of the RMSF plot, it was found that the LEV-BLG complex exhibited heightened fluctuation levels in specific residue ranges, namely 1–20, 39–61, 65–74, and 81–85. Our findings imply that a large number of BLG amino acid residues are correlated with heightened fluctuations, which may result in an alteration of secondary conformation during complexation.

To investigate changes in protein conformation, the time-dependent evolution of the Rg was assessed for both systems. The Rg parameter is an important metric associated with protein folding, compactness, and tertiary structural volume, and its value is indicative of the degree of structural expansion or compactness. Specifically, a decrease in the Rg parameter suggests compactness in structural dimensions, whereas an increase signifies a greater level of system expansion (Gupta, Bhat, Biswal, & Rana, 2020). Fig. S13 in Supplementary Data shows the plot of Rg for native and LEV-bound BLG during the simulation period. The findings of MD simulation indicated that the initial Rg value for BLG was observed to be approximately 1.4., which is fully supported by prior investigations (Sahihi & Ghayeb, 2014). Based on the results shown in Table 4, it is evident that the overall value of Rg for the native state of BLG in the range of 30–100 ns (i.e., the equilibrium state) was somewhat higher as compared to the ligand-bound BLG. This finding implies that the BLG protein experienced a degree of compaction as well as conformational changes upon conjugation with LEV. Based on these results, which demonstrate a decrease in RMSD and Rg values, aligning with the findings from CD analysis (indicating an increase in alpha-helix and beta-turn structures), it was concluded that the inclusion of LEV led to an augmentation in the stability of the BLG protein.

## 4. Conclusion

The present investigation represented a rigorous and sophisticated analysis of the binding properties exhibited by the LEV-BLG complex. The study employed a diverse array of spectroscopic methods, which were complemented by state-of-the-art computational modeling approaches. The outcomes of our investigation revealed that the bonding of LEV with BLG leads to a highly efficient suppression of fluorescence emission via a static quenching mechanism, which arises due to the establishment of a ground-state complex. Analysis of the binding characteristics represented a 1:1 complex formation between the BLG and LEV. The thermodynamic parameters obtained from fluorescence spectroscopy confirmed the spontaneity of the interaction. In this regard, a meticulous examination of the ΔH and ΔS parameters revealed that hydrophobic interactions are the primary forces that significantly contribute to stabilizing the LEV-BLG complex. Further, BLG 's thermal stability was strengthened as a result of its binding with LEV. The most favorable binding location of the LEV molecule on the BLG structure was

predicted by molecular docking simulations, which was in agreement with experimental data obtained from spectroscopic investigations. Also, MD simulation demonstrated the capability of LEV to form a stable complex with BLG. It is widely acknowledged that the presence of veterinary drugs and their corresponding metabolites in milk and dairy products may pose a considerable hazard to human health through direct exposure. In light of this, our investigation exhibited the potential for designing and developing highly selective and sensitive BLG-based biosensors in the identification of anthelmintic compounds in environmental and food samples. Taking into consideration the unique binding and transport capabilities of BLG towards drug residues, it may be feasible to predict the potential environmental fate of veterinary drugs and their metabolites. This predictive capability can play a vital role in guiding efforts aimed at reducing the negative impact of such substances on ecosystems and public health. In addition, gaining insights into the binding properties of drugs and their corresponding metabolites to carrier proteins like BLG could aid pharmaceutical companies in designing novel drug formulations that are efficiently absorbed and distributed within the body, while concurrently minimizing the possibility of drug transfer into milk. Also, by characterizing drug-carrier protein interactions, it is possible to determine the optimal dose of a drug required to achieve therapeutic levels, which can enhance the efficacy of the drug while simultaneously mitigating the likelihood of any adverse side effects. By considering our findings, regulatory agencies are expected to take into account the ability of BLG to bind and transport a variety of drugs when establishing and validating parameters such as maximum residue limit (MRL) and withdrawal periods (the time until a safe level of drug in milk is achieved). This might help to ensure that such residues are not present at concentrations posing a risk to human health. In regard to our study that showed the ability of BLG to bind with LEV, we recommend other researchers conduct more investigations regarding the interaction of other drugs with BLG protein while taking into account the limitations of previous studies to gain a comprehensive understanding of the binding properties of the BLG with various ligands. Also, it is suggested to study the impact of environmental factors such as temperature and pH on BLG-drug interactions to better understand their behavior in different settings. Further, it is of great interest to investigate the potential impact of BLG-drug interactions on the allergenicity of BLG to individuals with milk allergies, given that BLG is a known allergen in milk and can elicit adverse reactions in sensitive individuals. In conclusion, our data shed light on the underlying mechanism of LEV-BLG binding interaction, thus providing a useful and informative reference on the ability of carrier proteins to bind and carry hazardous agents.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137073.

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