Studies on the binding of isoalantolactone to human serum albumin by molecular spectroscopy and modeling

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The interactions between isoalantolactone (IAL) and human serum albumin (HSA) under simulative physiological conditions have been investigated by fluorescence, absorption and circular dichroism spectra. Fluorescence data reveal that the fluorescence quenching of HSA by IAL is the result of formation of the IAL–HSA complex. The thermodynamic parameters, enthalpy change and entropy change for the reaction are calculated to be -34.317 kJ mol\(^{-1}\) and -7.522 J mol\(^{-1}\) K\(^{-1}\), respectively, indicating that the hydrogen bonds and hydrophobic interactions play a dominant role in the binding of IAL to HSA. The conformational investigation shows that the presence of IAL decreases the \(\alpha\)-helical content of HSA and induces the remarkable unfolding of the polypeptides of protein. Furthermore, displacement experiments using warfarin and ibuprofen indicate that IAL could bind to site I of HSA, which is in agreement with the results obtained from molecular modeling.

Keywords: Bioinorganic chemistry, Isoalantolactone, Human serum albumin, Fluorescence spectroscopy, Molecular modelling, Binding sites

In traditional Chinese medicine, the dried roots of *Inula helenium* L. and *Inula racemosa* Hook f. are used commonly as folk medicine under the name of “Tumuxiang”. Several experimental studies have demonstrated that the major active component in *I. helenium* L. and *I. racemosa* Hook f. is isoalantolactone (IAL)\(^{1,2}\). Pharmacological investigations have shown IAL possesses the effects of anticancer\(^{3,6}\), significant anti-inflammatory and hepatoprotective activity similar to that of silymarin\(^7\), antidematophytic and antifungal activity\(^8,9\). These multiple pharmacological activities of IAL make it worth carrying out a further comprehensive study on the pharmacokinetic properties.

Human serum albumin (HSA), as a principal extracellular protein constituent of blood plasma, plays a dominant role in the transport and disposition of a variety of endogenous and exogenous substances such as drugs. HSA is a globular protein composed of 585 amino acid residues in three homologous \(\alpha\)-helical domains (I, II, III). Each domain contains 10 helices and is divided into six anti-parallel helices and four sub-domains (A and B)\(^{10}\). Two hydrophobic pockets are present in sub-domains II A and III A, which accommodate the bioactive molecules to bind reversibly\(^11\). The multiple binding sites underlie the exceptional ability of HSA to interact with many organic and inorganic molecules and make this protein an important regulator of intercellular fluxes, as well as the pharmacokinetic behavior of many drugs\(^12,13\). Therefore, detailed investigations of drug–protein interaction assumes significance for a thorough understanding of the pharmacokinetic behavior of a drug and for the design of analogues with effective pharmacological properties\(^14\).

It has recently been proved that serum albumin plays a decisive role in the transport and disposition of many lactone drugs like ochratoxin A and its derivatives\(^15\), camptothecin\(^16\), parthenolide\(^17\), digitoxin and acetyldigitoxin\(^18\), azithromycin\(^19\) and 9-aminocamptothecin glucuronide\(^20\). However, attempts have not been made so far to investigate the interactions between IAL and HSA. Therefore, in this work, the interaction between IAL and HSA has been studied under physiological conditions by fluorescence, circular dichroism spectra and
molecular modeling. Attempts have been made to investigate the interaction mechanism between the two, with respect to the quenching mechanism, specific binding site, type of interaction force, and the effect of IAL on the micro-environmental and conformational changes of HSA molecules.

Materials and Methods
HSA was obtained from Sigma Chemical Company, St. Louis, USA. The working solution of HSA (1.5 μM) was prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. HSA solution was prepared based on its molecular weight of 66,000.

Isoalantolactone (IAL) was isolated by the authors from the dried roots of Inula helenium and structure was characterized by chemical and spectroscopic methods (1H NMR, 13C NMR and MS) and compared with the data reported in literature. Analysis showed that its purity was above 99 % (HPLC). Ultra high purity water (UHP) was prepared with a Millipore-Q SAS 67120 system (Molsheim France). The working solution of IAL (1.0 × 10⁻⁴ M) was prepared by dissolving appropriate amount of IAL in 10 mL anhydrous ethanol. Warfarin and ibuprofen (obtained from China National Pharmaceutical Group Corporation) were initially dissolved in minimum amount of anhydrous ethanol and then diluted with distilled water. All other materials were of analytical reagent grade and doubly distilled water was used throughout. The experiments were carried out at room temperature, unless stated otherwise.

Spectral measurements
The fluorescence measurements were made on an F–7000 fluorescence spectrometer (Hitachi, Japan) equipped with a 150 W Xenon lamp, and a slit width of 5 nm. A 2.0 mL solution containing appropriate concentration of HSA was titrated manually by successive additions of IAL working solution (concentration of IAL ranging from 0.99 – 9.09 μM with the total accumulated volume of IAL less than 30 μL) with trace syringes, and the fluorescence intensity was measured (excitation at 280 nm and emission at 340 nm) at varying temperatures. The temperature of the sample was maintained by circulating water throughout the experiment.

Synchronous fluorescence spectra of HSA with various concentrations of IAL were obtained from 200 – 400 nm (Δλ = 15 nm and Δλ = 60 nm) with the excitation and emission slit widths of 5/5 nm, respectively.

The binding sites between IAL and HSA in the presence of two site markers (warfarin and ibuprofen) were measured using the fluorescence titration methods. The concentrations of HSA and IAL were stabilized at 1.5 and 4.7 μM. Warfarin/Ibuprofen was then gradually added to the IAL–HSA mixtures. An excitation wavelength of 280 nm was selected and the fluorescence spectra were recorded in the range of 290–450 nm.

The fluorescence spectra of IAL–HSA were recorded in presence and absence of various common ions, viz., Ca²⁺, Mg²⁺, Cu²⁺, K⁺, Ba²⁺, Al³⁺, Zn²⁺ and Fe³⁺, in the range of 290 – 400 nm upon excitation at 280 nm. The concentrations of HSA and common ion were fixed at 1.5 and 2.5 μM, respectively. The operations were carried out at room temperature.

Absorption spectra of the IAL–HSA solutions (concentration of IAL ranges from 0.99 – 9.09 μM with the total accumulated volume of IAL was less than 30 μL) were recorded in the range of 200 – 300 nm on a UV–2501PC spectrophotometer (Shimadzu, Japan).

Circular dichroism (CD) measurements were made on a J–810 spectropolarimeter (Jasco, Tokyo, Japan) with a quartz cell having path length of 0.1 cm. CD spectra of HSA (1.5 μM) in absence and presence of IAL (the molar ratio of HSA to IAL was varied as 1:2, 1:4, 1:6, 1:8 and 1:10, respectively) were made in the range of 200–260 nm, and the results were expressed in terms of mean residue ellipticity, MRE. Buffer solution as reference was subtracted from the sample spectra.

Molecular modeling
The crystal structure of HSA with a resolution of 2.50 Å was retrieved from the Protein Data Bank (PDB code:1H9Z). The molecular docking program GOLD4.0 was used to undertake the molecular docking studies. The docking scheme is summarized

![Diagram](image-url)
as follows. Firstly, the pre-process to HSA was carried out by using the programs of Discovery Studio 2.55 (Accelrys, Inc., USA), which includes removing water, adding hydrogen atoms and assigning CHARMM like force field. IAL was built and geometry optimized using Discovery Studio 2.55. Then the binding site was defined as a sphere containing the residues that stay within 10 Å from the ligand in this crystal structure and slightly adjusted manually. Finally, for the optional selection of the docking parameters that are best for individual HSA docking study, the ligand was taken out of the crystal structure and docked back again, and then the docking parameters were adjusted until the docked structures (both the poses and positions of heavy atoms) were as close as possible to their original in the binding site. The finally optimized docking parameters were as follows: number of dockings was set to 10 without using early termination option; the detect cavity was turn on; the optimized positions of polar protein hydrogen atoms were saved; the genetic algorithm (GA) parameter was set to “2 times speedup”; the top 50 scoring poses were saved for each compound and the GoldScore was selected as the score function. The other parameters were kept at their default settings.

Results and Discussion
Fluorescence quenching mechanism

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions, such as excited-state reactions, energy transfer, ground-state complex formation and collisional quenching. The different mechanisms of quenching are usually classified as dynamic quenching, static quenching or simultaneous static and dynamic quenching. Dynamic quenching depends upon diffusion. Since higher temperature results in larger diffusion coefficient, the bimolecular quenching constant is expected increase with increasing temperature. On the other hand, high temperature is likely to result in decreased stability of the complex, and thus lower the value of the static quenching constant.

Figure 1 shows the fluorescence spectra of HSA in the presence of varying concentrations of IAL at 298 K. It is noted that the fluorescence intensity of HSA decreases regularly with the increase of IAL concentration, along with a slight blue shift from 340 to 336 nm, indicating that IAL can interact with HSA and quench its intrinsic fluorescence. The ultimate percentages of ethanol in all the solutions were below 2 % (v/v). Since IAL was prepared in ethanol, the quenching effect of ethanol on HSA conformation was evaluated. Results showed that the effect of ethanol on the IAL interaction with HSA was negligible with the amount used in our experiments. For dynamic quenching, the decrease in intensity is usually described by the well-known Stern–Volmer equation, and corresponding data are listed in Table 1. The results show that the quenching constant \( K_{SV} \) is inversely correlated with temperature and the values of \( K_q \) are much larger than the maximum scattering collision quenching constant \( (2 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}) \), suggesting that the quenching mechanism is mainly due to the predominance of complex formation, while dynamic collision could be negligible in the concentration studied. According to Sharma et al., dynamic quenching affects the excited states of molecules rather than the absorption spectra of fluorescent substance. In order to confirm the

<table>
<thead>
<tr>
<th>( T ) (K)</th>
<th>( K_{SV} ) (10^4 M(^{-1}))</th>
<th>( K_q ) (10^2 M(^{-1}) S(^{-1}))</th>
<th>( K ) (10^5 M(^{-1}))</th>
<th>( n )</th>
<th>( \Delta H ) (kJ M(^{-1}))</th>
<th>( \Delta S ) (J M(^{-1}) K(^{-1}))</th>
<th>( \Delta G ) (kJ M(^{-1}))</th>
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<td>6.414</td>
<td>6.414</td>
<td>4.180</td>
<td>1.177</td>
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<td></td>
<td></td>
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<tr>
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<td>6.064</td>
<td>3.515</td>
<td>1.166</td>
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<td>-7.522</td>
<td>-32.075</td>
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<td>5.758</td>
<td>5.758</td>
<td>2.908</td>
<td>1.154</td>
<td>-34.317</td>
<td>-7.522</td>
<td>-32.075</td>
</tr>
<tr>
<td>310</td>
<td>5.463</td>
<td>5.463</td>
<td>2.454</td>
<td>1.144</td>
<td>-34.317</td>
<td>-7.522</td>
<td>-32.075</td>
</tr>
</tbody>
</table>
probable quenching mechanism, the absorption spectra of HSA were also recorded in the presence of different concentrations of IAL at room temperature. As seen from Fig. 2, the absorbance of HSA increased with the addition of IAL along with a blue shift from 278.8 to 273.8 nm. This result confirms that the quenching is mainly a static quenching process and a IAL–HSA complex with a certain new structure is formed.

**Identification of the binding site of IAL on HSA**

When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding parameters can be determined by the following equation,

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K + n \log [Q] \quad \ldots(1)
\]

where \(K\) is the binding constant, \(n\) is the number of binding sites, and \(F_0, F\) and \([Q]\) have the usual meanings, and the values of \(K\) and \(n\) calculated from the modified Stern–Volmer plots for the IAL–HSA system are presented in Table 1. The decreasing trend of \(K\) with increasing temperature is in accordance with that of \(K_{SV}\) stated above, resulting in a reduction of the stability of the IAL–HSA complex. The values of \(n\) are approximately equal to 1, indicating that there is one class of binding sites of IAL on HSA. The number of binding sites in the protein should be equal to the number of bound molecules per protein. Considering that only one drug molecule binds to each site, the results suggest that one molecule of the drug binds to HSA.

Crystal structure of HSA shows that HSA comprises three homologous domains (I–III): I (residues 1–195), II (196–383), III (384–585), each domain is a product of sub-domains that possess common structural motifs. There is a large hydrophobic cavity in sub–domain IIA that many drugs can bind\(^{27}\). According to He et al.\(^{28,29}\), warfarin has been demonstrated to bind to the sub–domain II A while ibuprofen is considered as a sub–domain III A binder.

The interaction of site markers with the IAL–HSA complex (IAL/HSA molar ratio = 3) was investigated at the excitation wavelength 280 nm. As shown in Fig. 3A, with addition of warfarin into HSA–IAL solution, the maximum emission wavelength of HSA–IAL has a remarkable red shift and the fluorescence intensity of the HSA–IAL, in equimolar concentrations, decreases gradually, indicating that the binding of IAL to HSA is affected by addition of warfarin. In contrast, in the presence of ibuprofen, the
fluorescence intensity of the IAL–HSA complex also decreases gradually with a slight blue shift (Fig. 3B). However, no obvious effect is observed, indicating that while ibuprofen does not prevent the binding of IAL in its usual binding location, there is weak interaction force between the residues of sub–domain III and IAL. In order to confirm the binding site, the complementary applications of the molecular docking program GOLD 4.0 were employed to understand the interaction between IAL and HSA. As shown in Fig. 4, IAL binds within the sub–domain IIA of the protein (the warfarin binding pocket). IAL is located within the binding pocket and the B and C rings are practically coplanar. The interaction between IAL and HSA is dominated by the hydrogen bonds between the ester bond of IAL and the residues Arg222 and Arg218 of HSA. However, there is also hydrophobic interaction between IAL and the residues Pro447, Cys448 and Lys444 of HSA, which may be responsible for change in the fluorescence intensity and maximum emission wavelength of IAL–HSA complex on addition of ibuprofen. Furthermore, there are also π → π* interactions between the double bond in C ring and the benzene ring in Phe206 of HSA, which may result in an increase of the absorption peak around 280 nm of HSA. The result of molecular modeling is in accordance with that of the experiments of site markers. As compared to non–covalent binding of serum albumin with small molecules of similar structure, the value of the binding constant for IAL was relatively larger. It can be seen from Fig. 4 there are more atoms in the plane of the heterocyclic ring and thus less steric hindrance would be involved in the binding process.

As a result, the binding of IAL to HSA became relatively easier. Moreover, ester bond in C ring may enhance the binding of the molecule significantly.

**Type of interaction force between IAL and HSA**

The acting forces between a small organic molecular and biomolecule may include hydrophobic force, electrostatic interactions, van der Waals interactions, hydrogen bonds. It is assumed that the interaction enthalpy change ($\Delta H$) does not vary significantly over the temperature range studied; the thermodynamic parameters can be estimated from Eq. (2).

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$ …(2)

The negative values of free energy ($\Delta G$), seen in Table 1, support the assertion that the binding process is spontaneous. The negative enthalpy ($\Delta H$) and entropy ($\Delta S$) values of the interaction of IAL and HSA indicate that since the binding is mainly enthalpy–driven and the entropy is unfavorable, hydrophobic interactions and hydrogen bonds play a major role in the reaction, which is accordance with the result of the molecular modeling.

**Energy transfer between IAL and HSA**

Energy transfer phenomena have wide applications in energy conversion process. According to Förster’s fluorescence resonance energy transfer theory, the energy transfer will occur under the following conditions: (a) the donor can produce fluorescence that has sufficiently long lifetime; (b) the fluorescence emission spectra of the donor and UV–vis absorption spectra of the acceptor have more overlap; (c) the distance between the donor and the acceptor is approachable and less than 7 nm. The efficiency of energy transfer ($E$) between the donor and the acceptor can be calculated by Eq. (3),

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6}$$ …(3)

where $r$ is the distance between the donor (HSA) and the acceptor (IAL), $R_0$ is the critical distance when the transfer efficiency is 50%, which can be obtained by Eq. (4),

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-1} \varphi J$$ …(4)

where $k^2$ is the the spatial orientation factor of the dipole, $N$ is the refracted index of medium, $\varphi$ is the
fluorescence quantum yield of the donor and \( J \) is the overlap integral of the fluorescence emission spectra of the donor and the absorption spectra of the acceptor, which can be calculated by Eq. (5),

\[
J = \frac{\sum \int F(\lambda)\varepsilon(\lambda)d\lambda}{\sum F(\lambda)\lambda}
\] (5)

where \( F(\lambda) \) is the fluorescence intensity of the donor at wavelength \( \lambda \), \( \varepsilon(\lambda) \) is molar absorptivity of the acceptor at wavelength \( \lambda \). In the present case, \( k^2 = 2/3 \), \( N = 1.36 \), \( \varphi = 0.15 \). The spectral overlap between HSA and IAL is shown in Fig. 5, and the calculated parameters are as follows, \( J = 1.265 \times 10^{-12} \) cm \(^3\) L mol \(^{-1}\), \( R_0 = 7.75 \) nm and \( r = 3.96 \) nm. Obviously, the distance between HSA and IAL is less than 7 nm, indicating that there is high possibility of the energy transfer from HSA to IAL. This is in accordance with conditions of Förster’s theory of non-radioactive energy transfer and again indicates a static quenching between IAL and HSA.

**Effect of metal ions on binding of IAL to HSA**

Metal ions, especially bivalent metals, are vital and play an essentially structural role in many proteins based in coordinate bonds. The presence of metal ions in plasma may affect interaction of drugs with HSA\(^{40}\). Effects of common metal ions (e.g. Mg\(^{2+}\) and Fe\(^{3+}\)) on binding constants of IAL–HSA system were investigated. The values of binding constant \( K \) in the presence of metal ions are listed in Table 2. It can be seen that while most of ions did not affect, or only interfered slightly, the binding constants, Fe\(^{3+}\), Cu\(^{2+}\), Mg\(^{2+}\) and Pb\(^{2+}\) produced obvious interference.

The effect of metal cations on the fluorescence spectra of IAL in ethanol was investigated (Fig. 6). It can be seen that the fluorescence intensity of IAL excited at 280 nm was decreased on addition of Fe\(^{3+}\), whereas the intensity was increased with the addition of Cu\(^{2+}\), Mg\(^{2+}\) and Pb\(^{2+}\) along with a slight red shift, suggesting that the metal ion–IAL or metal ion–HSA complexes via metal ion bridge was likely to be formed. The presence of Fe\(^{3+}\) may prolong storage period of IAL in blood plasma and enhance its maximum effects.

**Conformation investigation**

It has been ascertained that it is the binding of IAL to HSA which caused the fluorescence quenching of HSA. However, it is still not clear whether the binding affects the conformation and/or micro-environment of HSA. To further investigate this, the methods of circular dichroism (CD) and synchronous fluorescence spectroscopy were utilized.

**Table 2—Effects of metal ions on binding constants (\( K \)) of the IAL–HSA system. ([metal ions] = 1.5 \( \mu M \))**

<table>
<thead>
<tr>
<th>System</th>
<th>( K (10^5 M^{-1}) )</th>
<th>( R^2 )</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA+ IAL</td>
<td>4.847</td>
<td>0.9958</td>
<td>0.002</td>
</tr>
<tr>
<td>HSA+ IAL +Cu(^{2+})</td>
<td>4.242</td>
<td>0.9968</td>
<td>0.004</td>
</tr>
<tr>
<td>HSA+ IAL +K(^+)</td>
<td>4.642</td>
<td>0.9884</td>
<td>0.005</td>
</tr>
<tr>
<td>HSA+ IAL +Mg(^{2+})</td>
<td>2.466</td>
<td>0.9584</td>
<td>0.006</td>
</tr>
<tr>
<td>HSA+ IAL +Ag(^+)</td>
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<td>0.9979</td>
<td>0.001</td>
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<tr>
<td>HSA+ IAL +Al(^{3+})</td>
<td>4.303</td>
<td>0.9540</td>
<td>0.003</td>
</tr>
<tr>
<td>HSA+ IAL +Pb(^{2+})</td>
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<td>0.9885</td>
<td>0.023</td>
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<tr>
<td>HSA+ IAL +Cu(^{2+})</td>
<td>2.598</td>
<td>0.9556</td>
<td>0.015</td>
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<tr>
<td>HSA+ IAL +Zn(^{2+})</td>
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<td>0.9766</td>
<td>0.005</td>
</tr>
<tr>
<td>HSA+ IAL +Fe(^{3+})</td>
<td>12.970</td>
<td>0.9879</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* \( R \) is the correlation coefficient

![Fig. 5—Spectral overlap of the fluorescence spectra of HSA (F) with absorption spectra of IAL (A). ([HSA] = 1.5 \( \mu M \); [IAL] = 9.09 \( \mu M \); \( \lambda_{ex} = 280 \) nm).](image1)

![Fig. 6—Effects of common ions on the fluorescence spectra of IAL. ([IAL] = 1.01 \( \mu M \); \( \lambda_{ex} = 280 \) nm).](image2)
CD experiments of HSA in the absence and presence of IAL at room temperature were carried out. The CD spectra of HSA exhibited two negative bands in the UV region at 208 and 222 nm, characteristic of an \( \alpha \)-helical structure of protein\(^{41}\) (Fig. 7 curve a). The binding of IAL to HSA caused a decrease in band intensity with significant shift of the peaks, indicating a decrease of the \( \alpha \)-helical content in HSA. From the above results, it is apparent that the effect of IAL on HSA causes a conformational change of HSA, with the loss of \( \alpha \)-helical stability. Also, the loss of the \( \alpha \)-helix indicates IAL binds with the amino acid residues of the main polypeptide chain of protein and destroys their hydrogen bonding networks\(^{42}\).

When the wavelength interval (\( \Delta \lambda \)) between excitation wavelength and emission wavelength is 15 and 60 nm, the synchronous fluorescence spectra exhibit the characteristics of tyrosine and the tryptophan residues of HSA\(^{43}\). As shown in Fig. 8B, the maximum emission wavelength of tryptophan residues has a slight blue shift, while no obvious wavelength shift of tyrosine residues is observed in Fig. 8A. Results suggest that tryptophan residues are in a more hydrophobic environment, while the microenvironment around the tyrosine residues has no remarkable change during the binding process\(^{44}\).

The above phenomena combining with the results of the synchronous fluorescence and circular dichroism spectra reveal that the interactions between IAL and HSA induce a remarkable unfolding of the polypeptides of HSA, which results in a conformational change of HSA, leading to increased exposure of some unexposed hydrophobic regions.

**Conclusions**

In this paper, the interaction between IAL and HSA was studied by molecular spectra under simulative physiological conditions. The fluorescence experiment results showed that the fluorescence quenching of HSA induced by IAL was static quenching. The enthalpy change and entropy change for the reaction were calculated to be -34.317 kJ mol\(^{-1}\) and -7.522 J mol\(^{-1}\) K\(^{-1}\) respectively, indicating that the hydrogen bonds and hydrophobic interactions were the dominant intermolecular forces in stabilizing the complex. Site marker competitive experiment suggested that IAL binds to the sub-domain IIA of HSA, which was in accordance with the results of the molecular modeling. The distance, \( r \), between HSA and IAL was obtained to be 3.96 nm, indicating high possibility of energy transfer from HSA to IAL. CD and synchronous fluorescence spectra revealed that the conformation of HSA changed in the binding of IAL to HSA and the tryptophan residue in
HSA was placed in a more hydrophobic environment. This study provides important insights into the interactions of the physiologically important HSA with drugs. Besides, useful information can be also obtained about the effect of environment on the structural features of HSA, which may be correlated to its physiologically activity.

Acknowledgements

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References