Biophysical study on the interaction of etomidate and the carrier protein in vitro

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Abstract

Etomidate is a unique drug used for induction of general anesthesia and sedation, and is usually used through intravenous injection clinically. Before targeting to the receptor, etomidate binds proteins in blood when it comes into veins. Thus to study the interaction of etomidate and serum albumin would be of great toxicological and pharmacological importance. In this study, the interaction between etomidate and human serum albumin (HSA) was studied using fluorescence spectroscopy, UV–vis absorption spectroscopy, Fourier transform infrared spectroscopy (FT-IR), circular dichroism (CD) spectroscopy, site maker displacement and molecular modeling methods. Investigations of the binding constant (K = 3.35 × 10^5 M⁻¹, 295 K), the number of binding sites (n = 1.16), thermodynamic parameters (ΔG = 3.13 × 10⁻⁴ J mol⁻¹, ΔS = 364 J mol⁻¹ K⁻¹ and ΔH = -6.85 × 10⁻⁵ J mol⁻¹) for the reaction and changes to the binding sites and conformation in HSA in response to etomidate were presented. Results show that etomidate can bind HSA tightly through electrostatic forces, and the protein skeleton conformation and secondary structure changes thereby. This is the first spectroscopic report for etomidate–HSA interactions which illustrates the complex nature of this subject.

Keywords

Binding site, circular dichroism, etomidate, fluorescence, FT-IR, human serum albumin

Introduction

Etomidate (molecular structure shown in Figure 1) is a hypnotic drug without analgesic activity. Intravenous injection of etomidate produces hypnosis characterized by a rapid onset of action, usually within 1 min. It is a non-barbiturate hypnotic that acts at the level of the reticular-activating system to produce anesthesia1,2. Etomidate is an imidazole compound that appears to depress central nervous system function via γ-aminobutyric acid3. Duration of action is intermediate between thiopental and methohexital, and recovery from a single dose is rapid with little residual depression. Like the barbiturates and propofol, etomidate is does not induce analgesia4. Etomidate induces unconsciousness within one circulation time. Recovery is rapid as a result of extensive redistribution and rapid metabolism5,6.

Human serum albumin (HSA) is one of the main extracellular proteins, with a high concentration in blood plasma. HSA is a monomeric globular protein composed of three structurally similar domains (I, II and III), each containing two subdomains (A and B). Aromatic and heterocyclic ligands were found to bind within two hydrophobic pockets in subdomains IIA and IIIA, namely, sites I and II, respectively7,8. Site I is the primary binding site for drugs like warfarin and phenylbutazone analogs, whereas diazepam and ibuprofen are bound primarily to site II. The exceptional capacity of HSA to interact with many organic and inorganic molecules makes this protein an important regulator of intercellular fluxes9–13. For this reason, it is important to conduct detailed studies of the microenvironment of binding sites of HSA to understand HSA–drug interactions.

The study of protein–drug interaction has become the hot spot in the fields of chemistry, medicine, toxicology and biology because most drugs undergo a greater or less extent of reversible binding to plasma proteins and blood cells. There are various methods to evaluate protein–drug interactions and spectrometry is one of them. Etomidate was found to bind HSA in vivo14. The main contribution of this study is about the mechanism of interaction between etomidate and human serum albumin at molecular level. Biophysical and computational assays like fluorescence, UV–vis, CD, FT-IR spectroscopy and molecular docking were performed to elucidate the binding and the interactions responsible for stabilizing the HSA–etomidate complex.

Materials and methods

Materials

All reagents were of analytical grade, purchased from the Sigma-Aldrich Corporation (St. Louis, MO) and used without further purification. The HSA solution (1 × 10⁻⁵ M) was prepared at

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The interaction of etomidate with HSA, the thermodynamic parameters were calculated using the van’t Hoff equation:

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

where $K$ is the binding constant to a site, $R$ is the universal gas constant (8.314 J mol$^{-1}$ K$^{-1}$), $\Delta H$ and $\Delta S$ are the changes in enthalpy and entropy during quenching process. The free energy change ($\Delta G$) associated with the interaction of etomidate with HSA can be calculated from the following equation:

$$\Delta G = \Delta H - T\Delta S = -RT \ln K$$

According to the Forster’s theory, the energy transfer efficiency, $E$ was calculated using the following equation:

$$E = 1 - \frac{F_0}{F_0 - \frac{R_0^6}{R_0^6 + r^6}}$$

where $F$ and $F_0$ are the fluorescence intensities of HSA in the presence and absence of etomidate, $r$ is the distance between the HSA and etomidate and $R_0$ is the Forster critical distance at which 50% of the excitation energy is transferred to the acceptor. The value of $R_0$ can be deduced using the equation shown below:

$$R_0^6 = 8.88 \times 10^{-25} k^2 N^{-4} \Phi J$$

where $k^2$ is the spatial orientation factor of the dipole, $N$ is the refractive index of the medium, $\Phi$ is the fluorescence quantum yield of the donor and $J$ is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. $J$ is given by

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\Delta\lambda}{\sum F(\lambda)\Delta\lambda}$$

Figure 1. Molecular structure of etomidate.
at $1 \times 10^{-6}$ M. The fluorescence titration was used as before to determine the binding constants of etomidate with HSA in the presence of the site probes. Phenylbutazone (PB) and flufenamic acid (FA) are used as site probes of site I and II, respectively.

The docking studies were carried out with AutoDock 4.2 program (La Jolla, CA) by applying the Lamarckian Genetic Algorithm. The 3D structure of the ligand etomate was generated in Sybyl 91.1 (Tripos Inc., St. Louis, MO) and its energy-minimized conformation was obtained with the help of the MMFF94 force field using MMFF94 charges. The crystal structure of the HSA with identifier 1h9z was downloaded from the protein data bank (http://www.rcsb.org/pdb). Water was removed from the HSA file prepared for docking, and essential hydrogen atoms along with Gasteiger charges were added with the aid of AutoDock Tools. After HSA was enclosed in the grid and the fluorescence of tyrosine is almost totally quenched if it is ionized, or near an amino group, a carboxyl group or a hydrogen atom.

Results and discussion

Fluorescence quenching spectra

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample such as excited state reactions, energy transfers, ground-state complexes formation and collisional process. HSA has three intrinsic fluorophores: tryptophan, tyrosine and phenylalanine that can be quenched. In fact, as Sulowska said, because phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized, or near an amino group, a carboxyl group or a tryptophan, the intrinsic fluorescence of HSA is almost contributed by tryptophan alone.

The fluorescence spectra of HSA were recorded in the presence of increasing amount of drug as shown in Figure 2, the fluorescence intensity of HSA decreased regularly with increasing concentration of etomidate, while the emission maximum and shape of the peaks remained almost unchanged. This indicated that the drug could bind to HSA without altering the environment in the vicinity of the chromophore tryptophan residues.

Nature of the binding process

Fluorescence quenching is usually classified as dynamic quenching and static quenching. Dynamic quenching, or collisional quenching, results from collision between fluorophore and quencher. Static quenching is due to the ground-state complex formation between fluorophore and drug. The fluorescence lifetime of the biopolymer is approximately $10^{-6}$ s. Figure 3(A) shows the Stern–Volmer curves for the binding of etomidate with HSA at 295 K. As can be seen in the insets, the Stern–Volmer plot has a good linear relationship within the investigated concentration range. Table 1 summarizes the calculated $K_{SV}$ and $K_q$ at 280, 295 and 310 K. $K_{SV}$ decreased gradually within increasing temperature. Some references have pointed out that the maximum scatter collision quenching constant of various quenchers with biopolymer is $2.0 \times 10^{10}$ M$^{-1}$ s$^{-1}$ [27]. The rate constants for the quenching of HSA due to etomidate were larger than $K_q$ in the scatter mechanism, proving that quenching was caused by the formed complex and was not initiated by dynamic collision.

As discussed above, the resulting fluorescence quenching between etomidate and HSA is initiated by a static quenching process. Determination of the level of drug binding with serum albumin is critical and will directly correlate with the transport, disposition and in vivo efficacy of the drug. The binding constants ($K$) and binding sites ($n$) can be calculated by the double-logarithm equation. Plots of $\log(F_0 - F)/F$ versus $\log[Q]$ for etomidate–HSA was shown in Figure 3(B), and Table 1 listed the corresponding calculated results at diverse temperatures. Both the binding constants and binding sites at higher temperatures were smaller compared with those at lower temperatures, which indicated that lower temperature might facilitate the drug to bind HSA with more binding sites and higher affinity. Besides, the high binding constants suggest etomidate can bind HSA firmly and the drug may not be released from the binding protein easily. This may

Figure 2. Fluorescence emission spectra of HSA in the presence of etomidate. Inset is the fluorescence maximum of HSA in the presence of the drug at emission wavelength 338 nm. $C_{HSA}: 1 \times 10^{-6}$ M, $C_{etomidate}: 1–7$: 0, 1, 2, 3, 4, 5 and 6 $\times 10^{-6}$ M; pH 7.4, $T = 295$ K.

Figure 3. Stern–Volmer (A) and Hill (B) plots of HSA–etomidate interaction data at different temperatures. $C_{HSA}: 1 \times 10^{-6}$ M, $C_{etomidate}: 0$, 1, 2, 3, 4, 5 and 6 $\times 10^{-6}$ M; pH 7.4, $T = 280$, 295 and 310 K.
help explain why the serum albumin binding rate is as high as about 80% and there is still high dose of this drug at 6–8 h after induction.

The thermodynamic parameters were summarized in Table 1 and the van’t Hoff plot was drawn in Figure 4. According to the views of Ross, if \( \Delta H > 0, \Delta S > 0 \), the main binding force is hydrophobic interaction; if \( \Delta H < 0, \Delta S > 0 \), the main force is electrostatic interaction; if \( \Delta H < 0, \Delta S < 0 \), H-bond and van der Waals interactions play major roles in the binding process. As could be seen in Table 1, the negative \( \Delta H \) and positive \( \Delta S \) values indicated that the main binding forces between etomidate and HSA were electrostatic interactions. Moreover, the negative \( \Delta G \) showed the protein–drug interaction occurs spontaneously.

### Synchronous fluorescence measurements

The conformational changes of HSA in the presence of etomidate have been studied by synchronous fluorescence spectroscopy that involves simultaneous scanning of the excitation and emission monochromators maintaining a constant wavelength interval between them. The synchronous fluorescence spectroscopy gives information about the microenvironment of the chromophores such as Trp and Tyr as amino acid residues in case of proteins. This technique has several advantages. It can simplify the spectrum reducing its bandwidth and increasing sensitivity avoiding different perturbing effects. Usually, each protein has only one emission band in the normal fluorescence spectrum, which is due to the emission of both Trp and Tyr because their emission peaks merge in the normal fluorescence spectrum. In order to distinguish the emission peaks of Trp and Tyr residues, the synchronous fluorescence spectroscopic technique is used. It was reported that the shorter wavelength difference (\( \Delta \lambda = 15 \text{ nm} \)) signifies the microenvironment of Tyr residues and longer wavelength difference (\( \Delta \lambda = 60 \text{ nm} \)) indicates the microenvironment of Trp residues.

The fluorescence spectra of the Tyr and the Trp residues of HSA at various concentrations of etomidate have been shown in Figure 5(A) and (B). There is a little extent of red shift (3 nm) occurs but in case of \( \Delta \lambda = 15 \text{ nm} \) while no shift in emission maximum when \( \Delta \lambda = 60 \text{ nm} \), indicating that only the microenvironment around Tyr changes to some extent. The microenvironment around Trp remains unperturbed. The change suggests the decrease in hydrophobicity of Tyr only in presence of etomidate. The red shift observed in Figure 5(A) shows a probable rearrangement of tertiary structure around Tyr residue during the binding process.

### The energy transfer between HSA and etomidate

It is well known that the technique of fluorescence resonance energy transfer (FRET) is useful for measuring the distance between the donor fluorophore and acceptor in vitro and in vivo. A non-destructive spectroscopic method can be employed to monitor the proximity and relative angular orientation of the donor and acceptor. Transfer of energy may take place through a direct electrodynamic interaction between the primarily excited donor fluorophore and acceptor. Transfer of energy may take place through a direct electrodynamic interaction between the primarily excited donor fluorophore and acceptor.
molecule and its neighbors, which will take place under the following conditions:

(i) the donor can produce fluorescence light,
(ii) fluorescence emission spectrum of the donor and UV–vis absorbance spectrum of the acceptor have more overlap, and
(iii) the distance between the donor and the acceptor is lower than 8 nm.

Supplementary Figure S1 shows the overlap of the fluorescence spectrum of HSA with the absorption spectrum of etomidate. For this, equimolar concentrations of etomidate and HSA were employed. The value of \( J \) was calculated to be \( 2.593 \times 10^{-15} \text{ cm}^3 \text{ mol}^{-1} \). Using the values of \( K^2 = 2/3 \), \( N = 1.336 \) and \( \Phi = 0.15 \), the values of \( R_0 \), \( r \) and \( E \) were found to be 3.63 nm, 3.01 nm and 0.057, respectively. An essential criterion for energy transfer to take place is that the distance between donor and acceptor must be within 2–8 nm. This criterion is satisfied in the above case and hence quenching of Tyr fluorescence of HSA in the presence of the probe is attributed to energy transfer. The higher value of \( r \) compared to the value of \( R_0 \) revealed the presence of static quenching mechanism, and 3.01 nm between the bound etomidate and Trp residues in the proposed study suggested high possibility of energy transfer from HSA to the drug.

**Molecular binding site analysis**

Sudlow et al. have suggested two distinct binding sites on HSA, site I and site II, site I of HSA showed affinity for warfarin, phenylbutazone, etc. and site II for ibuprofen, flufenamic acid, etc. To determine the specificity of the drug binding, competition experiments were performed with phenylbutazone and flufenamic acid in connection with Sudlow’s classification of the binding sites. The table within Figure 6 shows the changes in fluorescence of etomidate bound to HSA on the addition of other drugs. Etomidate was not significantly displaced by flufenamic acid (site II). However, phenylbutazone (site I) gave a significant displacement of etomidate suggesting that etomidate binding site on HSA is site I, so the site I is the main binding site for etomidate binding to HSA through electrostatic force. Besides, this binding position was also confirmed by molecular docking study. Atomic coordinates of etomidate were generated using a minimal energy algorithm, and the molecule was found in the HSA structure at subdomain IIA. The site in subdomain IIA also binds phenylbutazone. The binding constant \( K \) and minimum free energy of the HSA–etomidate complex were found to be \( 3.11 \times 10^5 \text{ M}^{-1} \) and \( -3.41 \times 10^4 \text{ kJ/mol} \), respectively, which is in line with the fluorescence study.

**CD and FTIR studies**

The interaction between etomidate and HSA was characterized by infrared spectroscopy. As there was no major spectral shifting for HSA amide I band at around 1655 cm\(^{-1}\) (mainly C=O stretch) and amide II band at around 1550 cm\(^{-1}\) (C–N stretching coupled with N–H bending modes) in the presence of etomidate, the difference spectra [(protein + etomidate solution) – (protein solution)] were obtained to monitor the intensity changes of these vibrations, and their results are shown in Figure 7. When the molar ratio of HSA to etomidate was maintained at 1:3, the intensity changes were observed for HSA amide I band at 1654 cm\(^{-1}\) and amide II band at 1549 cm\(^{-1}\), and negative features were observed in the difference spectra for the amide I and II bands at 1655 and 1547 cm\(^{-1}\) in the HSA–etomidate system. For the HSA–etomidate complexes, decrease in intensities of the amide I and II bands suggested that the percentage content of \( \alpha \)-helical structure of proteins would decrease and destruction of protein tertiary structure at high etomidate concentration.

![Figure 6](image-url). The molecule docking of HSA–etomidate complex (upper), molecular structure of the site probing molecules flufenamic acid and phenylbutazone (middle) and corresponding binding constants (lower).

![Figure 7](image-url). CD spectra of HSA in the presence of etomidate at 295 K. \( C_{\text{HSA}}: 2 \times 10^{-7} \text{ M} \), \( C_{\text{etomidate}}: 0, 10 \) and \( 20 \times 10^{-7} \text{ M} \); pH 7.4, \( T = 295 \text{ K} \).
To further characterize the structural changes in a detailed manner, CD spectroscopy, a quantitative technique to investigate the conformation of proteins in aqueous solution was applied. A high content of α-helices in HSA was revealed by the two minima around 208 and 222 nm (Figure 8). A reasonable explanation is that the negative peaks between 208 and 209 nm and 222–223 nm are both contributed to by an electronic transition for a peptide bond within α-helix. The CD spectrum of HSA in the absence and presence of etomidate with varying concentrations are shown in Figure 8. The α-helices of HSA show a strong double minimum at 220 and 208 nm, which is the typical α-helix structure of HSA. The intensities of this double minimum reflect the amount of helicity of HSA. Upon addition of etomidate to HSA, the results displayed a reduction of α-helical structures from 58.4 to 54.5% at a molar ratio of HSA to etomidate of 1:0 and 1:1.5, respectively; and the reduction continues to be 48.0% at a ratio of 1:10 (Table 2). The decreased helicity suggests the binding of etomidate with HSA induces a slight adaptive unfolding of the constitutive polypeptides of protein, which results in a conformational change of the protein that increased the exposure of some hydrophobic regions that were previously buried. This is in agreement with the synchronous fluorescence results, and is also in accordance with the FT-IR data analysis.

**Conclusion**

In this report, we have studied the complex formed between etomidate and human serum albumin. The interactions have been investigated spectroscopically and theoretically by docking methods. We find that the binding constants obtained from the Stern–Volmer equation are in the 10^5 range, with a binding stoichiometry of 1. The studies with CD and FT-IR related to the secondary structural changes on complex formation corroborate each other indicating a slight reduction in the α-helix content. Thermodynamic parameters obtained from a van’t Hoff plot indicate the driving forces are electrostatic interactions. FRET calculations based on the observed spectral overlap reveal a donor to acceptor distance of about 3 nm indicative of efficient energy transfer. Site probing experiment coupled with docking studies testified that etomidate binds HSA most probably at subdomain IIA, Site I. This study is expected to provide guidelines into understanding how etomidate bind serum albumin before being delivered to the targeted organ.

**Declaration of interest**

The authors declare that they have no competing interests.

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Supplementary material available online
Supplementary Figure S1