Studies on the Interaction of Cefepime Hydrochloride with Bovine Serum Albumin by Fluorescence, Synchronous Fluorescence, Three-Dimensional Fluorescence and Circular Dichroism

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Abstract

The investigation on the binding mode between drug and protein is extremely important to understand biopharmaceutics, pharmacokinetics and toxicity of the drug as well as the relationship of structure and function of the protein. It is well proved that biological activity is a function of the chemical structure or structural properties. There is a study on the interaction of cefepime hydrochloride with serum albumin using in-silico molecular docking. But up to date, there is hardly any interaction investigation of cefepime hydrochloride with serum albumin utilizing fluorescence, synchronous fluorescence, three-dimensional fluorescence and circular dichroism. In this study, the interaction of cefepime hydrochloride with bovine serum albumin in aqueous solution has been investigated by molecular spectroscopy under different pH conditions. The quenching rate constant and binding constant calculated at pH 7.4 indicated the static quenching mechanism and medium binding force. The effect of cefepime hydrochloride on the conformation of bovine serum albumin was analyzed using fluorescence, synchronous fluorescence, three-dimensional fluorescence and circular dichroism. In addition, influence of pH on the binding of cefepime hydrochloride to bovine serum albumin was investigated and the binding ability of the drug to bovine serum albumin deceased under other pH conditions (pH 1.9, 3.5, and 9.0) as compared with that at pH 7.4. As compared with the binding ability of cefepime hydrochloride to native bovine serum albumin that of cefepime hydrochloride to denatured bovine serum albumin deceases dramatically. Furthermore, the effect of metal ions on the binding constant of cefepime hydrochloride with bovine serum albumin was investigated.

Keywords: Cefepime hydrochloride; Bovine serum albumin; Fluorescence quenching; Circular dichroism; Three-dimensional fluorescence

Introduction

The interaction of proteins with drugs has attracted great interest among researchers for many years [1-3]. Serum Albumin (SA), the most important transport protein, has been frequently used as a model protein among researchers for many years [1-3]. Serum Albumin (SA), the most important transport protein, has been extensively studied, for investigating the protein folding and ligand-binding mechanism. In this regard, Bovine Serum Albumin (BSA) has been extensively studied, partly due to its structural homology with Human Serum Albumin (HSA) [4-8]. BSA (Figure 1), composed of three linearly arranged, structurally homologous sub-domains (A, B), has two tryptophan residues Trp-134, which is located on the surface of sub-domain IB, and Trp-212, locating within the hydrophobic binding pocket of sub-domain IIA [9]. The principal regions of drugs binding sites of SA are often located in hydrophobic cavities in sub-domains IIA and IIA.

Cefepime hydrochloride (Figure 2) is a type of broad spectrum fourth-generation cephalosporin antibiotic. It has good antimicrobial activity against Gram-negative bacteria, including Enterobacter spp., Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus bacteria grapes. It is more active against some Gram-positive bacteria, such as Streptococcus pyogenes, Streptococcus pneumoniae compared with the three-generation cephalosporin [10].

In addition, investigation on the interaction between drug and protein under various pH conditions would provide information for profoundly understanding the pharmacological actions of the drug and the relationships of their structures and functions [11]. The binding affinity of drug with protein may be weaker when pH-induced conformational changes of the protein occurs [11], which would directly

Figure 1: Structure of bovine serum albumin, with Tryptophan residues shown in green color.

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influence the concentration of drug in the blood. Thus, the biological actions of the drug can be affected. It is necessary to investigate the interaction of drug with BSA under different pH conditions.

Furthermore, weaker interaction of drug with denatured BSA can lead to obvious increase of drug concentration in the blood. Sometimes this effect may cause toxic poisoning or even cause death [12]. Therefore, studies on the binding of drug to denatured BSA can improve interpretation of the metabolism and transporting process of drug, which is extremely helpful to understand the relationship between the structure and the function of the protein.

Moreover, there are also some metal ions present in blood, which can affect the binding of the drugs with protein [13]. They could participate in many biochemical processes. Some proteins have a variety of metal sites and metal ions-BSA binding can cause conformational alteration of BSA. So it can be deduced that metal ions may have obvious effect on the interaction of medicine molecules with BSA, and thus it would influence the distribution, pharmacological property, and metabolism of drug in blood.

Due to great importance on the binding study of drugs with proteins in pharmacy, pharmacology and biochemistry, our group has made many efforts in this direction in current years [13-24]. The interaction between BSA and several small molecules, such as baicalein [13], farrerol [18], B12 [19], rutin [20], chinodimine [21] and nevadensin [24] have been investigated in recent years. However, to date, there is no report on the binding of cefepime hydrochloride to BSA, especially on the effect of pH, urea and metal ions on the interaction of cefepime hydrochloride with BSA.

In this study, the interaction of cefepime hydrochloride with BSA reported was reported at physiological pH conditions. The static fluorescence quenching mechanism and medium binding constant was presented and the conformational change of BSA induced by cefepime hydrochloride was investigated using fluorescence and CD. In addition, influences of pH, urea and metal ions on the interaction of cefepime hydrochloride with BSA were systematically investigated by steady state fluorescence.

Materials and Methods

Materials and preparation of solutions

BSA was purchased from Sigma (USA). Cefepime hydrochloride was of analytical grade, and purchased from Sinopharm Group Chemical Reagent Co., Ltd. (China). All other reagents were of analytical grade. Double-distilled water was used throughout experiments. The pH of the phosphate buffer solution (20 mmol/L) was adjusted to 1.9, 3.5, 7.4 and 9.0. The concentration of BSA in the buffer was prepared using 66.5 kDa as molecular weight, and the final concentration was checked by measuring absorbance with the published value (optical absorbance at 280 nm) 0.531 (1 g/L). The stock solution (2 × 10⁻² mol/L) of cefepime hydrochloride was prepared by dissolving appropriate amount of cefepime hydrochloride in 10 mL anhydrous methanol. For the determination of fluorescence quenching, the quenching rate constants, and binding constants, the concentration of BSA was 4 μM. The molar ratio of metal ions to BSA was 1. Metal chlorides were used in the metal ion binding effect studies.

Apparatus and methods

Fluorescence measurements were performed on an F-4500 spectrophotofluorimeter (Hitachi, Japan) equipped with 1.0 cm quartz cells following an excitation at 295 nm. The widths of both the excitation slit and the emission slit were adjusted at 5 nm. The three-dimensional fluorescence spectra were performed under the following conditions: the emission wavelength scan range was recorded between 240 nm and 440 nm at 1 nm increments, and the excitation wavelength scan range was recorded from 200 to 360 nm at 5 nm increments. The number of scanning curves was 34, and the excitation and emission bandwidths were 5 nm. CD spectral measurements were run on an Olis DSM 1000 automatic recording spectrophotometer in a 1 mm cell.

A 3 mL buffer solution, containing appropriate concentration of BSA under different pH conditions was titrated by successive additions of a 2 mM solution of cefepime hydrochloride. Titrations were performed manually by using trace syringes. The effects of metal ions on the interaction of cefepime hydrochloride with BSA were investigated by the gradual addition of cefepime hydrochloride to the mixture of metal ions and BSA.

Results and Discussion

Binding mechanisms of cefepime hydrochloride with bovine serum albumin

Fluorescence of BSA originates from Tryptophan (Trp), Tyrosine (Tyr) and Phenylalanine (Phe) residues. Because the Phe residue has a relatively low quantum yield and the fluorescence of Tyr is almost totally quenched by nearby an amino group, a carboxyl group, or a Trp [25], the intrinsic fluorescence of BSA is mainly contributed by the Trp residue alone.

Figure 3 showed the fluorescence emission spectra of native BSA with various amount of cefepime hydrochloride at the excitation wavelength of 295 nm. It could be observed that the fluorescence intensity decreases regularly with the increase of cefepime hydrochloride concentration, indicating that the fluorescence quenching mechanism may be rationalized in terms of a static quenching process.

To confirm the quenching mechanism induced by cefepime hydrochloride, fluorescence quenching data are analyzed with the Stern–Volmer equation (Equation 1) [26-37]:

\[ F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \] (1)

where \( F_0 \) and \( F \) are the relative fluorescence intensities in the absence and presence of quencher, respectively, [Q] the concentration of quencher, \( k_q \) the biomolecular quenching rate constant, \( \tau_0 \) the average bimolecular life-time in the absence of quencher evaluated at about 5 ns [26] and \( K_{SV} \), the Stern–Volmer dynamic quenching constant that was determined by linear regression of a plot of \( F_0/F \) against [Q]. A plot of \( F_0/F \) versus [Q] was shown in Figure 4. The values for \( K_{SV} \) and \( k_q \) were presented in Table 1. The calculated \( k_q \) value is greater than the maximum dynamic...
collisional quenching constant \((2.0 \times 10^{10} \text{ L mol}^{-1} \text{s}^{-1})\) of quenchers with biopolymers \([38,39]\). The result implies that the fluorescence quenching was controlled by a static quenching mechanism.

For the static quenching process, the equilibrium between free and bound molecule is given by the following equation (Equation 2) \([40,41]\):

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

Where \(K\) is the binding constant and \(n\) the number of binding sites. The values for \(K\) and \(n\) can be calculated by a plot of \(\log (F_0 - F)/F\) versus \(\log [Q]\) (Figure 5) and presented in Table 1. The value of \(n\) indicates that one molecule of BSA combined with one molecule of the drug. The values for \(K\) and \(n\) indicated that cefepime hydrochloride was able to bind to BSA via medium binding force.

**Effect of cefepime hydrochloride on the conformation of bovine serum albumin**

Fluorescence and synchronous fluorescence spectroscopic studies of bovine serum albumin: In addition to the proximity of bound cefepime hydrochloride to Trp residue, fluorescence quenching might result from structural change of BSA upon cefepime hydrochloride binding \([42]\). As is well known, the maximum fluorescence emission wavelength \((\lambda_{em})\) of Trp residues is closely related to the polarity of the microenvironment around Trp residues. Therefore, the changes in \(\lambda_{em}\) of BSA will reflect the conformation changes of BSA. It was observed from Figure 3 that \(\lambda_{em}\) of BSA shifted from 343 nm to 351 nm after the addition of cefepime hydrochloride. It implies that the interaction of cefepime hydrochloride with BSA resulted in a significant conformational change of Trp residues micro-regions.

In addition, the change of BSA conformation upon addition of cefepime hydrochloride can be also demonstrated by synchronous fluorescence spectra. As is known, synchronous fluorescence spectroscopy is a very useful method to investigate the microenvironment of amino acid residues by measuring the emission wavelength \([43,44]\) and has several advantages such as sensitivity, spectral simplification, and spectral bandwidth reduction \([45]\).

As is known, synchronous fluorescence spectra show Trp residues of BSA only at the wavelength interval \((\Delta \lambda)\) of 60 nm and Tyr residues of BSA only at \(\Delta \lambda\) of 15 nm. As such, Figure 6A and 6B showed the effect of cefepime hydrochloride on the synchronous fluorescence spectra of Trp and Tyr residues in BSA, respectively. It can be observed from Figure 6 that emission maximum of Trp residues was red shifted while the emission maximum of Tyr residues kept constant at the investigated concentrations range. It indicated that the polarity around tryptophan residues increased, which suggested that tryptophan residues were

![Figure 3: Fluorescence emission spectra of bovine serum albumin in the presence of various concentrations of cefepime hydrochloride at pH 7.4. (1–6) The concentrations of cefepime hydrochloride are (μM): 0, 4, 12, 20, 28 and 36; [bovine serum albumin]=4 μM. λ_{em}=295 nm.](image)

![Figure 4: Plots of \(F_0/F\) for bovine serum albumin against cefepime hydrochloride concentration ranging from 4 to 36 μM; [bovine serum albumin]=4 μM.](image)

### Table 1: Fluorescence quenching constant and binding constant of bovine serum albumin–cefepime hydrochloride system under different pH conditions.

| pH    | Lys state | \(K\) (×10^4 L/mol) | \(n\) | \(R\) | \(K_{SV}\) (×10^4 L/mol) | \(k_q\) (×10^12 L/mol/s) | \(K_{SV}\) (×10^4 L/mol) | \(k_q\) (×10^12 L/mol/s) | \(R\) |
|-------|-----------|----------------------|------|------|----------------------|---------------------|----------------------|---------------------|------|
| 7.4   | Native    | 2.36                 | 0.99 | 0.99 | 2.50                 | 5.00                | 0.99                 | 2.50                 | 5.00 |
|       | Denatured | 0.10                 | 0.79 | 0.99 | 0.76                 | 1.52                | 0.99                 | 0.76                 | 1.52 |
| 1.9   | Native    | 0.10                 | 0.80 | 0.99 | 0.99                 | 0.97                | -                    | -                    | -    |
| 3.5   | Native    | 0.72                 | 0.93 | 0.99 | -                    | -                   | -                    | -                    | -    |
| 9.0   | Native    | 1.38                 | 0.79 | 0.99 | 0.94                 | -                   | -                    | -                    | -    |

![Figure 5: Plot of \(\log (F_0/F)\) for bovine serum albumin vs. \(\log [Q]\) [bovine serum albumin]=4 μM.](image)
that binding of cefepime hydrochloride to BSA causes a conformational change around Trp residues micro-regions while it did not affect significantly the conformational change around Tyr residues micro regions.

Three-dimensional fluorescence spectroscopic studies: Three-dimensional fluorescence is a popular fluorescence analysis technique in current years [46] since the three-dimensional fluorescence spectrum can provide more detailed information on the change of the configuration of proteins. In addition, the contour map can also provide much important information. Figure 7 showed the three-dimensional fluorescence spectra and contour ones of BSA or cefepime hydrochloride-BSA. Two typical fluorescence peaks (peak 1 and peak 2) of BSA could be observed in isometric three-dimensional projection or three-dimensional fluorescence contour map. As depicted in Figure 7, peak 1 mainly reveals the spectral characteristic of tryptophan and tyrosine residues. The fluorescence intensity of the peak decreased and the maximum emission wavelength resulted in red shift after the addition of cefepime hydrochloride (Table 2). This red shift effect indicates that conformational changes of BSA occurred since red shift led to the increase of the polarity around the Trp residues, which was in good agreement with the result obtained from fluorescence and synchronous fluorescence spectroscopy.

The excitation wavelength of peak 2 is 235 nm, being related to the conformation of the peptide backbone associated with the helix-coil. The emission intensity of BSA decreased and the emission wavelength has a little red shift upon addition of cefepime hydrochloride, which implied that the interaction of cefepime hydrochloride with BSA changed the polypeptide backbone structures of BSA.

Circular dichroism spectroscopic studies: CD is a sensitive technique to investigate the conformational changes of proteins upon the interaction with a ligand. Figure 8 displays the CD spectra of BSA before and after the addition of cefepime hydrochloride. They exhibit two negative bands at 209 nm and 222 nm, which is a typical characteristic of the α-helix structure of proteins [47]. As we know, the solvent used for CD studies has no CD signal. The negative bands at 208 nm and 222 nm are contributed by the n-π transition in the peptide bond [48]. It can be observed that there was an apparent reduction in both bands without any significant shift of the peaks, which implied that binding of cefepime hydrochloride to BSA causes a conformational change of the protein.

Influence of pH on the interaction of cefepime hydrochloride with bovine serum albumin

It is well known that the fluorescence of BSA is related to pH in located in a less hydrophobic environment. It implies that the interaction of cefepime hydrochloride with BSA led to a conformational change of the configuration of proteins. In addition, the contour map can also provide much important information. Figure 7 showed the three-dimensional fluorescence spectra and contour ones of BSA or cefepime hydrochloride-BSA. Two typical fluorescence peaks (peak 1 and peak 2) of BSA could be observed in isometric three-dimensional projection or three-dimensional fluorescence contour map. As depicted in Figure 7, peak 1 mainly reveals the spectral characteristic of tryptophan and tyrosine residues. The fluorescence intensity of the peak decreased and the maximum emission wavelength resulted in red shift after the addition of cefepime hydrochloride (Table 2). This red shift effect indicates that conformational changes of BSA occurred since red shift led to the increase of the polarity around the Trp residues, which was in good agreement with the result obtained from fluorescence and synchronous fluorescence spectroscopy.

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Influence of pH on the interaction of cefepime hydrochloride with bovine serum albumin

It is well known that the fluorescence of BSA is related to pH in

Figure 6: Synchronous fluorescence spectra of bovine serum albumin with various amounts of cefepime hydrochloride. (1–6) The concentrations of cefepime hydrochloride are (μM): 0, 4, 12, 20, 28 and 36; [bovine serum albumin]=4 μM; (A): Δλ=15 nm and (B): Δλ=60 nm.

Figure 7: The three-dimensional fluorescence projections and three-dimensional fluorescence contour map of bovine serum albumin before (A) and after (B) cefepime hydrochloride addition. (A): [bovine serum albumin]=4 μM; (B): [bovine serum albumin]=4 μM; [Cefepime hydrochloride]=16 μM.

Table 2: Three-dimensional fluorescence characteristics of bovine serum albumin and bovine serum albumin–cefepime hydrochloride system at pH 7.4.

| System                     | Peak 1   | Peak 2   |
|----------------------------|----------|----------|
| BSA                        | 280/342  | 235/343  |
| Cefepime hydrochloride/BSA (4:1) | 280/345 | 235/344  |

Figure 8: Circular dichroism spectra of the cefepime hydrochloride–bovine serum albumin system at pH 7.40 at 298 K: [bovine serum albumin]=4 μM; Cefepime hydrochloride to bovine serum albumin ratios: (a) 0.1, (b) 8.1.
Where $F_0$ and $F$ represent the relative fluorescence intensities of BSA before and after binding of cefepime hydrochloride to BSA under various pH conditions. Clearly, the extent of the fluorescence quenching can be well reflected using $Q$. Figure 9 shows the effect of pH on the fluorescence quenching fractions of BSA induced by cefepime hydrochloride at the molar ratio of the drug to protein was 1, 4 and 8, respectively. It was observed that the fluorescence quenching extent at pH 7.4 is the greatest in all the four pHs, which implies that the binding affinity of BSA for cefepime hydrochloride at pH 7.4 may be greater than that at the other pHs. This result can be successfully confirmed by the determination of binding constant ($K$) of cefepime hydrochloride with BSA under four different pH conditions (Figure 10). The values for $K$ at pH 1.9, 3.5, 7.4 and 9.0 are also listed in Table 1, which indicated that BSA indeed exhibited the highest binding force toward cefepime hydrochloride at pH 7.4. This phenomenon may result from two aspects, protein and drug. As depicted in Figure 11, the maximum emission wavelengths of BSA shift from 343 to 335 nm and 338 nm when pH 7.4 changes into 3.5 and 1.9, respectively. In addition, the fluorescence intensity of BSA was greater than that at pH 1.9, 3.5, or 9.0. The above results indicate that the influence of pH on the structure of BSA is relatively great, thus impacting binding capacity of drug to BSA [49,50]. Moreover, the drug may be a little sensitive to the pH because it would cause different concentration of ionization state. Thus, the binding ability of drug to BSA would be affected.

The interaction of urea-induced bovine serum albumin with cefepime hydrochloride

The denaturation of protein can be induced chemically by using urea, SDS, or acetone, etc. [38]. As is well known, higher than 8 M urea would lead to the complete denaturation of BSA [21]. To explore the interaction of completely denatured BSA caused by urea with cefepime hydrochloride, we investigated the fluorescence of denatured BSA with various amount of cefepime hydrochloride. The values for

![Figure 9: The effect of pH on the fluorescence quenching fractions $Q$ of bovine serum albumin induced by cefepime hydrochloride: (A) the drug to protein molar ratio is 1, (B) the drug to protein molar ratio is 4, (C) the drug to protein molar ratio is 8.](image)

![Figure 10: Plots of log ($F_0$-$F$)/$F$ vs. log $[Q]$ under different pH conditions; [bovine serum albumin]=4 μM.](image)

![Figure 11: Fluorescence emission spectra of bovine serum albumin in different pHs: (a) 7.4, (b) 9.0, (c) 3.5, and (d) 1.9; [bovine serum albumin]=4.0 μM.](image)

![Figure 12: Plot of $F/F_0$ for denatured bovine serum albumin against $[Q]$ of cefepime hydrochloride ranging from 4 to 36 μM under different pH conditions; [bovine serum albumin]=4 μM.](image)
of Zn\textsuperscript{2+} or Fe\textsuperscript{3+}, the binding constant of cefepime hydrochloride–BSA was reduced and the values for $K_{SV}$ increased a conformational change in BSA, being more favorable for cefepime hydrochloride binding to BSA. Thus, the increase in binding constant of cefepime hydrochloride–BSA in presence of the above ions prolongs storage compared with that without these ions. The higher binding constant may result from the fact that Zn\textsuperscript{2+} or Fe\textsuperscript{3+} induced period of cefepime hydrochloride in blood and weaken its maximum medicinal effects.

**Conclusion**

In the paper, influences of pH, urea, and metal ions on the interaction of cefepime hydrochloride with BSA were characterized by molecular spectroscopy. It can be elicited that the binding reaction of cefepime hydrochloride with the protein in blood is sensitive to the change in pH. The pH 7.4 is the optimal acidity. In addition, the denatured BSA resulted in weaker binding affinity of the protein towards cefepime hydrochloride. The effect of metal ions on the binding constant of cefepime hydrochloride with BSA was studied. This study can also provide important insight into the interactions of the physiologically important protein with drugs. This study will be also helpful to structure-based drug design.

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**Table 3**: Effects of metal ions on binding constants of cefepime hydrochloride-bovine serum albumin.
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