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Research Article

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Alteration of the nonspecific binding constant of chlorothiazide to bovine serum albumin induced by Cl⁻ and oleic Acid

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ABSTRACT

Serum albumin (SA) acts as a reservoir and transport protein for endogenous (e.g. long chain fatty acids (LCFAs) and chloride anion (CГ)) and exogenous ligands (e.g. drugs) in the blood. In this study, the alteration of the nonspecific binding constant (K values) of chlorothiazide (CTZ) to bovine serum albumin (BSA) induced by the presence of oleic acid (OLA) and/or CГ was evaluated by a second derivative spectrophotometric method at simulated physiological conditions (pH = 7.4 and 37 °C). The residual background impacts caused by the incomplete suppression of BSA signals have been entirely eliminated in the second derivative spectra. Since the therapeutic effects of a drug is closely related to its free fraction in the blood, which in turn may be altered in the presence of endogenous ligands, the results of the present study are very useful for providing safer and efficient pharmacological information in clinical settings.

Key words: Drug-albumin binding, Chlorothiazide, Oleic acid, Derivative spectrophotometry.

INTRODUCTION

Desire for better diuretics prompted the search for a carbonic anhydrase inhibitor with better diuretic impact and antihypertensive potency resulted in the appearance of chlorothiazide (CTZ; Fig. 1) [1-3]. The actual target of CTZ was shown to be mediated through inhibition of the NaCl transport system. CTZ is used clinically for the treatment of hypertension, congestive heart failure and edematous conditions. It has been reported to be excreted unmetabolized by the human kidney [4].



Figure 1: Structure of chlorothiazide (CTZ)

As the most abundant and the major soluble protein constituent of the circulatory system, serum albumin (SA) has many physiological and pharmacological functions [5]. It is mainly responsible for the maintenance of blood pH and osmotic pressure. Moreover, SA also plays an important role in the transport and disposition of endogenous and exogenous ligands present in blood [6,7]. Its tremendous potential to bind a vast array of drugs results in its prevailing role in drug pharmacokinetics and pharmacodynamics. Thus, data on SA binding are extensively used as

a parameter in pharmacokinetic modeling to predict absorption, distribution, metabolism, and excretion (ADME) of drugs in humans [8,9].

It is well recognized that only the free drug fraction can cross membrane barriers and be distributed to tissues. Also, it is accepted that the therapeutic effect of a drug is closely related to the free drug in plasma rather than to the total drug concentration [10,11]. The binding of a drug to plasma proteins, by regulating the free drug fraction, is thus considered an important parameter to be determined not only in the early stages of its discovery but in the state of designing its dosage forms as well [12]. Any alteration in the free drug fraction of the drug, of physiologically or pathologically origin, will change the binding constants with SA. The presence of other endogenous or exogenous ligands can affect the binding of the drug to SA. This effect, therefore, may significantly alter the pharmacokinetic and pharmacodynamic properties of the drugs.

Among endogenous substances of vital importance Cl⁻ and long chain fatty acids (LCFAs) possess a strong tendency to bind SA [13-15]. Accordingly, it is important to understand and predict the effect of Cl⁻ and LCFAs on the binding interactions of drugs with SA. Effect of fatty acids on the binding of drugs with SA has long been studied [16-21].

Several techniques such as equilibrium dialysis [22], ultracentrifugation [23], electrochemical technique [24], capillary electrophoresis [25], high-performance liquid chromatography [26], have also been utilized for the evaluation of binding mode and binding constants. However, the potential problems of such separative techniques are that relatively large quantities of drug are required and that the equilibrium between bound and unbound drug may shift during the experiment. Other methods require expensive instruments and skilled operators.

Spectroscopic methods mainly including UV-Vis, fluorescence, CD, FT-IR, nuclear magnetic resonance (NMR) and conductivity have been widely used to study the interaction of small ligands with SA [27-32]. Among them, spectrophotometry is still the most widely used owing to its exceptional simplicity, availability and abundant theoretical foundation.

Binding constants of drugs to SA can be obtained by analyzing the absorbance difference before and after the addition of albumin to aqueous drug solutions. But background signals caused by SA are so strong that the baseline is not always flat and zero-level, even if the used reference solutions contain the same amount of protein. Thus, these absorption changes of the drugs are not usually accurate enough for direct calculation of the binding constants. An alternative, valid, simple and direct procedure depending on the use of second derivative spectrophotometry has been previously introduced. Derivative spectrophotometry enhances the ability to detect minor spectral features and allows the resolution of overlapped bands [33,34]. The second derivative method is particularly useful because it eliminates the interferences of liposomes [35-38], β -cyclodextrin [39], human erythrocyte ghosts [40,41] and serum albumin [42-43].

In this work, we demonstrated the usefulness of second derivative spectrophotometry to investigate the influence of Cl⁻and OLA on the binding of CTZ to BSA at simulated physiological conditions (pH=7.4, 37 $^{\circ}$ C).

EXPERIMENTAL SECTION

Fundamentals

Like the absorbance, the derivative intensity is proportional to the solute concentration. Thus, if the effect of residual background signals is entirely eliminated in the derivative spectrum, the derivative intensity (D) of CTZ in a sample solution at a specific wavelength is represented as follows:

$$D = E_{\rm b}[CTZ_{\rm b}] + E_{\rm u}[CTZ_{\rm u}]$$

where $[CTZ_b]$ and $[CTZ_u]$ represent the molar concentrations of CTZ bound to BSA and unbound in the water phase, respectively, and E_b and E_u are their corresponding molar derivative intensities. With $E = E_b - E_u$ and $[CTZ_t] = [CTZ_b] + [CTZ_u]$, where $[CTZ_t]$ stands for the total molar CTZ concentration in sample solutions, *D* can be written as

$$D = E_{\rm u}[CTZ_{\rm t}] + E[CTZ_{\rm b}] \tag{1}$$

A new variable is introduced to represent the difference between D and $E_u[CTZ_t]$ as

$$\Delta D = D - Eu[CTZ_t] = E[CTZ_b]$$

(2)

The ΔD represents the experimentally-determined value for derivative intensity difference before and after the addition of BSA to a drug solution in the absence and presence of OLA and/or Cl⁻, and its value is proportional to [*CTZ*_b].

Thus, the fraction of a drug bound to BSA, α , can be given by

$$\alpha = \Delta D / \Delta D_{\rm max} \tag{3}$$

where $\Delta D_{\text{max}} = E[CTZ_t]$. The value of ΔD_{max} will be obtained by extrapolating the total concentration of BSA added, [*BSA*], to ∞ in the plot of $1/\Delta D$ versus 1/[BSA]. Thus, the values of $[CTZ_b]$ and $[CTZ_u]$ are given by $\alpha[CTZ_t]$ and $(1-\alpha)[CTZ_t]$, respectively, and the number of moles of the CTZ bound per mole of BSA, *r*, is given by $\alpha[CTZ_t]/[BSA]$. With these values, the interactions of the drug with BSA in the absence or the presence of each endogenous substance were analyzed using the Scatchard equation.

Instrumentation

Absorbance measurements were performed on a Shimadzu UV-1800 UV-Vis spectrophotometer (Kyoto, Japan), equipped with a Shimadzu CPS 240A thermostated multi-cell positioner with temperature stability of ± 0.1 °C. Eppendorf adjustable-volume automatic pipettes (100-1000 and 500-5000 µL) were used to deliver accurate volumes. All pH measurements, with an accuracy of ± 0.01 , were made on a calibrated Hanna digital pH-mV meter model 211 with a combined glass Ag/AgCl electrode. A thermostated water bath, GFL 1003 type (Burgwedel, Germany), with an accuracy of ± 0.1 °C, was also used. Ultrapure water was daily prepared with a Barnstead Nanopure diamond water purification system (Dubuque, IA, USA), and was used throughout. In addition, the spectrophotometer was connected with a personal computer through a USB cable. Using UVProbe control/data processing PC software makes it directly read data that is transferred to the PC. Further treatment of the spectral data and the calculations to obtain derivative spectra and *K* values were performed using a customized visual basic program [37,40].

Reagents

CTZ, BSA (essentially fatty acid-free) and sodium salt of OLA were purchased from Sigma (St. Louis, MO, USA). Analytical reagent-grade sodium chloride was used. Deionized water was used as a solvent to prepare the salt-containing sodium phosphate buffer (0.05 mol/l, pH 7.4). The pH of the salt-containing buffer was adjusted to 7.4 with H_3PO_4 or NaOH solutions at 37 °C. CTZ, OLA and BSA stock solutions were prepared by using the salt-containing buffer as a solvent. The concentration of BSA in a stock solution was determined by UV absorption using the absorptivity value $E_{1cm}^{1\%} = 6.67$ at 279 nm and the molecular weight of 66.4 kDa.

Preparation of Sample Solutions

For CTZ-BSA Binding Studies

Sample solutions were prepared in 10-ml volumetric flasks by adding a suitable aliquot of a stock solution of 0.2 mM CTZ to 2 ml of buffer solutions containing various amounts of BSA and the buffer was further added to the mark. Reference solutions were buffer solutions containing the same amount of BSA as the sample solutions.

For Cl⁻ Effect Investigation

To several 10-ml volumetric flasks, 2 ml of a salt-containing buffer and 3 ml of a 0.2 mM CTZ stock solution were added; therefore the final drug concentration was 60 μ M. Thereafter, a suitable aliquot of the BSA stock solution was added to each of the flasks, and the salt-containing buffer was added to the appropriate volume. The reference solutions were prepared as the same manner without the drug.

For OLA Effect Investigation

OLA-bound BSA buffer solution (OLA-BSA stock solution) was prepared by mixing adequate amounts of OLA and BSA stock solutions to attain a required OLA/BSA molar ratio of 0 to 7. To a 10-mL volumetric flask, 3 mL of the 0.2 mM CTZ stock solution and an aliquot of the OLA-BSA stock solution sufficient to yield a BSA concentration of 0 - 60 μ M were added, and followed the addition by 0.1 M NaCl sodium phosphate buffer to volume. The sample solutions thus obtained were 60 μ M CTZ-0.1 M NaCl sodium phosphate buffer solutions containing 0 to 60 μ M BSA, which binds OLA at an OLA/BSA molar ratio of 0 to 7. The reference solutions were prepared in the same manner as the sample solution without the drug.

Absorption and Derivative Spectral Measurements

Each flask was shaken for a short time and incubated at 37 °C for 30 min before the spectral measurements. The absorption spectrum of the sample solution was measured against its reference solution at 37 °C with a slit width 2

nm and a wavelength interval of 0.1 nm using 10 mm light-path cuvette. The spectral data were stored in a PC for further use and calculations. The second derivative spectra were calculated from the absorption spectra using a Visual Basic program based on the Savitzky-Golay method [46]. The wavelength interval ($\Delta\lambda$) of 1.0 nm was used in the calculation since it gave results of lowest standard deviations.

RESULTS AND DISCUSSION

Absorption Spectra

The absorption spectra of 40 μ M CTZ in buffer solutions in the presence of increasing amounts of BSA are depicted in Fig. 2 as typical results of the spectrophotometric titration experiments. The absorption spectrum of CTZ shows a bathochromic shift induced by the addition of BSA. On the basis of such measurable spectral changes the capability of CTZ to bind BSA was adequately ascertained. However, it is clear that the counterbalance of background signals of BSA in the sample and reference beams is incomplete, even though the solutions in the sample and reference cuvettes were prepared carefully to contain the same amount of BSA. All other spectral measurements obtained with Cl⁻ and OLA showed similar results. When the background signal level is very high, it is usually difficult to cancel the background signal effects completely to obtain a flat and zero-level baseline. Accordingly, quantitative spectral data for calculating the binding constants (*K* values) could not be obtained from these absorption spectra. To overcome such difficulties second derivative spectrophotometry has been applied since it offers the opportunity to remove the background signals effects without disturbing the equilibrium states of sample solutions with prior separation procedures.



Figure 2: Absorption spectra of 40 μ M CTZ in buffer solution (pH = 7.4 at 37°C) in the presence of several amounts of BSA. BSA concentration (μ M): 0, 6.08, 12.17, 18.25, 24.34, 30.42, 36.51 and 48.68, lines 1, 2, 3, 4, 5, 6, 7 and 8, respectively



Figure 3: Second derivative spectra of CTZ calculated from the absorption spectra of Fig. 2. The numbers in both Fig. 3 and Fig.2 are the same

Second Derivative Spectra

The second derivative spectra calculated from the absorption spectra in Fig. 2 are depicted in Fig. 3. Figure 3 shows a bathochromic shift induced by the increase in BSA concentration similar to that in the absorption spectra in Fig. 2. Furthermore, Fig. 3 clearly shows the distinctive derivative isosbestic points at 315.5, 324.9 and 337.3 nm. The results not only indicate that the residual background signal effects were entirely eliminated in the second derivative spectra, but also prove that CTZ exists in two states exhibiting different derivative spectra, i.e., a free state in the buffer phase and BSA bound phase [47]. Accordingly, accurate spectral data for calculating the K values could be obtained from the second-derivative spectra.

Double Reciprocal Plots

The ΔD values of CTZ for free BSA or in the presence of OLA and/or Cl⁻ at each concentration where measured in each corresponding spectrum at 334 nm; where the calculated ΔD_{max} and K values showed lowest standard deviation. Then the ΔD_{max} values were obtained by the extrapolation of the total concentration of albumin, [*BSA*], to ∞ in the plot of $1/\Delta D_{max}$ versus 1/[BSA]. Figure 4 shows the double reciprocal plots for determining ΔD_{max} of 40, 60 and 80 μ M CTZ as typical examples. The ΔD_{max} values for evaluating the individual, Cl⁻, and simultaneous, OLA and Cl⁻, impacts on the binding of CTZ on BSA were similarly calculated.



Figure 4: Double reciprocal plots for the calculation of ΔD_{max} of CTZ at several concentrations. CTZ concentration (μ M): 40 (\bullet), 60 (\blacksquare) and 80 (\bullet)



Figure 5: Scatchard plot for the interaction of CTZ with BSA. (A) at several concentrations of CΓ. [Cl⁻] (M): zero (•), 0.05 (■), 0.1 (•) and 0.15 (▲). (B) at several OLA/BSA molar ratios in the presence of 0.1 M CΓ. OLA/BSA: (•) zero, (■) 1, (▲) 2, (•) 3, (○) 4, (□) 5, (△) 6 and (•) 7

Scatchard's Plot

With the obtained ΔD_{max} values the fractions of CTZ in either the absence or the presence of OLA and/or Cl-, α values were calculated by using Eq. (3). Using the calculated α values the interactions of CTZ with BSA were examined by Schatchard's plot. The results (the concentration of each is indicated in figure legend) are depicted in Fig. 5, which shows straight lines parallel to the abscissa either in the absence or the presence of OLA and/or Cl⁻.

It has been reported that the binding interaction of small organic ligands to SA can be explained by specific and nonspecific binding model [43,44]. The results in Fig. 5 show that the interaction of CTZ to BSA can be explained by the nonspecific binding model, i.e., the r/[CTZu] values are constant and do not depend on the BSA concentration.

For partition-like nonspecific binding, the binding constant, K, can be given as shown in Eq. 4.

$$\frac{r}{[CTZ_u]} = \frac{[CTZ_b]}{[BSA][CTZ_u]} = K$$
(4)

Thus $r/[CTZ_u]$ values are constant and independent on the BSA concentration. Using ΔD value, *K* can be written as follows:

$$K = \frac{(\Delta D / \Delta D_{\text{max}})}{(1 - \Delta D / \Delta D_{\text{max}})[BSA]}$$
(5)

From Eq. 5, we obtain

$$\Delta D = \frac{K[BSA]\Delta D_{\max}}{(1+K[BSA])}$$
(6)

The *K* values were calculated by fitting the observed data regarding the ΔD value and the BSA concentration to Eq. 6 using a nonlinear least-squares method with a Taylor expansion (by a customized Visual Basic program) [37,40]. In the calculation, the ΔD_{max} value was also treated as a parameter, because the ΔD_{max} values at hand had been obtained by the extrapolation.

Calculation of K Values and Fractions of CTZ Bound to BSA

The *K* values were calculated using 40, 60 and 80 μ M of CTZ based on Eq. 6 with a nonlinear least-squares method. For each *K* value, four independent experiments were performed. All of the *K* values were obtained with satisfactory precision (relative standard deviation, < 6%), Table 1. It is obvious that the *K* values are almost constant for all of CTZ's concentrations studied. These results provide a further confirmation on the validity of applying the partition-like nonspecific binding model to the interaction of CTZ to BSA.

Table 1: K and ΔD_{max} values of the binding of CTZ at various concentrations, to BSA

[CTZ] (µM)	K ^a ×10 ⁻³ M ⁻¹	$\Delta D_{max}^{a} \times 10^{3}$		
40	13.42 ± 0.39	2.01 ± 0.08		
60	13.75 ± 0.53	3.19 ± 0.15		
80	12.93 ± 0.60	2.79 ± 0.14		
^{<i>a</i>} Mean \pm standard deviation (n=4)				

The calculation was also done with the ΔD values measured at several wavelengths in order to see to what extent the *K* values are affected by these wavelengths. The results for 40 µM CTZ are summarized in Table 2 and show that the wavelength at which the ΔD values are measured did not largely affect the *K* values but the values measured at 334 nm gave a little better precision than the other wavelengths. Thus all of the *K* values in this study were calculated with the ΔD values measured at this wavelength. Since, it has been well-recognized that, the wavelength interval ($\Delta \lambda$) has a significant impact upon the calculation of a derivative spectrum from an absorption one [23], the effect of changing $\Delta \lambda$ value on the calculated binding constant should be investigated. Accordingly, the *K* values of 60 µM CTZ were calculated using the ΔD of the derivative spectra calculated by using the $\Delta \lambda$ values of 0.4, 0.6, 0.8, 1.0 and 1.2 nm. The results summarized in Table 2 show that the $\Delta \lambda$ values examined do not largely affect the *K* values. These results also indicate the validity of the proposed derivative method.

$\mathrm{K}^{\mathrm{a}} imes10^{-3}\mathrm{M}^{-1}$					
$\Delta\lambda$ (nm) λ (nm)	a) 332	334	340	342	
0.4	12.89 ± 0.68	13.57 ± 0.66	13.57 ± 0.72	12.80 ± 0.51	
0.6	13.08 ± 0.68	14.12 ± 0.78	13.34 ± 0.47	13.54 ± 0.53	
0.8	13.16 ± 0.51	13.42 ± 0.39	12.82 ± 0.71	13.83 ± 0.50	
1.0	12.89 ± 0.73	12.91 ± 0.62	14.12 ± 0.62	14.08 ± 0.59	
1.2	13.32 ± 0.65	13.74 ± 0.70	14.09 ± 0.69	14.15 ± 0.58	
^{<i>a</i>} Mean \pm standard deviation (n = 4)					

Table 2: Calculated K values of 40 μ M CTZ from ΔD values measured at different wavelengths and using various wavelength intervals

To confirm the accuracy of the obtained *K* values, the fraction $(\alpha = \Delta D/\Delta D_{max})$ of CTZ bound to BSA in the presence of individual Cl⁻ (0, 0.05 and 0.15 M), Fig. 6(A), and the simultaneous presence of Cl⁻ (0.1 M) and OLA/BSA at various molar ratios, for 0, 1:1, 2:1, 3:1 and 6:1, Fig. 6(B), was calculated with the obtained *K* and ΔD_{max} values at each experimental BSA concentration, based on Eq. 6. The results shown in Fig. 6 reveal that all of the plotted experimental values fell close to the calculated curves, which in turn demonstrates the reliability of the obtained *K* values.



Figure 6: Fraction of CTZ bound to BSA. The solid lines show the theoretical curves calculated using Eq. (6) with the obtained K and ΔD_{max} values and the symbols are the experimental values. (A) at several Cl⁻ concentrations. [Cl⁻] (M): zero (\bullet), 0.05 (\blacksquare) and 0.15 (\blacktriangle). (B) at several OLA/BSA molar ratios in the presence of 0.1 M Cl⁻. OLA/BSA: (\bullet) zero, (\circ) 1, (\bigstar) 2, (\bullet) 3 and (\blacksquare) 6



Figure 7: *K* values of CTZ to BSA in the presence of various concentrations of CF. Each histogram represents the mean value of four determinations and the error bars represent the standard deviations

Influences of OLA and/or Cl⁻ on the Binding of CTZ to BSA

The effect of Cl^- on the binding of CTZ to BSA is illustrated in Fig. 7 as a function of representative sodium chloride concentrations. Previously, we emphasized that the effects of both NaCl and KCl on the nonspecific

binding of triflupromazine and chlorpromazine to BSA are quite similar and these effects is only derived from their anion; $CI^{-}[14]$. Figure 7 reveals that CI^{-} resulted in decreases in the *K* value, depending on the salt concentration. It is obvious that the inhibitory effect of chloride anion on the binding of CTZ to BSA were about 18.53, 21.21 and 22.57% for 0.05, 0.1 and 0.15 M Cl⁻, respectively. The results indicate that 0.1 and 0.15 M Cl⁻ have a similar impact on the *K* values and as a result 0.1 M Cl⁻ has been used for further investigation of the influence of OLA on the binding of CTZ to BSA. Further investigation on the effect of OLA has been performed in the presence of 0.1 M Cl⁻ by calculating the *K* values at various OLA/BSA molar ratios. Interestingly, the results revealed that the *K* values are increased with the increase of the molar ratio OLA/BSA from 1 to 5 and decreased with larger ratios.

Figure 8 clarifies that when OLA/BSA was 3, the *K* value was enhanced by 30% compared to the value for BSA in the presence of 0.1 M Cl⁻. Accordingly, the influence of OLA on the binding CTZ-BSA is considered to be dependent on its content, i.e. it enhances the binding of CTZ at an OLA/BSA molar ratio of 1 to 5. Higher molar ratios (e.g. 6 or 7) result in an inhibitory effect. As it is known, since the LCFAs content varies with the physical condition of the body [1], it can be suggested that the concentration of free CTZ in the blood may vary according to the physical condition of the body, e.g., suffering from diabetic and duration from last meal, etc. This in turn may be resulted from the competition of LCFAs with the administrated drug to bind SA.



Figure 8: K values of CTZ to BSA in the presence of 0.1 M CГ at several OLA/BSA ratios. Each histogram represents the mean value of four determinations and the error bars represent the standard deviations

CONCLUSION

Drug-albumin interaction on which many studies has been focused over long time is still an interesting research area not only because such studies offer the opportunity for understanding the pharmacokinetic and pharmacodynamic properties of the drugs but also because these studies have been recently applied for the therapeutic and diagnostic purposes [5]. Basically, under normal pathological and physiological conditions, the LCFAs content of SA is 1-2, but during fasting or after hard exercise, or in subjects suffering from diabetes, SA can bind LCFAs at the highest level of 6-7. The results of the present study suggest that the concentration of unbound CTZ in the blood, which relates closely to its therapeutic activities, may be influenced by the condition of the body via its LCFAs content in SA. Therefore, the results of the present study are pharmacokinetically, pharmacologically, and clinically very important.

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