ANALYSIS OF CHINOFORM BINDING TO HUMAN SERUM ALBUMIN BY AN IMPROVED PARTITION EQUILIBRIUM METHOD

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Previous methods such as equilibrium dialysis to measure chionoform binding to human serum albumin are flawed with problems due to the drug's poor solubility in neutral aqueous solutions and its strong adsorption onto the surfaces of dialysis membrane and other apparatus. Accordingly, we adopted a previously reported improved partition equilibrium method to keep off such difficulties, and developed additionally a rapid method using oil-wells. Both of these methods were used in this study. The equilibration of both partition and binding was attained after 6 h by the standard method and after 2 h by the rapid method. Two binding sites were found on the albumin molecule. One had an extremely large affinity constant of 10^8 M^{-1} and the other had a moderate one of 10^6 M^{-1} . These values obtained with both methods agreed well and were sufficiently reproducible. The bound percentage of chionoform in serum was estimated at 99.999% under the condition where chionoform reveals its nervous toxicity.

The binding constant had a maximum at pH 7.6, the physiological pH of serum. The pH profile suggested the participation of a histidine residue in the binding site.

The inhibition experiment with palmitic acid revealed that the first binding site of chionoform was not identical with that of palmitic acid.

The present method is applicable to analyses of protein binding by other drugs showing similar physicochemical properties as chionoform.

Keywords — chionoform; binding; human serum albumin; improved partition equilibrium; oil-well; adsorption; sparing water-solubility; pH effect; palmitic acid inhibition

INTRODUCTION

The elevation of protein-bound iodine after administration of iodinated drugs has been known for a long time. This fact suggests that these drugs bind serum proteins.

Chionoform (CF; clioquinol, 5-chloro-8-hydroxy-7-iodoquinoline) has been widely used as a preventive medicine for amoebic dysentery and as a remedy and a preventive medicine for diarrhea. Liewendahl *et al* observed a binding of radioactivity to serum albumin after oral administration of tracer doses of [^{125}I]CF to hyperthyroid and euthyroid patients.^{1,2)} He also pointed out from the *in vitro* experiment, using equilibrium dialysis, that [^{125}I]CF bound human serum proteins.³⁾ In 1974, Kira showed by equilibrium dialysis that about 1.2 moles of [^{131}I]CF bound 1 mol of human serum albumin (HSA) in veronal buffered saline at pH 8.6.⁴⁾ However, these reports did not succeed in determining the number of binding sites and their affinity constants. The main cause was thought to be large errors in measurements due to poor solubility of

CF in neutral aqueous solution and to its strong adsorption onto dialysis membrane and glassware, which are also investigated in this work.

We have already developed a precise and convenient method to determine partition ratios of CF.⁵⁾ In this paper, we report a precise analysis of CF binding to HSA using the improved partition method which is not affected by sparingly soluble drugs with strong adsorption characteristics, and also report the development of a more rapid method using oil-wells. Furthermore, the effect of pH of the medium on the binding and the inhibition with palmitic acid were investigated.

MATERIALS AND METHODS

Chemicals — [^3H]CF (7.0 mCi/mmol) was synthesized from [^3H]glycerol (ethanol solution, Amersham International, Buckinghamshire, England) and 2-amino-4-chlorophenol.⁵⁾ HSA was Cohn's fraction V (ICN NBC Laboratories, Cleveland, Ohio) or fatty acid free fraction V (Miles Laboratories, Elkhart, Indiana). The concentration of these solutions was determined

from the absorbance at 280 nm using the $E_{280}^{\%}$ of 5.8.⁶⁾ *n*-Decane, having a fairly high boiling point (174 °C), was used as the organic phase to prevent the errors caused by evaporation. *n*-Decane of reagent grade (Tokyo Kasei, Tokyo) was purified by shaking several times with concentrated sulfuric acid until the acid phase became colorless and by distillation under reduced pressure. The buffer used in this work was 0.02 M phosphate-0.13 M NaCl containing 5 mM EDTA (pH 7.4; PBS) to mask interfering metal ions. The scintillation cocktail consisted of 5 g 2,5-diphenyloxazole (Nakarai Kagaku, Kyoto; PPO) and 0.134 g 2,2'-*p*-phenylene-bis-(4-methyl-5-phenyloxazole) (Dimethyl POPOP; Eastman Kodak, Rochester, N.Y.) per liter of toluene. Commercially available [^{14}C]-palmitic acid (New England Nuclear, Boston, Mass.) was used after extraction with the buffer. All other chemicals were of reagent grade.

Determination of Partition Ratios and Protein Binding of CF—Partition ratio of CF between *n*-decane and aqueous buffer was determined previously⁵⁾ by an improved partition equilibrium utilizing a disposable injector and a glass tube.

CF binding to HSA was measured according to a previous partition method except for the volume of both layers and the incubation time. The aqueous layer was 1.0 ml of *n*-decane containing [^3H]CF. Figure 1 shows that the equilibration of both partition and binding was at-

tained after 6 h when the set tubes containing the mixture were gently shaken by a reciprocating shaker 73 times per min at 25 ± 0.5 °C. The time required for this equilibration was twice as long as that required for only partition equilibrium.

To shorten the incubation time still further, we developed the following rapid method. Figure 2 illustrates the teflon plate used in the

FIG. 1. Time Course of the Equilibrium by the Standard Method

The aqueous layer was 1.1 ml of PBS, pH 7.4, containing 15.8 nmol HSA and the organic layer was 1.0 ml of *n*-decane containing 10 nmol [^3H]CF. Equilibration was carried out at 25 °C.

FIG. 2. Teflon Plate Used in the Rapid Method

The teflon plate has many cylindrical wells as illustrated in the horizontal section (right), 6 mm depth and 5 mm i.d., in the vertical section (left), and is covered with a glass plate during the incubation to prevent the loss of *n*-decane by evaporation.

FIG. 3. Time Course of the Equilibration by the Rapid Method

Aqueous phase was 20 μ l of PBS, pH 7.4, containing 0.4 nmol HSA and organic phase was 50 μ l of *n*-decane containing 0.2 nmol [3 H]CF. Equilibration was carried out at 25 $^{\circ}$ C.

Key: (○) without rotation, (●) with rotation 12 times per min.

rapid method which has many cylindrical wells, 6 mm depth and 5 mm i.d. Fifty μ l of various concentrations of *n*-decane solution of [3 H]CF and 20 μ l of PBS containing 20 μ M HSA were added gently to each well and then, the aqueous phase formed a droplet in the organic phase.

When the four corners of the teflon plate was moved up and down in the phase lag of 90 $^{\circ}$ by 90 $^{\circ}$ by a rotating apparatus we made, the droplet rotated around the inside wall of the well, and the equilibration of both partition and binding was attained after 2 h (Fig. 3). Sampling of the aqueous phase was performed carefully with a glass micropipette.

The concentrations of bound and unbound CF were calculated from the radioactivities in both phases and from the partition ratio of CF of 1750 under the condition used. The apparent binding constant, k' , was estimated graphically as the slope of the Scatchard's plot.

pH Effect of the Medium on the Binding

—Fatty acid free HSA was used in this experiment. The aqueous layer was 1.1 ml of 0.02 M of various kinds of buffer—0.13 M NaCl—5 mM EDTA adjusted at different pH and containing 10 μ M HSA and the organic layer was 1 ml of *n*-decane containing 25 μ M [3 H]CF.

Inhibition Analysis with Palmitic Acid—Fatty acid free HSA was also used in this experiment. The aqueous layer was 2 ml of PBS containing 10 μ M HSA and the organic layer was 1 ml of *n*-decane containing various combinations of concentrations of CF and palmitic acid. Two sets having the same combination of the concentra-

FIG. 4. Permeability of CF through a Visking Tube

(A) Time Course of the [3 H]CF Concentration in Both Sides

Outside solution was 8 ml of PBS, pH 7.4, initially containing [3 H]CF and inside solution was 3 ml of PBS. Dialysis was performed at 25 $^{\circ}$ C. A level line drawn at 19.8 nM CF shows the expected concentration in both sides after the equilibration without any adsorption.

(B) Time Course of the Recovery of [3 H]CF

Solution: Recovered from outside and inside solutions, glassware: extracted from the inside of the glassware, visking tube: extracted from the visking tube, unrecovered: total CF — [solution + glassware + visking tube].

tion were prepared and [^3H]CF was used in one set and [^{14}C]palmitic acid used in the other set as a radioactive sources. The concentrations of bound and unbound CF in the aqueous layer were calculated using the former set and that of palmitic acid in the aqueous layer was measured using the latter set in the same manner.

RESULTS

Adsorption of CF to Materials during Equilibrium Dialysis

Phosphate-buffered saline, pH 7.4, was used. When 8 ml of outside [^3H]CF solution (27 nM) was dialyzed against 3 ml of inside buffer, the outside concentration did not become identical to the inside concentration even after 22 h as shown in Fig. 4A. Figure 4B shows that the sum of the radioactivities recovered from the solutions of both sides was only 30% after 22 h and that CF was strongly adsorbed mainly to the visking tubing. Even when the initial concentration of CF was 25 times higher (about 700 nM),

FIG. 5. Scatchard Plot of the CF-HSA Binding by the Standard Method (A) and by the Rapid Method (B)

(A) The aqueous layer was 1.1 ml of PBS, pH 7.4, containing $14.4\ \mu\text{M}$ HSA and the organic layer was 1.0 ml of *n*-decane containing [^3H]CF. Equilibration was carried out at $25\ ^\circ\text{C}$. The apparent binding constants obtained from (A) were $1.94 \times 10^8\ \text{M}^{-1}$ (k'_1) and $2.7 \times 10^6\ \text{M}^{-1}$ (k'_2).

(B) The aqueous phase was $20\ \mu\text{l}$ of PBS, pH 7.4, containing $20\ \mu\text{M}$ HSA and the organic phase was $50\ \mu\text{l}$ of *n*-decane containing [^3H]CF. Equilibration was carried at $25\ ^\circ\text{C}$. The apparent binding constants obtained from (B) were $1.75 \times 10^8\ \text{M}^{-1}$ (k'_1) and $6.0 \times 10^6\ \text{M}^{-1}$ (k'_2).

TABLE I. Estimated Values of the Bound Percentage, the Bound Molar Ratio and the Concentration of Unbound CF in Serum

$[D_t]$ (μM)	Bound percentage (%)	r	$[D_F]$ (pM)	Relative $[D_F]$
10	99.999188	0.0154	80.4	1
30	99.999169	0.0462	249	3.10
100	99.999063	0.154	937	11.7

These calculations were made according to the equation of $k' = r/(n-r) \cdot [D_F]$ on the assumption that participation of the second class of sites could be neglected under these conditions, where the drug concentrations were less than that of HSA in normal state ($650\ \mu\text{M}$).

Key: $[D_t]$, total CF concentration in serum; r , the bound molar ratio of CF to HSA; $[D_F]$, the concentration of unbound CF in serum.

similar results were obtained. Adding 5 mM EDTA to the buffer resulted in an increase of the recovery to 60%. Therefore, adsorption of CF mainly to the visking tubing is thought to be caused by hydrophobic and hydrogen bonding and by chelation with metal ions which may be present in very low amounts in the system.

Determination of the Binding Parameters

Figure 5 shows two Scatchard's plots obtained from the standard (A) and the rapid method (B). The apparent binding constants obtained from the former, k'_1 and k'_2 , were $1.94 \times 10^8 \text{ M}^{-1}$ and $2.7 \times 10^6 \text{ M}^{-1}$ at 25°C , respectively. The coefficient of variation of these values was about 2%. The rapid method gave $k'_1 = 1.75 \times 10^8 \text{ M}^{-1}$ and $k'_2 = 6.0 \times 10^6 \text{ M}^{-1}$ at 25°C . Both results show the presence of no less than two classes of sites of CF on the HSA molecule. One of the affinity constants of serum albumin for the drug, k'_1 , was extraordinary large and the other, k'_2 was moderate.

The bound percentage, the bound molar ratio, r and the concentration of unbound CF, $[D_F]$, in serum were estimated theoretically

from these binding parameters as shown in Table I. These calculations were performed assuming that participation of the second class of sites could be neglected under these conditions, where the drug concentrations were less than that of serum albumin in normal state ($6.5 \times 10^{-4} \text{ M}$). The bound percentage of CF in serum was estimated at 99.999% within the range of the total concentration from 10^{-5} to 10^{-4} M , which was the range for the outbreak of SMON, subacute-myelo-optico-neuropathy. The concentration of unbound CF was suggested to be almost in proportion to that of total CF within this range. The effect of various factors which may affect the binding such as long-chain fatty acids were neglected and thus, these values in Table I are estimates of the binding parameters.

pH Effect of the Medium on the Binding

Figure 6A shows that r was very small under an acidic condition and increased as pH became higher. Figure 6B illustrates that among the unbound CF, the concentration of undissociated species was almost constant at every pH value

FIG. 6. pH Effect of the Medium on the CF-HSA Binding (A) Effect on the Bound Molar Ratio, r , (B) Effect on the Concentration of Unbound CF in Aqueous Layer, $[D_F]$, (C) Effect on the Apparent Binding Constant, k'

Aqueous layer was 1.0 ml of 20 mM of various kinds of buffer containing 0.13 M NaCl, 5 mM EDTA and $10 \mu\text{M}$ HSA. The organic layer was 1.0 ml of *n*-decane containing $25 \mu\text{M}$ $[^3\text{H}]$ CF. Equilibration was carried out at 25°C .

The solid line in (B) shows the unbound concentration of CF in the aqueous layer calculated from the concentration of CF in the organic layer and the partition ratio at each pH.⁵⁾ The dotted line in (B) shows the undissociated concentration of CF in the aqueous layer calculated from the unbound concentration and the pK'_1 and pK'_2 of CF .⁵⁾

Key: ($\blacktriangledown$) glycine-HCl, ($\circ$) acetate, ($\bullet$) phosphate, ($\triangle$) Tris-HCl, ($\blacksquare$) glycine-NaOH.

FIG. 7. Effect of Palmitic Acid on the CF-HSA Binding (A) Effect on the Bound Molar Ratio, r , (B) Effect on the Concentration of Unbound CF in Aqueous Layer, $[D_F]$, (C) Lineweaver-Burk Plot of the Inhibition with Palmitic Acid

The aqueous layer was 2 ml of PBS, pH 7.4, containing $10 \mu\text{M}$ HSA and the organic layer was 1 ml of *n*-decane containing various combinations of the concentration of CF and palmitic acid. Equilibration was carried out at 25°C . Each different key in (A) and (B) corresponds to the different concentration of CF added.

Key in (C): ($\bullet$) without palmitic acid, palmitic acid added by ($\circ$) $10 \mu\text{M}$, ($\times$) $20 \mu\text{M}$, ($\blacksquare$) $30 \mu\text{M}$, ($\blacktriangle$) $40 \mu\text{M}$.

but that of phenolate ions, which increased with pH from $\text{p}K'_2$ (7.9) of CF. Therefore, the total concentration of unbound CF in the aqueous phase also increased largely under the same condition. Approximate calculations from each datum in Fig. 6A and 6B were made according to the same equation as in Table I. These approximation gave us information that the apparent binding constant may have the maximum value at pH 7.6 as shown in Fig. 6C.

Inhibition Analysis with Palmitic Acid

Figure 7A shows that the decrease of the bound molar ratio of CF, r , was gradual at first and became steeper in proportion to the increase of palmitic acid and the inhibition curves attained a reverse sigmoidal shape. The unbound concentration of CF in the aqueous layer, $[D_F]$, increased gradually as shown in Fig. 7B. Figure 7C shows Lineweaver-Burk plot based on the calculations that r and $[D_F]$ were estimated from the points of intersection after drawing perpendicular lines at every ten μM of palmitic acid in (A) and (B). It suggests that the first binding site of CF is not identical with that of palmitic acid. Non-competitive inhibition was ob-

served when the molar ratio of palmitic acid to HSA was less than 2, and competitive inhibition was observed when its ratio was higher than 2.

DISCUSSION

Various techniques have been proposed for the determination of the binding parameters. Spectrophotometric methods utilizing the competition with pigment^{7,8)} or fluorescent⁹⁾ probes have the merits of momentary achievement of equilibration because supports and special devices are not used. However, these methods are indirect and not reliable when plural binding sites are present. Our preliminary experiments using 8-anilino-1-naphthalenesulfonic acid gave unreasonable results in that the bound concentration was calculated to be higher than the total concentration. Equilibrium dialysis has the merit of directly measuring the concentrations in both inside and outside solutions. However, it cannot be applied to drugs that strongly adsorb to the membrane. The supports of electrophoresis and the resins of chromatography can also adsorb these drugs strongly. Thus, the binding constant of CF obtained by Hobara

and Taketa¹⁰⁾ using electrophoresis on agarose gel, $5.6 \times 10^4 \text{ M}^{-1}$ at pH 8.6, was about two-thousandths of the value we obtained at the same pH.

On the other hand, the present method has some value in measuring the drug concentrations directly in both phases and requires no supports or special glassware. However, the influences of the organic solvent used in this method should be determined.

In the first place, the influence of binding of the organic solvent itself to the protein was considered using [^{14}C] *n*-decane. The solubility of *n*-decane in PBS and in $14.4 \mu\text{M}$ HSA solution, pH 7.4, was found to be $7.41 \mu\text{M}$ and $14.4 \mu\text{M}$ at 25°C , respectively. The number of *n*-decane bound per mole of albumin was then calculated to be 0.49. The apparent binding constant of *n*-decane was estimated to be $1.3 \times 10^5 \text{ M}^{-1}$ assuming $n = 1$. This value is about one-thousandth of the primary binding constant of CF. Therefore, the influence of the *n*-decane molecule is negligible.

Next, conformational change of albumin by *n*-decane during the incubation should be considered. Goodman reported that denaturation of HSA was not observed visually and by ultracentrifugation in partition equilibrium using *n*-heptane.¹¹⁾ Moreover, Spector *et al.* observed no spectral changes of bovine serum albumin after the incubation with and without adding low molecular substances in the partition equilibrium using *n*-heptane.¹²⁾ Performed tests were; (a) absorption spectrum after adding 2-[(4'-hydroxyphenyl)-azo] benzoic acid, (b) fluorescence spectrum after adding 8-anilino-1-naphthalenesulfonic acid, (c) fluorescence spectrum and (d) UV spectrum of bovine serum albumin itself. These data do not completely exclude the possibility of conformational changes. However, if conformational changes do occur, they are very small.

The following speculation is offered to explain the binding of CF to HSA. The binding constant of CF to HSA has a maximum value at pH 7.6 (Fig. 6C), isoelectric point of HSA was reported as 4.9¹³⁾ and the $\text{p}K'_1$ and $\text{p}K'_2$ of CF were determined to be 3.2 and 7.9, respectively, in the previous paper.⁵⁾ From these facts, therefore, CF molecules are thought to be inaccessible to albumin molecules due to electrostatic repulsion at pH under 3 and over 8. However, the pH

profile of the affinity constant under the acidic condition suggests the need for an additional consideration. The $\text{p}K_a$ value of the imidazole residue of histidine on proteins is reported to be 5.6 to 7.2.¹⁴⁾ Assuming the participation of histidine in this binding, this discrepancy may be explained as follows: the hydrogen bonding between the hydrogen in the undissociated phenol of CF and the nitrogen atom in the undissociated imidazole of histidine is destroyed by the protonation of imidazole under acidic condition. Thus, it is proposed that CF binds albumin by both hydrophobic and hydrogen bondings.

Free long-chain fatty acids were reported to be present in normal serum in amounts of one half to two times the moles of HSA. Therefore, it is assumed from the inhibition experiment that they do not exert a large influence on the CF binding under these conditions. However, if the concentration of free long-chain fatty acids increases abnormally, then the unbound fraction of CF will also increase largely in serum.

Thus, the present method should be considered as being reliable and useful for the analyses of protein binding by drugs or serum components which are sparingly water-soluble and adsorb strongly to inert materials.

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