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Interaction between Methacycline and Human Serum Albumin

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The effects of pH, chloride ion, and fatty acids on the interaction between methacycline (MTC) and human serum albumin (HSA) were fluorometrically examined. The fluorescence intensity and binding constant (K) of MTC–HSA complex increased with pH from 6.4 to 8.4, suggesting that MTC binding to HSA is significantly affected by conformational change of HSA in this pH range. Chloride ion effectively displaced MTC from its binding site when HSA was in the basic conformation. The binding affinity of MTC for fatty acid–free HSA depended upon the concentration of fatty acids (oleic and palmitic acids) at physiological pH. Specific binding sites for MTC on HSA were also examined in connection with Sudlow's classification. It was found that the primary binding site of MTC on HSA is site **1**.

Keywords——methacyline; human serum albumin; methacycline–human serum albumin binding; fluorescence technique; human serum albumin conformation; pH effect; chloride ion effect; fatty acid effect; methacycline binding site

The interaction between tetracyclines and albumin has been investigated by various methods such as equilibrium dialysis,[1] absorption spectrophotometry,[2] and fluorescence measurement.[3] Fluorescence spectroscopy is one of the most sensitive and convenient methods for studying the interaction of drugs with proteins. Kohn[4] first discovered that the fluorescence of tetracycline chelates of calcium and zinc was enhanced in the presence of deoxyribonucleic acid and serum albumin. Popov *et al.*[3b] also studied the effects of metal ions, albumin concentration, and pH on the fluorescence of tetracyclines and of tetracycline–bovine serum albumin (BSA) complexes, and determined fluorometrically the thermodynamic parameters of binding of tetracyclines to BSA using the van't Hoff equation. In addition, they suggested that tetracyclines might be generally suitable for use as fluorescent probes. Furthermore, Luzzi *et al.*[3c] found two high-affinity binding sites on BSA for demeclocycline and oxytetracycline by the fluorescence method. In our preliminary experiments, methacycline (MTC) among various tetracyclines showed the greatest enhancement of fluorescence in aqueous albumin solution.

Thus, we investigated the effects of pH, chloride ion, and fatty acids on the binding of MTC to human serum albumin (HSA) by the fluorescence technique with the aim of elucidating the nature of the binding. Furthermore, the displacement of MTC from its primary binding site by other commonly prescribed drugs was investigated at various drug-to-protein ratios in order to identify the primary binding site.

Experimental

Materials——HSA (Lot. No. A-2386) was obtained from Sigma Chemical Company, St. Louis, Mo. Methacycline hydrochloride (Taito Pfizer Ltd.), phenylbutazone, oxyphenbutazone (Ciba-Geigy Co.), ibuprofen (Kaken Pharmaceutical Co., Ltd.), diazepam (Sumitomo Chemicals), digitoxin and digoxin (Mitsubishi Yuka

Pharmaceutical Co.) were gifts of the manufacturers. All other chemicals used were of reagent grade, and deionized and distilled water was used throughout. All of the buffers used were prepared with sodium phosphate dibasic and potassium phosphate monobasic. The pH values were checked at 25 °C using a suitabley standardized pH meter.

Apparatus and Methods——Fluorescence spectra were measured with a Hitachi 650-60 fluorescence spectrophotometer. All HSA and MTC solutions were prepared in 1/15 M phosphate buffer at 25 °C.

Fluorometric titrations: HSA solution at an appropriate concentration was titrated by successive additions of a solution of MTC (to give a final concentration of 4.0×10^{-5} M) and the fluorescence intensity was measured (excitation at 400 nm and emission at 502 nm).

Data treatment: The fraction of MTC bound, X, is usually determined by using equation (1), where Fp and Fo are the fluorescence intensities of a given concentration of MTC in a solution of low HSA concentration and in a

$$X=\frac{Fp-Fo}{Fb-Fo} \tag{1}$$

solution without HSA, and Fb is the fluorescence of the same concentration of fully bound MTC. To determine the values of Fb for a given concentration of MTC, fluorescence titrations were carried out for several albumin concentrations. Fb is taken to be the fluorescence intensity of the substrate in the presence of excess albumin. After values for the fraction of bound MTC had been found for all points along the titration curve, the results were plotted according to the Scatchard equation:[5]

$$r/Df=nK-rK \tag{2}$$

where r is the number of mol of MTC bound per mol of protein, n is the number of binding sites, K is the binding constant and Df is the concentration of free MTC.

Results and Discussion

Effect of pH on MTC–HSA Interaction

The fluorescence intensity of MTC was greatly enhanced by binding to HSA. The emission maximum was shifted toward the blue following interaction with HSA. Fluorometric titration at various pHs were done by altering the MTC concentration as shown in Fig. 1. Fluorescence intensities for two titrations with high HSA concentrations (1.0×10^{-4} M and 2.0×10^{-4} M) at pH 8.4 were identical (straight line a), suggesting that MTC added was fully bound at both protein concentrations. A similar results was obtained at pH 7.4 (straight line c). For this treatment to be valid, the fluorescence intensity of the bound MTC must be a linear function of concentration. This is the case only when the absorbance of the complex at the exciting wavelength is low. A correction for this absorbance can be made by the method of Naik *et al.*[6] and was made for all the data when the absorbance at 400 nm was greater than 0.02. The straight lines (lines a and c in Fig. 1) were obtained after correcting the observed fluorescence intensities for the absorbance effect (the second term in the correction equation was considered). When the titrations were carried out at low concentrations, MTC was only partially bound (curves b and d). The plateau in each titration

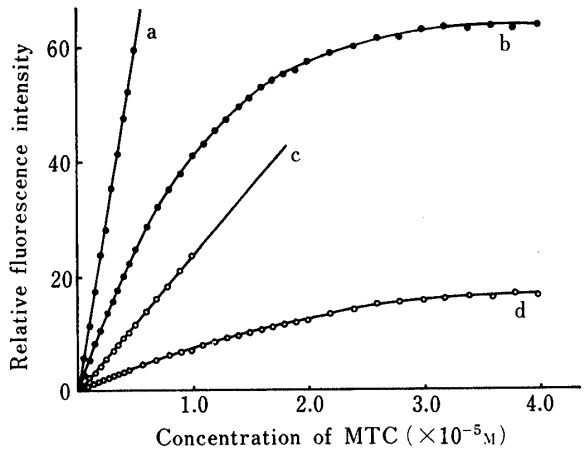

Fig. 1. Relative Fluorescence Intensity Due to the MTC–HSA Interaction as a Function of the Concentration of MTC

HSA concentration: a, 1.0×10^{-4} and 2.0×10^{-4} M; b, 1.0×10^{-5} M; c, 1.4×10^{-4} and 1.5×10^{-4} M; d, 1.4×10^{-5} M. ●—●, pH 8.4; ○—○, pH 7.4.

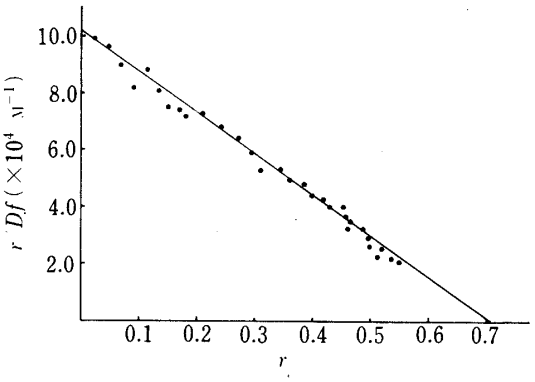

Fig. 2. Scatchard Plots of the MTC–HSA Interaction at pH 8.4

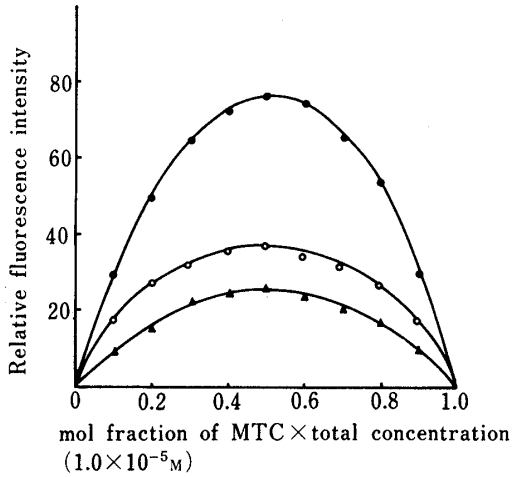

Fig. 3. Job's Plots of Relative Fluorescence Intensity for the MTC–HSA Interaction at Various pHs

The total concentration of [HSA] + [MTC] was kept at 1.0×10^{-5} M. ●—●, pH 8.4; ○—○, pH 7.4; ▲—▲, pH 6.4.

TABLE I. Binding Parameters for the Interaction of MTC with HSA at Various pH Values

pH	Parameter	
	n	K, M^{-1}
6.4	0.66	0.21×10^5
7.4	0.68	0.47×10^5
7.9	0.69	0.53×10^5
8.4	0.70	1.46×10^5

curve obtained at low protein concentration indicates saturation of the HSA binding site. As shown in Fig. 1, the fluorescence intensity of MTC–HSA complex at pH 8.4 was significantly greater than that at pH 7.4, indicating that the fluorescence intensity (quantum yield of the MTC–HSA complex) or the binding affinity of the MTC–HSA interaction increases with pH.

The linearity of Scatchard plots for the MTC–HSA system indicates that MTC binds to one class of sites on HSA (Fig. 2). To check the maximum number of binding sites, Job's plots[7] were also prepared for the MTC–HSA system by keeping the total concentration of MTC and HSA at 1.0×10^{-5} M. These plots are shown in Fig. 3. The inflection points for these plots are near 0.5, the value expected for 1 : 1 complex formation. The n values estimated from the Scatchard plots were in fair agreement with those from Job's plots. Slight differences between the n values in both methods may be based on the differences of data treatment. Therefore, it is reasonable to conclude that the maximum number of binding sites contributing to the fluorescence of MTC–HSA interaction at all pHs examined is one.

The binding parameters for MTC–HSA interaction estimated from Scatchard plots are summrized in Table I. Although no significant changes in the n values were observed in the pH range from 6.4 to 8.4, the binding constants K for the MTC–HSA system increased with pH. Colaizzi and Klink[8] reported that at around the physiological pH, tetracyclines exist in various ionized forms, *i.e.*, in cationic form at more acidic pH, in anionic form at more alkaline pH, and in zwitterion form at relatively neutral pH. On the other hand, it is well-known that within the narrow pH range of 6—9, HSA exists in two conformational forms, the

so-called N-form (at neutral pH) and B-form (at alkaline pH).[9] Thus, it is presumed that MTC predominantly exists in anionic form at pH 8.4 and HSA in the B-form having anionic charges in the molecule. In such a situation, the interaction of MTC with HSA may be weekened by charge repulsion.

Therefore, it is reasonable to consider that the enhancement of fluorescence intensity of MTC–HSA complex at pH 8.4 (Fig. 1) and the increase of K values with pH (Table I) can be attributed to the N–B transition of HSA.

Effect of Chloride Ion on MTC–HSA Interaction

Figure 4 shows the relative changes in the fluorescence intensities of the MTC–HSA complex as a function of pH in the presence and absence of sodium chloride. Chloride ion quenched the fluorescence of MTC–HSA complex at all pHs measured (sodium ion has no effect on the fluorescence). The degree of quenching was great over the pH range of the N–B transition as shown in Fig. 4, indicating that the affinity of MTC for HSA is decreased over this pH range. These results suggest that chloride ion effectively displaces MTC when HSA is in the B-conformation. On the other hand, Perrin *et al.*[10] reported that the effect of chloride ion in the interaction of benoxaprofen with HSA is greater when HSA is in the N-conformation. The difference in behavior of the complexes of benoxaprofen and MTC with HSA in the presence of chloride ion may be related to the HSA conformation and the specificity of drug binding sites on HSA but further work is required to elucidate the mechanism of their interaction. The effect of calcium ion, which greatly alters both the conformation of HSA[9b-e,10] and the fluorescence of tetracyclines,[3b,4] was also examined in the present system. However, unfortunately, no clear effect could be observed because of solubility problems due to calcium phosphate formation.

Effect of Fatty Acids on MTC–HSA Interaction

It has been reported that fatty acids interact with HSA and that their binding affects the drug binding to HSA through competitive displacement and the allosteric effect.[11] In the present studies, oleic and palmitic acids were examined to investigate the effects of fatty acids on the MTC–HSA interaction. Figure 5 shows the effect of oleic acid on the fluorescence

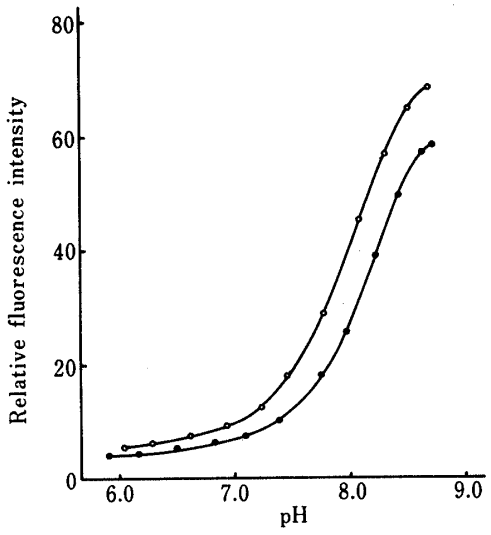

Fig. 4. Effect of Chloride Ion on the Fluorescence Intensity of the MTC–HSA Interaction as a Function of pH

Concentrations: HSA, 5.0×10^{-5} M; MTC, 5.0×10^{-6} M; chloride ion, 1.0×10^{-1} M. ●, with chloride ion; ○, without chloride ion.

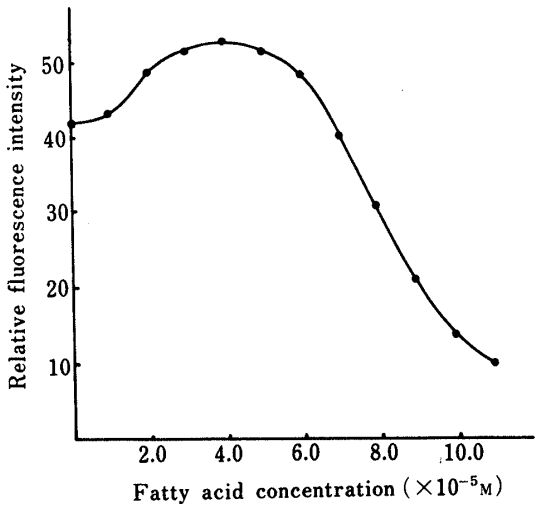

Fig. 5. Effect of Oleic Acid on the Fluorescence Intensity due to the MTC–FFHSA Interaction at pH 7.4.

MTC concentration, 3.0×10^{-5} M; FFHSA concentration, 1.5×10^{-5} M.

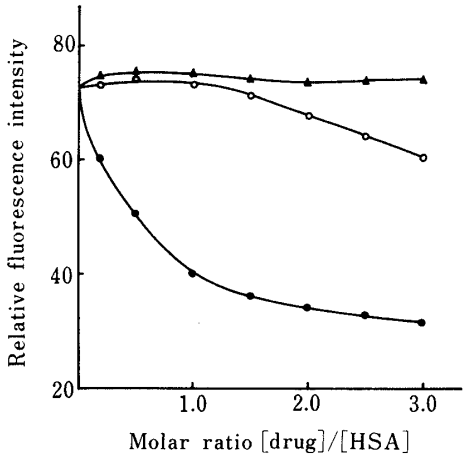

Fig. 6. Effects of Various Drugs on the Fluorescence Intensity of MTC (7.5×10^{-6} M) in the Presence of HSA (1.5×10^{-5} M)

●, phenylbutazone; ○, ibuprofen; ▲, digitoxin.

intensity of the MTC-fatty acid free HSA (FFHSA) system, as an example. The fluorescence intensities of the MTC–FFHSA system were enhanced at low oleic acid concentration and quenched with increasing concentration of the fatty acid. This suggests that fatty acids at low concentration increases the affinity of MTC for FFHSA by altering the conformation, but at high concentration it displaces MTC form FFHSA after saturation of its binding site. Similar results were also obtained for palmitic acid.

Determination of MTC Binding Site on HSA

The specific drug binding sites on HSA have been established as sites **1**, **2**, and **3** by Sudlow *et al.*[12] and Sjöholm *et al.*[13] Although a number of drugs have been classified in terms of binding to those sites, the specific binding sites for tetracyclines have not yet been investigated. To identify the MTC binding site on HSA in relation to Sudlow's classification, the competitive displacements by various drugs were examined. Figure 6 shows the changes in the fluorescence of the MTC–HSA system on addition of various drugs. As shown in Fig. 6, MTC was not displaced by digitoxin (a site **3** drug), whereas phenylbutazone (a site **1** drug) caused significant displacement of MTC at small molar ratios of drug to HSA. Furthermore, ibuprofen (a site **2** drug) caused no displacement of MTC at [drug]/[HSA] ratios less than 1.0 but showed slight displacement at molar ratios more than 1.0, suggesting that when site **2** on HSA was saturated with ibuprofen, the excess ibuprofen displaced MTC bound to HSA.

It is concluded from these results that the primary binding site of MTC on HSA is site **1**. The MTC binding site was also confirmed by competitive displacements observed with other drugs, *e.g.*, oxyphenbutazone (site **1**), diazepam (site **2**), and digoxin (site **3**). Further studies of the binding sites are planned with modified HSA.

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