Protein Binding of Quinolonecarboxylic Acids. II.[1,2] Spectral Changes on the Interaction of Cinoxacin, Nalidixic Acid and Pipemidic Acid with Human and Rat Albumins

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The interaction of cinoxacin (CINX), nalidixic acid (NA), and pipemidic acid (PPA) with human and rat serum albumins (HSA and RSA) was studied by UV difference absorption and circular dichroism (CD) spectroscopy. CINX and NA bound to the albumins and generated difference absorption and induced CD (ICD) spectra. The difference absorption spectral data explained reasonably our previous observations that CINX bound to HSA more weakly than NA, but to RSA as strongly as NA. We used a quantity $\Delta\varepsilon/\varepsilon$, designated as relative molar difference absorbance, at positions corresponding to the longest wavelength peaks in the difference spectra. The quantity was found to correlate linearly with percent bound to both HSA and RSA, but with different slopes, from which the binding site for CINX and NA in RSA was supposed to provide a much more nonpolar environment than that in HSA. The magnitude of ICD bands observed at 371 nm for CINX and at 342—348 nm for NA corresponded to the binding degrees of these drugs to both albumins. Anisotropy factors for the ICD bands at 350—271 nm for CINX and 320—348 nm for NA were approximately similar between HSA and RSA, suggesting a similar ability to generate the ICD spectra in these wavelength regions upon binding to the albumins. Spectral results for PPA in albumin solutions showed little or no binding of this drug to HSA and RSA. PPA existed as a betaine form in neutral solution and its positively charged group acted as an unfavorable factor for binding to both albumins.

Keywords protein binding; cinoxacin; nalidixic acid; pipemidic acid; betaine; human albumin; rat albumin; relative difference absorbance; induced CD; anisotropy factor

In the preceding paper[2] we reported on studies of protein binding of cinoxacin (CINX), nalidixic acid (NA), and pipemdic acid (PPA) by ultrafiltration and equilibrium dialysis methods. Some marked differences in binding characteristics have been observed among these three drugs: 1) CINX binds to human serum albumin (HSA) more weakly and also has lower protein binding in human serum than NA, but in rat serum, CINX shows protein binding as high as NA, owing to its higher affinity for rat serum albumin (RSA) than for HSA; 2) PPA has no significant binding to HSA, though it shows 20—30% protein binding in both human and rat sera.

Such differences seem to be of particular interest in relation to the difference in binding site nature between HSA and RSA as well as that in physicochemical properties among the ligands. We extended our investigation on protein binding of the three drugs by using different approaches, ultraviolet (UV) difference absorption and circular dichroism (CD) spectroscopy. Ligand binding to albumin has been reported to generate difference absorption and induced CD (ICD) spectra.[3]

In the present paper, the difference in binding characteristics among the quinolonecarboxylic acids and that between HSA and RSA were discussed on the basis of spectral analyses. The spectral data of CINX and NA explained well the characteristics of their binding to both albumins. It was found that PPA exists as a positively charged species (betaine), which is an unfavorable factor for binding to albumins.

Results and Discussion

Drug–Ethylene Glycol (EG) System Binding of drugs to protein affects their electronic transitions and hence generates difference absorption spectra.[3] Since the chromophores of the drugs are generally surrounded by a less polar environment upon binding to protein, similar effects are expected when the drugs are transferred from a purely aqueous solvent to an aqueous organic solvent. Thus we first investigated solvent perturbation difference absorption spectra of CINX, NA and PPA, using EG as a perturbant. EG has frequently been used for studies on protein chromophores and model compounds by solvent perturbation difference spectrophotometry.[4,5]

Figure 1 shows typical difference absorption spectra of the three drugs produced by EG. Each of the drugs gave a characteristic difference spectrum. The longest wavelength peaks for NA, CINX and PPA were oserved at 347, 371 and 351 nm, respectively. They were all positive and corresponded to red shifts of the original absorption bands (Fig. 4A3—C3) by 1—2 nm (Table I). The respective peak intensities increased with increasing EG concentration without significant change in the spectral shape. We deal hereafter only with these peaks for analysis, because they seem to be almost free from overlapping by the transitions at shorter wavelengths.

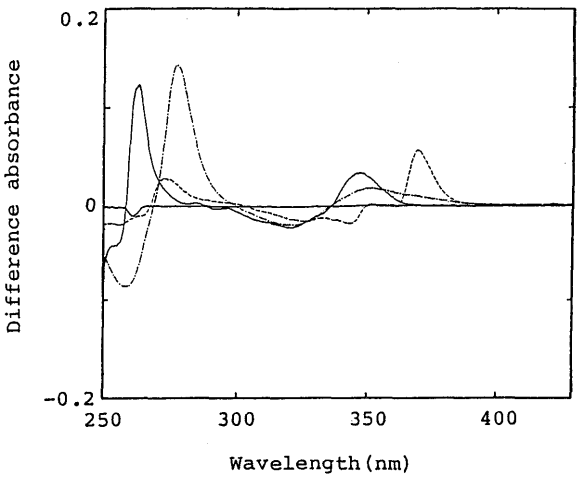


Fig. 1. Difference Absorption Spectra of NA (——), CINX (-----) and PPA (— - —) Produced by 50% EG at Apparent pH 7.9

Drug concentration (μg/ml): NA, 10.0; CINX, 9.85; PPA, 14.9.

In an attempt to evaluate the perturbation effect of EG on the absorption bands of the different quinolonecarboxylic acids, we adopted a quantity $\Delta\varepsilon/\varepsilon$, relative molar difference absorbance: $\Delta\varepsilon$ is the molar difference absorbance for the difference spectral peak and ε is the molar absorbance for the original absorption spectrum at the corresponding wavelength.

The values of $\Delta\varepsilon/\varepsilon$ obtained for the three drugs at their corresponding peak wavelengths were plotted against EG concentration. As shown in Fig. 2, the dependence of $\Delta\varepsilon/\varepsilon$ on EG concentration for the three drugs was found to form a single straight line, although $\Delta\varepsilon$ and ε themselves were different from one another among these compounds. The linearity was maintained up to EG concentrations of 50, 80 and 90% for PPA, NA and CINX, respectively.[6] Regression analysis for all the points in these ranges (Fig. 2) gave the following equation:

$$Y=0.0348X-0.00376 \quad (1)$$
$$n=20,\ r=0.997,\ s=0.00644$$

The applicability of such a single linearity suggests that the solvent perturbation effects on the electronic transitions concerned are similar among the quinolonecarboxylic acids.

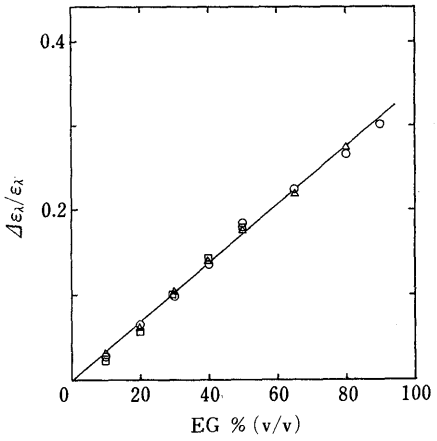


Fig. 2. EG-Concentration Dependence of Relative Molar Difference Absorbance at the Longest Wavelength Difference Spectral Maxima for CINX (○), NA (△) and PPA (□)

CINX- and NA–HSA System A) Difference Absorption Spectra Figures 3 and 4 (A1—C1 as solid lines) show difference spectra of the three drugs produced by HSA at protein concentrations of 0.18 and 4.0%, respectively. NA and CINX gave difference spectral patterns very similar to those in Fig. 1. The HSA-generated difference spectra showed characteristic peaks at 348—349, 321—322 and 263—264 nm for NA and 371—373 and 346—347 nm for CINX, commonly under the two different conditions (Fig. 3 and Fig. 4A1, B1). These peak wavelengths were almost the same as the EG-generated ones, 347.5, 321 and 263 nm for NA and 370.5 and 343 nm for CINX (Fig. 1).

The difference spectral data at the longest wavelength maxima for NA and CINX in the EG- and HSA–drug systems are summarized in Table I, together with the values of percent bound to HSA. Wavelength shifts ($\Delta\lambda$) of the

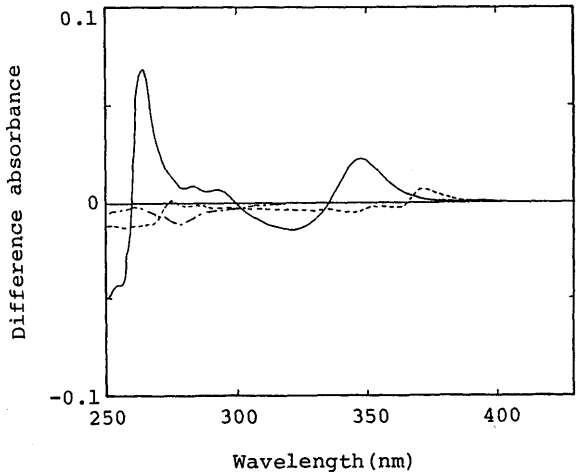


Fig. 3. Difference Absorption Spectra of NA (——), CINX (------) and PPA (— - —) Produced by 0.18% HSA

Drug concentration: 10 μg/ml. Molar ratios of drug/HSA are 1.4, 1.6 and 1.1 for CINX, NA and PPA, respectively.

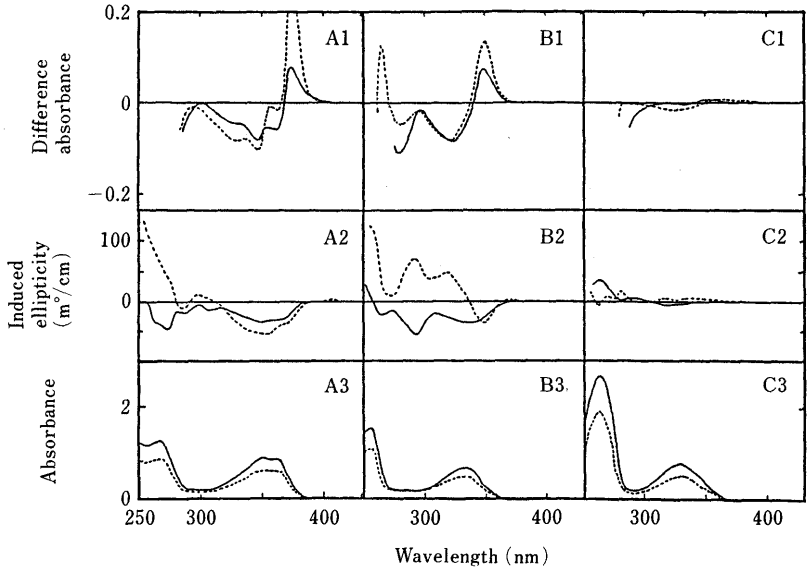


Fig. 4. Difference Absorption (1) and ICD (2) Spectra of CINX (A), NA (B) and PPA (C) Generated in the Presence of 4% HSA (——) and 2.7% RSA (------) at pH 7.4 and UV Absorption Spectra (3) of the Three Drugs

Molar ratio of drug/albumin = 1. Spectra (3) are UV absorption spectra of the drug solutions at the same concentrations as those in the respective drug–albumin systems.

TABLE I. Molar and Relative Molar Difference Absorbances at the Longest Wavelength Difference Spectral Maxima and Some Other Related Data for CINX and NA in Albumin and EG Solutions

Perturbant	Concentration (%)	CINX						NA					
		c_t[a] (μM)	$\Delta\lambda$[b] (nm)	λ[c] (nm)	$\Delta\varepsilon$	$\Delta\varepsilon/\varepsilon$	% bound[d]	c_t[a] (μM)	$\Delta\lambda$[c] (nm)	λ[c] (nm)	$\Delta\varepsilon$	$\Delta\varepsilon/\varepsilon$	% bound[d]
HSA	4.0	621	*ca.* 1	373	1300	0.183	44.9	617	3	349	1260	0.301	75.7
	0.18	38.1	<1	371	180	0.025	6.1	43.1	*ca.* 1	348	520	0.114	26.3
RSA	2.7	411	6	374	6650	1.163	84.1	418	4	349	3270	0.998	87.4
	0.09	18.3	1.5	374	2380	0.484	32.8	21.2	1.5	348	1360	0.394	30.7
EG[e]	50	38.1	2	370.5	1560	0.186	—	42.4	*ca.* 1	347.5	910	0.175	—

a) Total drug concentration. *b*) Shifts of the original absorption bands for CINX (363 nm) and NA (333 nm) by perturbants. *c*) The position of the longest wavelength difference absorption spectral peak. *d*) Calculated using the binding parameters in ref. 2. *e*) Corresponding data obtained with PPA (c_t=41.6 μM) were as follows: $\Delta\varepsilon$=980, $\Delta\varepsilon/\varepsilon$/0.177 ($\lambda$=351 nm) and the shift of the original absorption band (330 nm) $\Delta\lambda$=1.5 nm.

corresponding absorption bands are also listed.

The results show that the absorption bands of both drugs are perturbed to produce the difference spectral maxima, depending on their binding degrees to HSA. Similarities between the HSA- and EG-generated difference spectra indicate that these drugs bind to the binding site with less polar environment in the HSA molecule. In this system, the perturbant is the HSA molecule, or more precisely the less polar environment of the binding site, and the perturbant concentrations correspond to the binding degrees.

The values of $\Delta\varepsilon/\varepsilon$ observed at different concentrations of CINX, NA and HSA were plotted against percent bound to HSA. As shown in Fig. 5, $\Delta\varepsilon/\varepsilon$ was found to correlate linearly with percent bound. Regression analysis gave a straight line (Fig. 5) expressed by the following equation:

$$Y=0.00403X+0.00475 \quad (2)$$

$$n=6,\quad r=0.998,\quad s=0.00803$$

Difference spectra of two other analogues **1** and **2**[7] in 4% HSA solution and their corresponding binding degrees were separately measured. The $\Delta\varepsilon/\varepsilon$ values obtained were on the straight line, as shown in Fig. 5 (closed points).

The linearity of $\Delta\varepsilon/\varepsilon$ *vs.* percent bound allows us to obtain binding degrees of these quinolonecarboxyl acids at any concentrations of the compounds and HSA, simply by difference spectral measurements. In addition, Eqs. 1 and 2 yield some further information on the binding site of HSA. Extrapolation of Eq. 2 to 100% bound to HSA gives a $\Delta\varepsilon/\varepsilon$ value of 0.408, which results in an EG concentration of 118% EG by substitution into Eq. 1. This suggests that the binding site for the quinolonecarboxylic acids in the HSA molecule has a hydrophobic environment corresponding to 118% EG. Donovan[4] has assumed that the environment of the protein interior is equivalent to 120% EG, based on a comparison of the solvent perturbation difference spectra between a protein and model compounds. Thus the binding site in HSA for the quinolonecarboxylic acids seems to provide an environment like the protein interior.

B) Induced CD Spectra Since the drugs used are all optically inactive in the unbound state, the ICD spectra should originate from the bound drug species. ICD spectra of the three drugs in 4% HSA solution are shown in Fig. 4A2—C2 (solid lines). CINX and NA gave characteristic ICD spectra (Fig. 4A2 and B2). The magnitude of the ICD bands ($\Delta\theta/l$) at wavelengths above 280 nm and the corresponding anisotropy factor (g values)[3a,8] are summarized in Table II.

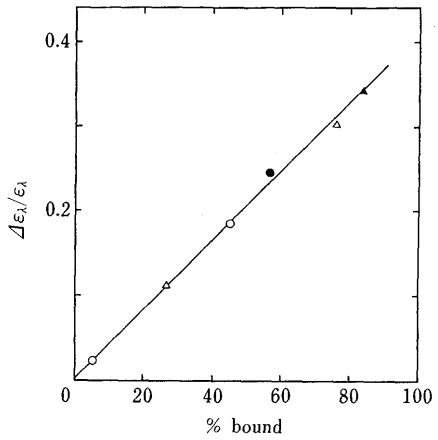
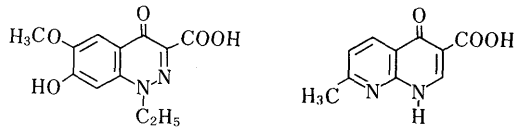


Fig. 5. Correlation between Percent Bound to HSA and Relative Molar Difference Absorbance at the Longest Wavelength Difference Spectral Maxima for CINX-(○) and NA-(△) HSA Systems at pH 7.4

The data for **1** (●) and **2** (▲) are also plotted.



1 **2**

Chart 1

TABLE II. Ellipticities and Anisotropy Factors for ICD Bands of CINX and NA Generated in Albumin Solutions

Albumin	c_p[a] (%)	CINX			NA		
		λ (nm)	$\Delta\theta/l$ (m°/cm)	$g\times10^4$	λ (nm)	$\Delta\theta/l$ (m°/cm)	$g\times10^4$
HSA	4.0	371	−28.4	−3.3	342	−34.3	−2.6
		350	−35.4	−2.7	295	−56.1	−11.1
		290	−17.4	−6.1	—	—	—
RSA	2.7	405	+1.88	[b]	370	+2.46	[b]
		371	−36.8	−3.4	348	−37.5	−5.7
		352	−54.9	−3.2	319.5	+46.2	+4.4
		295	+13.0	+3.8	294	+73.5	+19.6

a) Protein concentration. *b*) Absorbances at these wavelengths were too low to obtain exact g values under the UV spectral conditions employed.

As shown in Table II, the absolute $\Delta\theta/l$ value of CINX at 371 nm (28.4) was smaller than that of NA at 342 nm (34.3). This is consistent with the order (CINX < NA) of percent bound of the two drugs to HSA (Table I) under the same conditions as in the CD measurements. It seems likely that

the binding degrees of CINX and NA are approximately reflected by the magnitude of the ICD bands at these two wavelengths.

Differing from the case of the $\Delta\theta/l$ values, on the other hand, g values at the corresponding ICD bands (342—371 nm) were similar for the two drugs. The g value at a CD band for an optically active compound is defined generally as[3a,8]:

$$g=[\theta]/3300\varepsilon=(\theta/lc)/33\varepsilon \quad (3)$$

where $[\theta]$ is the molar ellipticity in degree·cm^2·dmol^{-1} calculated by $[\theta]=(\theta/lc)\times 100$, θ is the observed ellipticity in degrees, l is the cell-length in cm, and c and ε are the molar concentration and the molar absorbance of the compound, respectively. In the case of ICD bands for the drug–HSA system, c corresponds to the concentration of the bound drug (c_b) and θ must be replaced by $\Delta\theta$ as follows:

$$\Delta\theta=\theta(\text{albumin–drug mixture})-\theta(\text{albumin}) \quad (4)$$

Then,

$$g=(\Delta\theta/lc_b)/33\varepsilon \quad (5)$$

This is the g value for the ICD band, and represents the magnitude of the band as normalized with respect to the binding degree and the molar absorbance. Such values permit us, therefore, to evaluate the ICD spectral amplitudes upon ligand binding between ligands with different binding degrees and different molar absorbances.

Thus the similar g values for CINX and NA obtained at 342—371 nm suggest that both drugs have a similar ability to generate the ICD spectra upon binding to HSA, at least in the longer wavelength region.

However, in the shorter wavelength region, the CD data could not be interpreted in a similar manner: NA (295 nm) showed an absolute g value much higher than CINX (294 nm). It is unknown at present whether this might reflect some difference in the electronic transitions between the two drugs, some contribution of the chromophores in HSA, such as tryptophan and tyrosine side chains, or other factors.

CINX– and NA–RSA System A) Difference Absorption Spectra Difference spectra of the three drugs in 2.7% RSA solution are shown in Fig. 4 (A1—C1 as broken lines). CINX and NA showed difference spectral patterns very similar to those with HSA, with the longest wavelength peaks at 374 and 349 nm, respectively (Fig. 4A1 and B1). However, the peak intensities of both drugs were much higher than those observed for the drug–HSA system.[9]

Table I summarizes the difference spectral data for CINX and NA, together with corresponding binding degrees calculated using their binding parameters.[2]

Figure 6 shows the plot of $\Delta\varepsilon/\varepsilon$ values obtained at various concentrations of the two drugs and RSA against percent bound. In this case, however, the plot could not be represented by a single straight line. Regression analyses gave two linear Eqs. 6 and 7 for CINX and NA, respectively, with slopes much higher than that in Eq. 2.

$$Y=0.0151X+0.032 \quad (6)$$
$$n=8,\ r=0.982,\ s=0.0793$$

$$Y=0.0104X+0.034 \quad (7)$$
$$n=8,\ r=0.991,\ s=0.0415$$

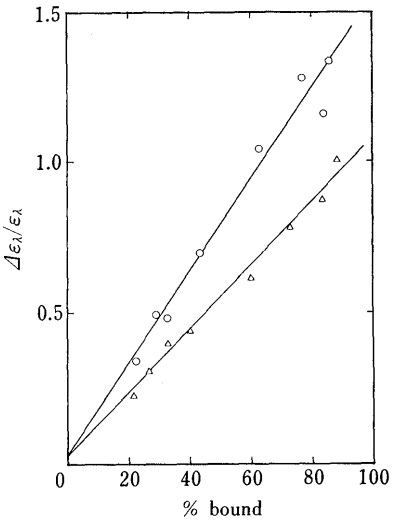


Fig. 6. Correlation between Percent Bound to RSA and Relative Molar Difference Absorbance at the Longest Wavelength Difference Spectral Maxima for CINX–(○) and NA–(△) RSA Systems at pH 7.4

The absence of any significant difference between the intercept value and zero was confirmed by the t test. Although $\Delta\varepsilon/\varepsilon$ values for CINX and NA show such different dependences on percent bound to RSA, Eqs. 6 and 7 can be used for obtaining binding degrees of the respective drugs to RSA from UV difference spectral measurements.

These results probably reflect one of the characteristics in the RSA–drug interactions that binding of the two drugs to RSA can no longer be interpreted merely in terms of their lipophilicity,[2] *i.e.*, their hydrophobic interactions in the binding site: even the less lipophilic CINX can bind to RSA as strongly as the more lipophilic NA.[2] In the case of CINX, additional factors such as hydrogen bonding may be involved in its interaction with RSA, contributing largely to the difference spectra. While NA shows very similar difference spectral patterns above 300 nm between RSA and HSA (Fig. 4B1), CINX gives negative shoulders at 363 nm with considerably different intensities from HSA to RSA (Fig. 4A1).

From extrapolation of Eqs. 6 and 7 to 100% bound to RSA, $\Delta\varepsilon/\varepsilon$ values of 1.54 and 1.07 were obtained. Even the latter value for the NA–RSA system is much higher than the corresponding value for the drug–HSA system (0.408). It seems, therefore, that the binding site for the two drugs in RSA provides an environment with a much more nonpolar nature for the bound species than that in HSA.

B) Induced CD Spectra Figure 4 (A2—C2) shows ICD spectra of the three drugs in 2.7% RSA solution. CINX and NA generated characteristic spectra with RSA. The ICD spectra observed for these drugs in the wavelength regions below 310 nm for CINX and 335 nm for NA were positive, whereas those for the drug–HSA system were negative. In the longer wavelength region, slightly positive ICD bands were observed at 405 and 370 nm for CINX and NA, respectively. The numerical data for the ICD bands at wavelengths above 280 nm are listed in Table II.

The absolute $\Delta\theta/l$ values of the ICD bands for CINX at 405, 371 and 352 nm were comparable with those for NA at

370, 348 and 320 nm, respectively. These results are consistent with the similar binding degrees to RSA between CINX and NA (Table I). Furthermore, similar $\Delta\theta/l$ values are observed for CINX (−36.8) and NA (−37.5) in RSA at 371 and 348 nm, respectively, and for NA (−34.3) in HSA at 342 nm (Table II), corresponding to similar binding degrees, 84, 87 and 76% (Table I), respectively. Therefore, the magnitude of the ICD bands (absolute $\Delta\theta/l$ values) at these wavelengths can be regarded at least qualitatively as a measure of the binding degrees of the two drugs to RSA as well as to HSA.

The absolute g values at 371 and 350 nm for CINX and at 348 nm for NA were of approximately the same order, suggesting a similar ability to generate the ICD spectra upon binding to RSA between the two drugs in these longer wavelength regions, as in the case of the drug–HSA system.

Different Features between HSA and RSA When the ICD spectral results are compared between HSA and RSA, some different features can be seen. One is the appearance of the small positive CD bands for both CINX (405 nm) and NA (370 nm) in RSA solution. This is probably related to large red shifts of 4—6 nm induced by RSA at the longest wavelength absorption bands for CINX and NA, as compared with small shifts of 1—3 nm by HSA and EG (Table I). PPA and its derivatives in RSA solution also generated small positive ICD bands in the wavelength range of 320—385 nm (Figs. 4C2 and 8B), although such bands were not observed with HSA. Moreover, RSA showed high affinities even to drugs with relatively low lipophilicity such as CINX, despite the fact that HSA had rather low affinities for them.[2] These results may be attributed to the more nonpolar nature of the binding site in RSA.

Another difference is the inversion in the sign, negative (HSA) to positive (RSA), of the ICD spectra for both drugs between HSA and RSA at wavelengths below 335 nm. The extent of inversion was especially marked for NA.

The magnitude and the sign of ICD bands generated by ligand binding to albumin depend on the distance and the spatial relationships between the ligand chromophore and the asymmetric center in the binding site, respectively, as well as on the rigidity of the ligand–albumin complex formed.[3a] It seems likely from this viewpoint that the marked inversion observed with NA sensitively reflects subtle differences in the structure and environment of the binding site and in the bound state of the NA molecule between HSA and RSA.

PPA and Other Positively Charged Compounds in the Albumin System In contrast to CINX and NA, PPA generated little difference absorption and ICD spectra (Fig. 4C1 and C2) in albumin solutions. This is consistent with no significant binding of this drug to HSA.[2] Such peculiar characteristics of PPA were investigated in more detail with reference to the ionization state of this drug and related compounds in aqueous solution, in addition to their CD spectral response in albumin solutions.

A) Betaine Form of PPA in Solution It is known that the structure of PPA in the solid state is a betaine form **4a**.[10] Since PPA might isomerize to an uncharged form **4b** in

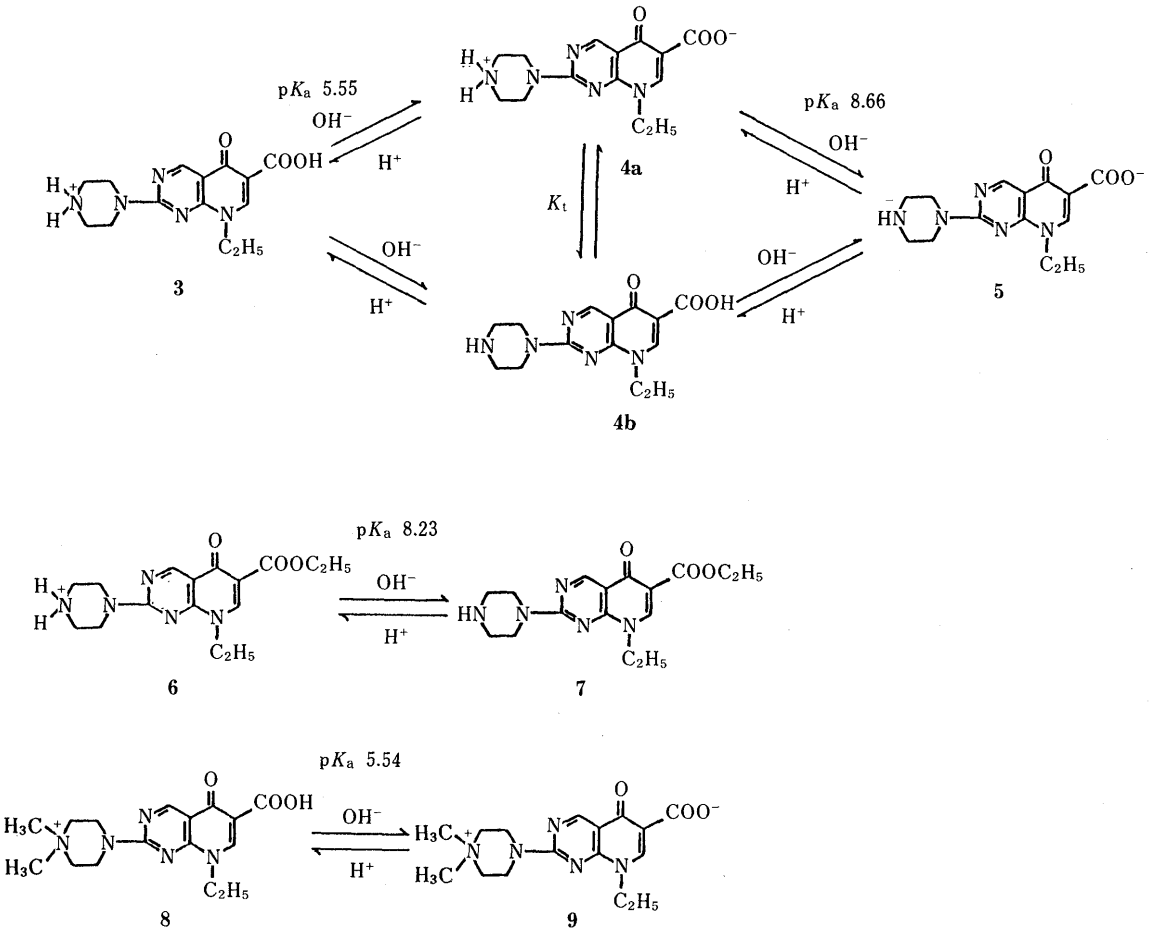


Chart 2

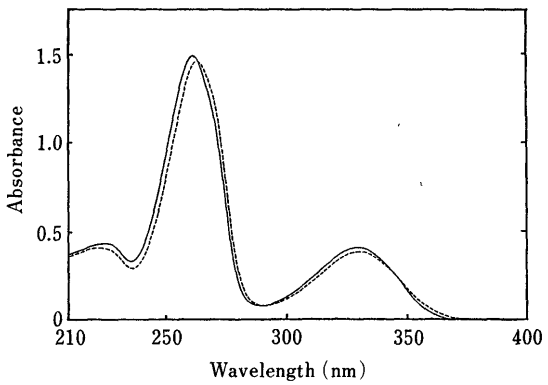


Fig. 7. UV Absorption Spectral Comparison between PPA **4** (------) and Its Dimethylpiperazinyl Betaine **9** (——) at pH 7.4

Concentration: 10 μg/ml.

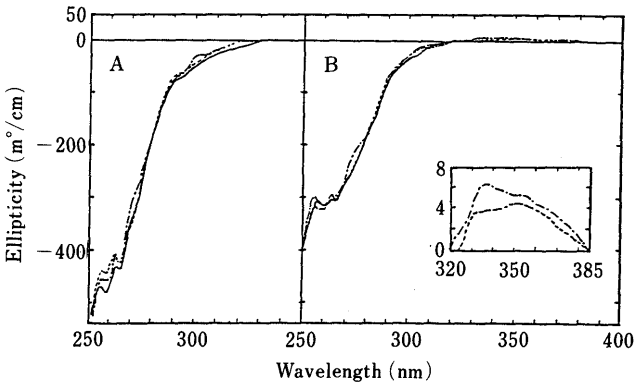


Fig. 8. Effects of Two PPA Derivatives **6** and **9** on the CD Spectra of 4.0% HSA (A) and 2.7% RSA (B) at pH 7.4

Albumin alone (——); albumin + **9** (------); albumin + **8** (— - —). Molar ratio of ligand/albumin = 1. CD spectra at 320—385 nm in the insert are shown on a magnified scale.

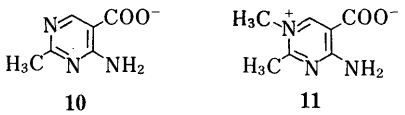


Chart 3

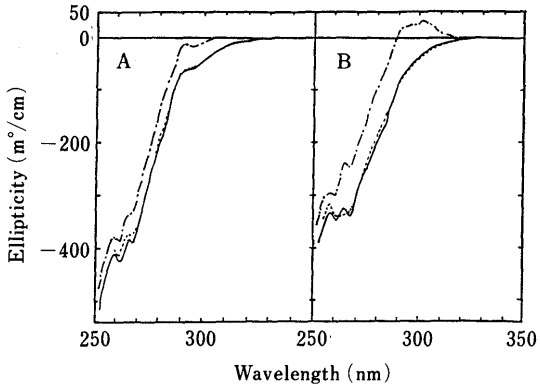


Fig. 9. Effects of **10** and **11** on the CD Spectra of 4% HSA (A) and 2.7% RSA (B) at pH 7.4

Albumin alone (——); albumin + **11** (------); albumin + **10** (— - —). Molar ratio of ligand/albumin = 5.

solution, we examined which form mainly exists in the tautomeric equilibrium. Each tautomer **4a** and **4b** forms corresponding acid–base equilibria by protonation and dissociation, as shown in Chart 2.

The two pK_a values of 5.55 and 8.66[11] for PPA have been obtained titrimetrically.[2] The former value corresponds to the dissociation constant of the cation **3**. To decide to which tautomer this cation would dissociate, pK_a values of two PPA derivatives **7** and **9** were measured. These derivatives with either of the ionizable groups of PPA being blocked by alkyl substituents were used as model compounds of **4a** and **4b**. They form protonation equilibria similar to those of the two tautomers. The pK_a value of **7** (8.23)[11] was quite different from that of PPA (5.55). On the other hand, the pK_a value of **9** (5.54) was in good accord with that of PPA. The results show that PPA exists mainly in the betaine form **4a**, not in the uncharged form **4b**. Therefore, another pK_a value of PPA 8.66 corresponds to the dissociation of the piperazinyl group.

The presence of **4a** form was also supported by the UV spectra. The spectrum of PPA at pH 7.4 was in close agreement with that of **9**, as shown in Fig. 7 . Protonation of PPA and **9** by increasing acidity caused similar changes in the respective spectra. The results show that PPA exists in the betaine form (**4a**) in aqueous solution just as in the solid state. The betaine form (**4a**) of PPA at pH 7.4 is probably one of the main reasons why little or no binding occurs to albumins, as was also suggested in the preceding paper.[2]

B) CD Spectral Response with HSA CD spectra of the two PPA derivatives **7** (mainly existing as form **6** at pH 7.4) and **9** in 4% HSA solution at pH 7.4 were measured. As shown in Fig. 8A, they were almost the same as the CD spectrum of 4% HSA solution alone. This indicates little or no binding of these PPA derivatives to HSA. Thus the positively charged group in the quinolone compounds can be regarded as an unfavorable factor for their binding to HSA, irrespective of the presence or absence of the negatively charged moiety.

Such an effect seems to explain well the lower serum protein binding (20—30%)[12] observed for ciprofloxacin,[12a] pefloxacin,[12b] and enoxacin,[12c] whcih probably also have positively charged groups as betaine forms in neutral solution.

A simpler betaine, 4-amino-2-methyl-5-pyrimidinecarboxylic acid 1-methyl betaine[13] (**11**) provided a similar example. As shown in Fig. 9, **11** did not affect the CD spectrum of 4% HSA solution at pH 7.4. On the other hand, **10**, lacking such a positively charged group, altered the CD spectrum of HSA, which shows that a negatively charged group alone favors the binding to HSA.

C) CD Spectral Responses with RSA CD spectra of **7**, **9**, **10** and **11** in 2.7% RSA solution were also measured. The results are shown in Figs. 8B and 9B. The effects of these compounds on the CD spectrum of 2.7% RSA were essentially the same as those in the case of 4% HSA. Consequently, the conclusion derived from the CD results with HSA also applies to the case of RSA: the positively charged groups in these ligands act as an unfavorable factor for binding to RSA as well.[14]

However, the PPA derivatives in RSA solution produced slightly positive CD bands in the longer wavelength region (320—385 nm) as shown in Fig. 8B (insert). In addition, the CD spectrum of **10** in RSA solution (Fig. 9B) was larger than that of HSA solution (Fig. 9A). The more non-

polar nature of the binding site in RSA may result in such differences.

Conclusions

In summary, we have reached the following conclusions. 1) The use of difference absorption and CD spectral responses as performed in this work should be effectively applicable to protein binding studies for other drugs with absorption above 300 nm. 2) A quantity $\Delta\varepsilon/\varepsilon$ at the longest wavelength difference absorption spectral maxima for some quinolonecarboxylic acids in albumin solution can be used as a measure of percent bound to HSA and RSA. 3) The fact that little or no binding of PPA occurs is due to the positively charged center in the betaine structure, and this may also be the case for other quinolone drugs having low serum protein binding.

Experimental

Materials HSA and RSA were purchased from Sigma Chemicals Co. EG used was of chromatographic grade. The sources of CINX, PPA, NA, and other chemicals used were the same as described in the preceding paper.[2] Compounds **10** and **11** were synthesized according to Mizukami and Hirai.[15] Compounds **7** and **9** were newly synthesized as described below. HSA and RSA were dissolved in 0.1 M phosphate buffer (pH 7.4) containing 0.2% NaCl. Protein concentrations were determined spectrophotometrically using $E^{1\%}_{1cm}$ values of 5.3 (279 nm)[16] for HSA and 6.9 (280 nm)[17] for RSA. Preparation of drug or ligand solutions and pK_a determination of the two PPA derivatives were performed in the reported manner.[2]

Preparation of 7 Sulfuric acid (10 ml) was added dropwise to a solution of 3 g of PPA in 100 ml of EtOH in an ice bath. The mixture was refluxed for 6 h. After filtration, the filtrate was neutralized with $NaHCO_3$ in an ice bath and evaporated to dryness under reduced pressure. The residue was dissolved in H_2O and the solution was filtered. Concentration of the filtrate and then addition of EtOH–Et_2O deposited crystals. Recrystallization from EtOH–Et_2O gave 0.52 g of white plates, mp 159—160.5 °C. IR (Nujol) cm^{-1}: 3450, 3200, 1730, 1680, 1620. *Anal.* Calcd for $C_{16}H_{21}N_5O_3$: C, 57.99; H, 6.39; N, 21.14. Found: C, 57.85; H, 6.20; N, 20.79.

Preparation of 9 A solution of 3 g of PPA in 500 ml of $CHCl_3$ was treated with CH_3I (50 ml). The mixture was refluxed on a steam bath for 11 h, then the precipitates were filtered off, washed with ether and dried *in vacuo*. The methiodide was obtained in 85% yield, pale yellow plates, mp 290 °C (dec.). IR (Nujol) cm^{-1}: 3400 (br), 1700. *Anal.* Calcd for $C_{15}H_{20}N_5O_3 \cdot 1/2\,H_2O$: C, 39.66; H, 4.66; N, 15.42. Found: C, 39.81; H, 4.33; N, 15.57. A solution of 2.5 g of the methiodide in H_2O was treated with $NaHCO_3$ and then Amberlite IRA-400 resin until iodide ion was completely eliminated. After filtering off the resin, the filtrate was evaporated to dryness under reduced pressure. The white and hygroscopic residue (3.1 g) was dissolved in H_2O and neutralized with 1 N HCl. The solution was evaporated to dryness under reduced pressure, and a solution of the residue in 90 ml of MeOH was treated with 30 ml of CH_3I. The mixture was refluxed for 2 h, then filtered, and the filtrate was evaporated under reduced pressure. The products were taken up in H_2O and purified by HP-20 column chromatography. The eluate was evaporated under reduced pressure. Recrystallization from H_2O–EtOH gave 0.27 g of colorless needles, mp > 300 °C. IR (Nujol) cm^{-1}: 1640, 1620. *Anal.* Calcd for $C_{16}H_{21}N_5O_3$: C, 57.99; H, 6.39; N, 21.14. Found: C, 57.72; H, 6.23; N, 20.96.

Difference Spectrophotometry For difference spectral measurements, three sets of solutions were prepared using pH 7.4 buffer as a solvent: a ligand (CINX, NA or PPA) solution and a perturbant (EG or albumin) solution and a ligand-perturbant mixture which contains each component at the same concentration as the respective ligand and perturbant solutions. Difference spectra were measured with a pair of matched cells at room temperature, using a Shimadzu UV-240 spectrophotometer equipped with an option unit OPI-1, in the following way. The spectrum of the ligand solution was first recorded and memorized by use of the option unit. The difference spectrum was then obtained by recording the spectral difference between the ligand-perturbant mixture *vs.* the perturbant solution and the ligand solution *vs.* buffer, using the memorized spectrum for the latter. Cells with shorter (1-mm) and longer (5- or 10-mm) pathlengths were used for solutions at higher and lower solute (albumin and ligand) concentrations, respectively.

CD Spectroscopy CD spectra of albumins and albumin-ligand mixtures in pH 7.4 buffer were measured at room temperature with a Jasco J-40 spectropolarimeter using 0.5 and 2 mm cells for wavelength regions below and above 330 nm, respectively.

In this paper, CD spectra were represented by the observed ellipticities as normalized with respect to different cell-lengths, θ/l (millidegree/cm) for simplicity, though they are usually represented by molar ellipticities. ICD spectra for ligands were constructed by subtracting the CD spectrum of albumin solution alone from those of corresponding albumin–ligand mixtures. The g values were calculated from Eq. 5.

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References and Notes

1) A part of this work was presented at the 105th Annual Meeting of the Pharmaceutical Society of Japan, Kanazawa, April 1985. The following abbreviations are used: CINX = cinoxacin, NA = nalidixic acid, PPA = pipemidic acid, HSA = human serum albumin, RSA = rat serum albumin, EG = ethylene glycol, ICD = induced circular dichroism.
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6) Deviation of $\Delta\varepsilon/\varepsilon$ for PPA and NA from the straight line was observed at higher EG concentrations. This was due to spectral changes originating probably from the unionized species produced in the media.
7) This compound (**2**) is assumed to be a keto form but not an enol form in neutral solution, since we have observed a similar UV spectral pattern between **2** and NA at pH 7.4.
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10) I. Fonseca, S. Martinez and S. Blanco, *Acta Crystallogr., Sect. C*, **42**, 1618 (1986).
11) Both PPA and **7** showed UV spectral changes with increasing pH. Using the spectral data, we obtained pK_a values of 8.67 and 8.34 for their respective piperazine moieties. These are in good agreement with those obtained by titrimetry, 8.66 and 8.23, respectively. These results are suggestive of some effect of protonation and dissociation at the piperazine nitrogen on the pyridopyrimidine skeleton, producing absorption spectral changes.
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14) Tryptophan shows high affinities to both HSA and RSA at pH 7.4 [F. C. I. Fellows and F. J. R. Hird, *Arch. Biochem. Biophys.*, **216**, 93 (1982)], although it exists as a zwitterionic form in neutral solution. There seems to be little effect of its positively charged group on the binding to both albumins. This is probably because tryptophan has a highly lipophilic side chain, being quite different from the betaine compounds used in this work.
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