Protein Binding of Quinolonecarboxylic Acids. I. Cinoxacin, Nalidixic Acid and Pipemidic Acid[1]

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Binding of cinoxain (CINX), nalidixic acid (NA) and pipemidic acid (PPA) to serum proteins was investigated by equilibrium dialysis, ultrafiltration and circular dichroism (CD) spectroscopy. CINX and NA were found to bind mainly to albumin in human serum, the latter interacting with the protein about ten times as strongly as CINX at pH 7.4 and 37 °C. PPA showed little or no significant binding to human serum albumin (HSA), α1-acid glycoprotein, and globulins, but showed 20—30% binding to protein in human serum. The CD results were suggestive of some weak interaction of PPA with human apotransferrin. Binding of the three drugs to HSA was found to depend on the lipophilicity of their substituents at the 7-position. The degree of protein binding for human, dog and rat sera at 37 °C was in the order of NA (92—97%) > CINX (68—90%) > PPA (20—30%) at drug concentrations of 10—30 μg/ml. CINX showed relatively large species dependence in serum protein binding, which seemed to be due to different affinities of this drug to the respective albumins. CINX was found to bind to rat serum albumin as strongly as NA.

Keywords cinoxacin; nalidixic acid; pipemidic acid; albumin; binding parameter; substituent effect; lipophilicity; pK_a; serum-protein binding; species dependence

Since the first discovery of the quinolone drug nalidixic acid (NA),[2] a number of its congeners such as oxolinic acid,[3] piromidic acid,[4] cinoxacin (CINX),[5] and pipemidic acid (PPA)[6] have been developed. Recently fluoroquinolones[7c,d] have come to be widely used. Concurrently, various characteristics of the drugs have been studied.[7] However, their binding properties are not fully understood: only serum protein binding data have been reported for several drugs.[5,8] Thus, we initially selected CINX, NA and PPA to investigate their interaction with serum proteins in relation to their physicochemical properties. In this paper we show that the properties of the substituent at the 7-position of the quinolonecarboxylic acids result in a great difference in their binding to serum proteins.

Experimental

Materials The sources of materials used in this work were as follows: CINX, NA, and PPA from Shionogi, Nihon Sieber Hegner, and Dainippon Pharmaceutical Co., Ltd., respectively, human serum albumin (HSA), α1-acid glycoprotein (AGP), essentially iron-free transferrin (TF) and γ-globulin (Cohn fraction II) from Sigma Chemicals Co., human α-globulins (Cohn fractions IV-1 and IV-4) from ICN Pharmaceuticals Inc., and human β-globulin (Cohn fraction III) from Wako Pure Chemical Industries, Ltd. Rat serum albumin (RSA) was a product (fraction V) of Sigma Chemicals Co. 1-Octanol was purchased from E. Merck Co. Human serum was obtained from healthy male volunteers. Dog and rat sera were collected from beagle dogs and Sprague Dawley rats, respectively. Other chemicals used were of reagent grade.

Protein and Drug Solutions HSA, RSA, AGP, TF and globulins were dissolved in 0.1 M phosphate buffer containing 0.2% NaCl at pH 7.4. Protein concentrations were determined spectrophotometrically using $E^{1\%}_{1cm}$ values of 5.3 (279 nm),[9] 6.9 (280 nm),[10] 8.9 (278 nm),[11] and 14.1 (280 nm)[12] for HSA, RSA, AGP and TF, respectively. For each globulin solution, insoluble components were removed by centrifugation and/or filtration and the protein concentration was determined on the dry weight basis. Drug was dissolved in 0.1 or 0.02 M borate buffer containing 0.2% NaCl at pH 8.9 to make 1 mg/ml and then diluted with pH 7.4 buffer to make 0.1—0.3 mg/ml. These solutions were further diluted with pH 7.4 buffer to required concentrations.

Equilibrium Dialysis As an inner solution, 1 ml of 4% HSA solution was taken into Visking tubing (6.4 mm i.d.) and dialyzed against 3 ml of 2.5—26 μg/ml drug solution as an outer solution at 37 °C for 15 h. In control experiments pH 7.4 buffer was used instead of 4% HSA solution. In some cases solution volumes of 3 and 10 ml were used as inner (V_i) and outer (V_o) volumes, respectively (Visking tubing, 14.3 mm i.d.). Drug concentrations of the outer solutions after equilibration (c_f and c_r for HSA-drug solutions and control, respectively) were quantitated spectrophotometrically at the positions of the longest wavelength absorption extrema (351, 333 and 330 nm for CINX, NA and PPA, respectively). The volumes of inner solution were also measured after completion of the dialysis. Concentrations of bound drug (c_b) were calculated from the relationship $c_b = (c_f - c_r) \times (V_i + V_o)/V_i$.

Equilibrium dialysis experiments for RSA–drug system were also performed at a protein concentration of 2.7% in a similar manner.

Ultrafiltration Into an Amicon ultrafiltration cell (model 12) equipped with a Diaflo membrane (PM 10), 10 ml of human, dog or rat serum was introduced and 1 ml of drug solution at a concentration of 0.1—0.3 mg/ml was added. The cell was set in a water jacket at 37 °C and allowed to stand for 10—15 min with stirring. Then ultrafiltration was started at an average rate of 0.1 ml/min under a nitrogen gas pressure of 0.2—0.4 kg/cm^2. The ultrafiltrate was fractionated and collected into four pre-weighed test tubes. The volumes of the four fractions (*ca.* 0.5 ml) were determined accurately by weighing each fraction. Drug concentrations of the fractions were quantitated spectrofluorometrically using a Hitachi MPF-2A spectrofluorometer: the mixture of each fraction with 2.5 ml of 0.15 N HCl was excited at 355, 323 and 316 nm with resulting emission at 432, 448 and 354 nm for CINX, PPA and NA, respectively. Ethanol solutions of anthracene (0.042 μg/ml) were used as a standard for fluorescence intensities. Free drug concentrations were obtained as average values of the drug concentrations for the last three fractions. Ultrafiltration of albumin and globulin solutions was also performed in a similar manner. Protein concentrations of the globulin solutions used were 0.34, 0.91, 0.66, and 0.85% for α-globulins, Cohn fraction IV-1 and IV-4, β-globulin, and γ-globulin, respectively.

Circular Dichroism (CD) Spectroscopy CD spectra of AGP– and TF–PPA systems at pH 7.4 were measured with a Jasco J-40 spectropolarimeter using a 0.5 or 1 cm cell at room temperature. Concentrations of AGP, TF, and PPA were 1.0 mg/ml, 0.7 mg/ml, and 10 μg/ml, respectively. For construction of CD curves, ellipticities corrected for cell length θ_λ/l (m°/cm) were used, where θ_λ is the observed ellipticity in millidegrees at a wavelength λ and l is the cell-length in cm.

pK_a Measurements The pK_a values for the carboxylic acid groups of CINX and NA were determined at room temperature (25±2 °C) by a spectrophotometric method using Britton and Robinson's buffer system[13] at ionic strength 0.1, and were corrected thermodynamically as usual.[14] A titrimetric method was applied to pK_a determination of PPA, using a Radiometer DTS 833 potentiometric titrimeter. The pH values were measured with a Radiometer PHM 60 pH meter. Spectral measurements were carried out at sample concentrations of *ca.* 10 μg/ml, using a Shimadzu UV-240 spectrophotometer.

Partition Coefficient Measurements Octanol/water partition coefficients (log P) were determined using Britton–Robinson buffers at pH's near the respective pK_a for CINX and NA and at an intermediate pH between the two pK_a's for PPA. Drugs were dissolved in the respective buffers saturated with 1-octanol at concentrations of 10—20 μg/ml. The drug solutions were equilibrated with buffer-saturated 1-octanol at three volume ratios, 3:7, 5:5, and 7:3 (ml/ml), in 12-ml glass-stoppered vessels by inverting them about 60 times by hand for 2 min.[15] After centrif-

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ugation, drug concentrations in the aqueous phase (c_w) were determined spectrophotometrically and those in the 1-octanol phase (c_o) were calculated. The partition coefficients at the three volume ratios were calculated from the relationship $P=c_o/c_w$ and then averaged.

Results and Discussion

Serum Protein Binding for Human, Dog and Rat Table I shows results of protein binding of the three drugs in human serum together with those for dog and rat sera at 37 °C in the drug concentration range of 10—30 μg/ml. The degrees of protein binding to human serum for these drugs were in the order of NA > CINX > PPA. A similar tendency of serum protein binding was also observed for animals (dog and rat). However, it should be noted that serum protein binding of CINX is characterized by species dependence (68, 80 and 90% on average for human, dog and rat, respectively), while those of NA (92—97%) and PPA (20—32%) are within relatively narrow ranges. In rat serum, furthermore, the binding degree of CINX becomes almost equal to or somewhat higher than that of NA at lower drug concentrations (Table I).

We thus investigated binding of these drugs to HSA and RSA in buffered solutions (pH 7.4) at protein concentrations near the normal level[16] in each serum by equilibrium dialysis at 37 °C.

Binding to HSA and RSA The equilibrium dialysis results were analyzed by means of a double reciprocal plot, $1/r$ *vs.* $1/c_f$, based on the equation:

$$1/r = 1/n + 1/nkc_f$$

where r is mol of bound drug per mol of albumin, n is the number of binding sites, k is the binding constant, and c_f is the free drug concentration. The plots for HSA–drug and RSA–drug systems are shown in Figs. 1 and 2, respectively. The binding parameters obtained from these plots are summarized in Table II.

The binding affinity to HSA is higher by one order of magnitude for NA than for CINX, whereas both drugs show similarly high affinities to RSA. This is suggestive of a considerable difference in the nature of the binding site between HSA and RSA.

In the drug concentration range of 10—30 μg/ml, the average binding degrees of CINX and NA to 4% HSA were 63 and 93% and those to 2.7% RSA were 95 and 94%, respectively. These values of percent bound correspond well to the results of serum protein binding for human and rat (Table I). It can therefore be concluded that at least for human and rat sera (and possibly also for dog serum), CINX and NA bind mainly to albumin among serum proteins.

On the other hand, PPA showed little or no difference in its concentration between the control (PPA alone) and sample solution (PPA–HSA) in the equilibrium dialysis, indicating that PPA has no significant binding to HSA. This was also supported by ultrafiltration results for the 4% HSA–20 μg/ml PPA system at pH 7.4: no significant difference between the drug (PPA) concentrations of the ultrafiltrate in the presence and absence of 4% HSA was observed. The possibility of binding of PPA to HSA at higher pH was also ruled out by ultrafiltration experiments separately performed for the 4% HSA–20 μg/ml PPA system at pH 7.8. Although there was no significant binding of PPA to HSA, this drug did show 23—27% serum protein binding, in agreement with the results (about 30% at pH 7.2) of Shimizu *et al.*[8b] These results imply that PPA binds to human serum protein(s) other than HSA.

TABLE I. Protein Binding of CINX, NA, and PPA to Sera from Human, Dog, and Rat at 37 °C

Serum[a]	CINX		NA		PPA	
	C_t[b] (μg/ml)	% bound	C_t[b] (μg/ml)	% bound	C_t[b] (μg/ml)	% bound
Human	9.43	64.6	9.07	95.7	9.49	26.6
	19.31	71.1	18.67	92.8	18.95	23.2
	29.08	69.0	28.34	94.0	—	—
Dog	9.47	80.8	9.38	96.9	9.38	31.7
	19.25	79.2	19.31	95.1	18.66	30.0
	29.04	79.0	29.27	95.3	28.05	31.7
Rat	9.60	94.1	9.33	91.9	9.26	23.0
	19.44	89.2	19.26	92.2	18.48	19.6
	29.23	85.6	29.16	91.6	—	—

a) pH values of serum samples were 7.8, 7.6 and 7.9 for human, dog and rat, respectively. *b*) Total drug concentration.

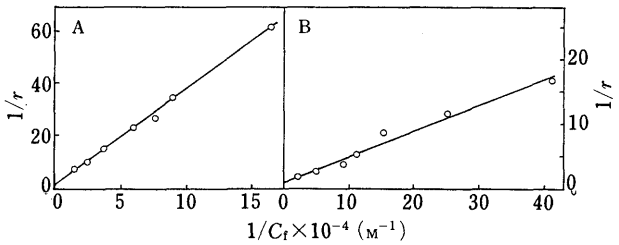

Fig. 1. Double Reciprocal Plots for CINX–HSA (A) and NA–HSA (B) Systems at pH 7.4 and 37 °C

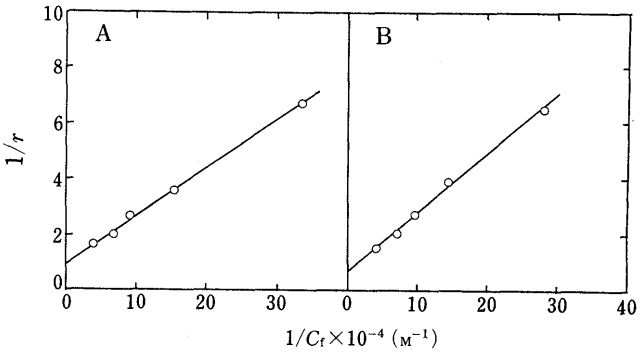

Fig. 2. Double Reciprocal Plots for CINX–RSA (A) and NA–RSA (B) Systems at pH 7.4 and 37 °C

TABLE II. Binding Parameters of CINX and NA in the Interaction with Human and Rat Albumins at pH 7.4 and 37 °C

Serum albumin	CINX		NA	
	n	$k\times10^{-4}$ (M^{-1})	n	$k\times10^{-4}$ (M^{-1})
Human	0.9±0.3	0.29±0.09	0.9±0.4	2.6±1.0
Rat	1.1±0.1	5.3 ±1.0	1.4±0.2	3.4±0.5

Binding of PPA to Other Human Serum Proteins

We examined binding of PPA at a concentration of 20 μg/ml to human globulins by the ultrafiltration method. However, PPA showed no significant binding to α-, β- and γ-globulins.

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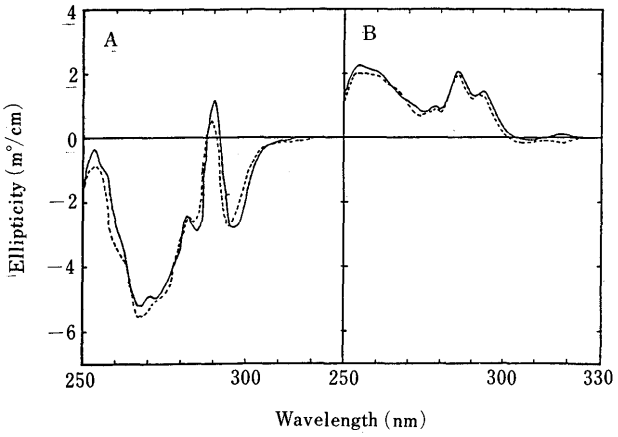

Fig. 3. CD Spectra of TF (A) and AGP (B) in the Presence (– – – –) and Absence (——) of PPA at pH 7.4 and Room Temperature

TF, 0.7 mg/ml; AGP, 1.0 mg/ml; PPA, 10 μg/ml.

In order to examine which protein is responsible for the observed protein binding of 20—30% in human serum, we measured the CD spectra of two human serum proteins available in purified form, TF (0.7 mg/ml) and AGP (1.0 mg/ml), in the presence and absence of 10 μg/ml PPA. As shown in Fig. 3B, the presence of PPA produced little change in the CD spectrum of AGP, indicative of little binding of this drug to AGP. On the other hand, the CD spectrum of the TF–PPA system showed a slight change in both band wavelengths and intensities as compared with that of TF alone (Fig. 3A). This is suggestive of some weak interaction of PPA with TF, irrelevant to the function (Fe-carrier) of this protein. Similar weak interactions of PPA with proteins in serum might result in 20—30% protein binding in total, as observed.

Correlation of Binding to Albumin with Physico-Chemical Properties of Drugs Table III shows pK_a and log P values for the three drugs and π values[17] for their 7-substituents ($\pi(7)$), together with ionization degrees of ionizable groups (–COOH, >NH) at pH 7.4. Since the carboxylic acid group of each drug molecule is almost wholly ionized at pH 7.4 as shown in Table III, factors other than ionization of the group must be determinants of the different binding properties of these drugs. It is apparent that the binding affinity to HSA increases with increasing $\pi(7)$ as well as log P values. Thus the lipophilicity of the 7-substituent seems to be one of main factors determining the binding properties, which explains the lower affinities of CINX and PPA to HSA. In the case of PPA, however, its poor binding to HSA seems to result also from another factor, *i.e.* the positively charged structure in the betaine form[18] at pH 7.4 for this drug molecule. Some cationic drugs show specific affinities not to albumin, but to other serum proteins such as AGP.[19]

As shown in Table II, CINX binds much more strongly to RSA than to HSA, with an affinity almost equal to or more than those of NA to HSA and RSA. This indicates that the affinities of CINX and NA for RSA can no longer be interpreted solely by the simple lipophilic dependence as seen for the HSA–drug system.

Although many drugs show species differences in serum protein binding,[20] little is known about the case where one of a series of analogous compounds binds with markedly different affinities to human and rat albumins or sera. Phenoxyacetic acid[21] and mannosylaglycone of ardacin[22] are similar examples. Phenoxyacetic acid binds more strongly to RSA than to HSA, whereas each of its more lipophilic chloroderivatives has similarly high affinities to both albumins.[21] This is also the case for rat and human serum protein binding of mannosylaglycone as compared with its parent compound ardacin A and ardacinaglycone: the former compound shows higher protein binding to rat serum (88%) than to human serum (73%), while the latter two more lipophilic compounds exhibit similar protein binding (92—98%) in human and rat sera.[22]

From inspection of all three cases, it seems likely that a compound having relatively low lipophilicity and relatively low affinity to HSA as compared with its analogues may turn out to be a ligand having a higher affinity to RSA. It would be of interest to investigate further the binding behavior of various ligands from this viewpoint in relation to understanding of the difference in the nature of the binding site between rat and human albumins.

TABLE III. Ionization and Lipophilicity Properties of Quinolone Drugs

Compound	pK_a	log P'[a]	(pH)	log P[b]	$\pi(7)$[c]	Ionization degree at pH 7.4
NA	6.12±0.07[d]	1.26±0.01	(6.19)	1.64	0.56	0.961
CINX	4.70±0.10[d]	0.17±0.01	(4.65)	0.50	−0.025	0.998
PPA	5.55,[e] 8.66[e]	−2.17±0.25	(6.90)	−2.15	−1.74	0.955[f]

a) Apparent values observed at the respective pH's. *b*) Values for neutral form calculated from log P' using pK_a and pH values. *c*) π values of the 7-substituents taken from ref. 15. *d*) Determined spectrophotometrically. *e*) Determined titrimetrically. Standard deviations of both pK_a values were within 0.01. *f*) Betaine (ref. 18).

Conclusion

In summary, the affinity of the three drugs to HSA is in the order of NA>CINX>PPA, depending on the lipophilic properties of their 7-substituents and this is also the case for human serum protein binding. PPA shows low serum binding in human, but has no significant binding to HSA, which seems to arise from its existence in the betaine form at neutral pH. The same situation may apply to serum protein binding of PPA in dog and rat. The higher protein binding of CINX observed in rat serum is due to its higher affinity to RSA. The lower lipophilicity might contribute at least in part to the relatively large species-dependent difference in serum protein binding observed only for CINX among the three analogous drugs. Such different binding characteristics should be taken into consideration when comparing pharmacological effects between rat and human.

References and Notes

1) A part of this work was presented at the 104th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, March 1984. The following abbreviations are used: CINX=cinoxacin, NA=nalidixic acid, PPA=pipemidic acid, HSA=human serum albumin, RSA=rat serum albumin, AGP=human α1-acid glycoprotein, TF=human transferrin (apo-form).
2) G. Y. Lesher, E. J. Froelich, M. D. Gruett, J. H. Bailey and R. P. Brundage, *J. Med. Chem.*, **5**, 1063 (1962).
3) D. Kaminsky and R. I. Meltzer, *J. Med. Chem.*, **11**, 6 (1968).
4) S. Minami, T. Shono and J. Matsumoto, *Chem. Pharm. Bull.*, **19**, 1426 (1971).

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5) W. E. Wick, D. A. Preston, W. A. White and R. S. Gordee, *Antimicrob. Agents Chemother.*, **4**, 415 (1973).
6) M. Pesson, M. Antonie, S. Chabassier, S. Geiger, P. Girard, D. Richer, P. de Lajudie, E. Horvath, B. Leriche and S. Patte, *Eur. J. Med. Chem.*, **9**, 591 (1974); J. Matsumoto and S. Minami, *J. Med. Chem.*, **18**, 74 (1975).
7) *a*) R. Albrecht, *Prog. Drug Res.*, **21**, 9 (1977); *b*) G. C. Crumplin, J. M. Midgley and J. T. Smith, "Topics in Antibiotic Chemistry," Vol. 3, ed. by P. G. Sammes, John Wiley & Sons, New York, 1980, pp. 9—38; *c*) J. S. Wolfson and D. C. Hooper, *Antimicrob. Agents Chemother.*, **28**, 581 (1985); *d*) D. C. Hooper and J. S. Wolfson, *ibid.*, **28**, 716 (1985).
8) *a*) K. Shimizu, T. Harada, M. Hatayama, O. Kunii, T. Jindate, E. Yamada and K. Shimada, *Chemotherapy* (Tokyo), **12**, 384 (1964); *b*) M. Shimizu, S. Nakamura, N. Kurobe and Y. Takase, *ibid.*, **23**, 2724 (1975); *c*) R. Wise, J. M. Andrews and L. J. Edwards, *Antimicrob. Agents Chemother.*, **23**, 559 (1983); *d*) G. Montay, Y. Goueffon and F. Roquet, *ibid.*, **25**, 463 (1984); *e*) R. Wise, J. M. Andrews and G. Danks, *J. Antimicrob. Chemother.*, **13**, 237 (1984).
9) P. Clark, M. R. Rachinsky and J. F. Foster, *J. Biol. Chem.*, **237**, 2509 (1962).
10) T. Peters, Jr., *J. Biol. Chem.*, **237**, 1181 (1962).
11) K. Schmid, M. B. MacNair and A. F. Burgi, *J. Biol. Chem.*, **230**, 853 (1958).
12) P. Aisen, R. Aasa, B. G. Malstrom and T. Vanngard, *J. Biol. Chem.*, **242**, 2507 (1967).
13) H. T. S. Britton and R. A. Robinson, *J. Chem. Soc.*, **1931**, 1456.
14) A. Albert and E. P. Serjeant, "The Determination of Ionization Constants. A Laboratory Manual," Chapman and Hall Ltd., 1971.
15) W. P. Purcell, G. E. Bass and J. M. Clayton, "Strategy of Drug Design: A Guide to Biological Activity," John Wiley & Sons, New York, 1973, pp. 126—143.
16) P. L. Altman and D. S. Dittmer, "Biology Data Book," 2nd ed., Vol II, Federation of American Societies for Experimental Biology, Bethesda, 1973, p. 1040; J. J. Kaneko, "Standard Values in Domestic Animals," 3rd revised ed., Department of Clinical Pathology, University of California, Davis, 1973, p. 11; both cited in T. F. Liao and F. W. Oehme, *Toxicol. Appl. Pharmacol.*, **57**, 226 (1981).
17) H. Koga, *Kagaku No Ryoiki Zokan*, **136**, 177 (1982).
18) T. Izumi, T. Nagayama and T. Kitagawa, *Chem. Pharm. Bull.*, **37**, 746 (1989).
19) *a*) L. D. De Leve and K. M. Piafsky, *Trends Pharmacol. Sci.*, **2**, 283 (1981); *b*) J.-P. Tillment, G. Honin, R. Zini, S. Urien, E. Albengres, J. Barre, M. Lecomte, P. D'Athis and B. Sebille, *Adv. Drug Res.*, **13**, 59 (1984).
20) W. J. Jusko and M. Gretch, *Drug Metab. Rev.*, **5**, 43 (1976).
21) S. C. Fang and F. T. Lindstron, *J. Pharmacokinet. Biopharm.*, **8**, 583 (1980).
22) R. W. Wittendorf, J. E. Swagzdis, R. Gifford and B. A. Mico, *J. Pharmacokinet. Biopharm.*, **15**, 5 (1987).

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