Serum Protein Binding of Lomefloxacin, a New Antimicrobial Agent, and Its Related Quinolones

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Abstract □ The serum protein binding of lomefloxacin (LFLX), a new quinolone (pyridonecarboxylic acid), and its related analogues was studied by an ultrafiltration technique. The extent of binding of quinolones was independent of the concentration of quinolones below 100 μg/mL in rat serum; but, above this concentration, the binding decreased with increased drug concentration in the case of nalidixic acid and analogue **3**. The extent of binding in rat serum differed widely among the quinolones examined [i.e., from 15% (norfloxacin) to 84% (nalidixic acid) at concentrations of 0.4–10.0 μg/mL]. Lomefloxacin was bound to serum proteins to the extent of 28.1, 20.1, and 20.6% in the sera of rats, dogs, and humans, respectively. The binding of nalidixic acid with rat serum albumin, which was very similar to that in rat serum, was concentration dependent. Some quinolone derivatives with a piperazinyl group or a relatively large-sized substituent at the 7-position exhibited a percentage unbound of ~70–80%, while some derivatives with small-sized substituents gave a low percentage unbound of 20–30%. This suggests that there is a steric effect of the substituents at the 7-position of quinolones on their binding characteristics with serum proteins. The results of the present study indicate that quinolones bind mainly with albumin among serum proteins and that the remarkable difference of the extent of binding of quinolone analogues is related to the size of the substituent at the 7-position of the molecule, possibly due to its steric effect.

Serum protein binding of antimicrobial agents is one of the important factors that determine their pharmacokinetic and antibacterial behaviors in vivo. Recently, several quinolones (pyridonecarboxylic acids) with wide antibacterial spectra against gram-negative and gram-positive organisms have been developed. In these antimicrobial agents, pharmacokinetic deficiencies of the first generation drugs, including nalidixic acid, such as high extent of serum protein binding, poor tissue distribution, and extensive metabolism, have been improved.[1] Lomefloxacin (LFLX) is a newly developed fluorinated quinolone derivative characterized by the presence of a methyl group at the 3-position of piperazine moiety (Table I).

It has been reported that the serum protein binding of enoxacin and pefloxacin, which have bulky substituent groups at the 7-position of the quinolone ring, was relatively low (20–35%)[2,3] compared with that of miloxacin (86%),[4] which has a small substituent group. To confirm the substituent effect on the extent of serum protein binding, we synthesized various quinolone analogues (e.g., **1**–**5**, Table I).

Lomefloxacin has a broad spectrum of activity covering both gram-positive and gram-negative organisms.[5] In humans, LFLX is almost completely absorbed after an oral dose, eliminated predominantly by renal excretion, and metabolized only to a small extent.[6] The new quinolones, enoxacin,[2] norfloxacin,[7] ofloxacin,[8] pefloxacin,[3] and LFLX,[9] distribute well into tissues. Generally speaking, the unbound fraction of a drug in serum (f_p), as well as the unbound fraction in tissues (f_t), are important factors in determining the distribution volume of the drug. In particular, if $f_{t,i}$ is constant, tissue distribution will depend on f_p, as verified in rats for quinolones.[10] Moreover, serum protein binding is important for the antimicrobial activity and toxicological response, which are associated with unbound drug concentrations in serum. With respect to quinolones, however, there appears to be no report on the structure–serum protein binding relationship or the mechanism of serum protein binding.

The present study describes the binding characteristics of LFLX and its related analogues with serum for a wide concentration range.

Experimental Section

Materials—Nalidixic acid (NA), ofloxacin (OFLX), and [^{14}C]OFLX (57 μCi/mg) were kindly supplied from Daiichi Pharmaceutical, Tokyo, Japan. Norfloxacin (NFLX), lomefloxacin (LFLX), [^{14}C]lomefloxacin ([^{14}C]LFLX; 9.29 μCi/mg), analogues **1**–**5**, and pipemidic acid (PPA) were synthesized in Central Research Laboratory, Hokuriku Seiyaku Co., Fukui, Japan. The structures of the quinolones used in this study are shown in Table I. Rat serum albumin (RSA, Fraction V) was purchased from Sigma Chemical (St. Louis, MO). All other reagents were commercially available and of analytical grade.

Serum and Rat Serum Albumin Samples—Blood was obtained from rats, dogs, and healthy human volunteers, to whom no drugs or anticoagulants were given, and centrifuged at 3000 rpm for 10 min to obtain serum samples. Sera samples thus obtained were pooled and stored at −20 °C until use in binding experiments. The RSA was dissolved in Krebs-Ringer bicarbonate buffer (pH 7.4)[11] to produce an albumin concentration of 3.95 g/100 mL.

Drug Solutions—An exactly weighed quinolone was dissolved with 0.3 M NaOH and the solution was diluted with distilled water. Then, 0.3 M HCl was added to obtain a drug solution with a pH value of 7–8.

Binding Experiments—Serum protein binding was determined by an ultrafiltration method. Unless otherwise mentioned, 300 μL of the drug solution was added to 6 mL of the pooled serum or RSA solution to obtain an exact drug concentration ranging from 0.1 to 400 μg/mL. In the case of LFLX and OFLX, the drug solution contained the corresponding ^{14}C-labeled compounds. The mixture was incubated for 30 min at 37 °C and then an aliquot of 1 mL was ultrafiltered using a micropartition system (MPS-1, Amicon, Lexington, MA) with a membrane filter (YMT-membrane, Amicon) at 2500 rpm for 20 min at 37 °C. The ultrafiltrate was assayed by counting radioactivity or by HPLC, as described later. The concentration of quinolone bound to proteins was calculated by subtracting the concentrations of the drug in the ultrafiltrate from the known total concentration in serum. The percent of total volume collected as filtrate was ~30–40%. Since the nonspecific adsorption of the drug to the membrane was small (<6%), no correction for nonspecific binding was made.

Analytical Procedures—The concentrations of LFLX and OFLX in the ultrafiltrate were determined by liquid scintillation counting by adding 100 μL of filtrate to 16 mL of liquid scintillation fluid (ASC-II, Aloka, Tokyo, Japan), and counting the radioactivity in a spectrometer (LSC-700, Aloka).

The concentration of the other quinolones in the ultrafiltrate was

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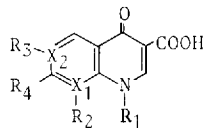
Table I—Quinolone Analogues



Analogue	X_1	X_2	R_1	R_2	R_3	R_4
Enoxacin (ENX)	N	C	C_2H_5	—	F	—N NH
Lomefloxacin (LFLX)	C	C	C_2H_5	F	F	—N NH CH_3
1	C	C	C_2H_5	F	F	OH
2	C	C	C_2H_5	F	F	$-N(CH_3)CH_2CH_2OH$
3	C	C	C_2H_5	F	F	$-NH\cdot CH_2CH_2OH$
4	CH	C	C_2H_5	—	F	$-CH_2N(C_2H_5)_2$
5	CH	C	C_2H_5	—	F	$-OC_2H_5$
Miloxacin (MLX)	CH	CH	OCH_3	—		$-OCH_2O-$
Nalidixic acid (NA)	N	CH	C_2H_5	—	—	CH_3
Norfloxacin (NFLX)	CH	C	C_2H_5	—	F	—N NH
Ofloxacin (OFLX)	CH	C	$-CH(CH_3)CH_2O$	—	F	—N N—CH_3
Pefloxacin (PFLX)	CH	C	C_2H_5	—	F	—N N—CH_3
Pipemidic acid (PPA)	N	N	C_2H_5	—	—	—N NH

determined by HPLC assay. A portion (50 μL) of the ultrafiltrate that had been passed through a membrane filter (0.45 μm, Nihon Millipore Kogyo, Yonezawa, Japan) was injected onto an HPLC analytical column.

For analysis of NA and analogues **1**–**5**, a solvent delivery system (BIP-1, Japan Spectroscopic, Tokyo, Japan), equipped with an ultraviolet spectrophotometer (UVIDEC-100V, Japan Spectroscopic) and a strong anion exchange column (2.1 mm × 50 cm, Zipak SAX, Shimadzu, Kyoto, Japan), was used. The mobile phase was aqueous boric acid solution of pH 9.0. The concentration of boric acid was chosen arbitrarily from 0.02 to 0.05 M so that a good separation between the blank peak obtained for the ultrafiltrate of the pooled serum and each drug peak could be established. The spectrophotometer was set at 258, 277, 284, 279, 252, and 270 nm for NA, **1**, **2**, **3**, **4**, and **5**, respectively.

For analysis of NFLX and PPA, a solvent delivery system (BIP-1), equipped with a spectrofluorometer (FP-110, Japan Spectroscopic) and a reversed-phase column (3.9 mm × 30 cm, μ-Bondapak C_{18}, Waters Associates, Milford, MA), was used. The mobile phase was acetonitrile:0.05 M citric acid:1 M ammonium acetate (16:83:1 v/v% for NFLX; 13:86:1 v/v% for PPA). The spectrofluorometer was set at the excitation:emission wavelength of 330:420 nm for NFLX and 340:420 nm for PPA. For both HPLC systems, the flow rate was 2 mL/min and the peak areas were recorded with a Chromatopac C-R3A (Shimadzu). The unknown concentrations in the ultrafiltrate samples were calculated by comparing the peak areas for the samples containing the known concentrations of the standard drug dissolved in the ultrafiltrate of the pooled serum. For this calculation, two sets of calibration curves were prepared: 0.05–10 and 10–100 μg/mL. Both curves were shown to be linear. The mean recoveries obtained were 94.0–103.3% for all compounds, and the coefficients of variation were <5.3%.

Results and Discussion

The present study was carried out to give insight into the binding characteristics of several quinolones with sera of different animal species. The concentration range (0.1–400 μg/mL) of quinolones employed in this study covered both the therapeutic serum concentrations (1–20 μg/mL) in human subjects and the serum concentrations in toxicological studies using experimental animals, which is estimated to be >100 μg/mL at a subacute dose of 1 g/kg in rats from previous pharmacokinetic data.[12]

The percentages of unbound quinolones with rat serum proteins are listed in Table II. For example, the percentages of unbound LFLX were 67.1 ± 0.83, 66.6 ± 0.59, 69.6 ± 2.32, 73.9 ± 0.81, 71.4 ± 1.16, 74.8 ± 1.83, 74.6 ± 0.59, and 77.5 ± 0.85 (mean ± SEM, n = 5) at the total LFLX concentrations of 0.1, 0.4, 1, 4, 10, 40, 100, and 400 μg/mL, respectively.

All the quinolones tested showed linear behavior in their serum protein binding in the therapeutic serum concentration range. On the other hand, the serum protein bindings of NA and **3** were nonlinear in the toxic concentration range, while those of other quinolones were still linear, as shown in Figure 1. These results suggest that nonlinearity of serum protein binding should be considered in acute and subacute toxic studies of some quinolones using experimental animals, and raise a possibility that the quinolones to be developed in the future may exhibit nonlinear pharmacokinetics due to their nonlinear serum protein binding in a therapeutic concentration range. Thus, possible concentration dependency of serum protein binding of quinolones is a matter of clinical importance.

The extent of binding of clinically usable quinolones in rat serum was in the order NA > LFLX > OFLX > PPA > NFLX. As expected from the data listed in Table II, quinolone derivatives with a piperazinyl group (NFLX, LFLX, OFLX, PPA) or a relatively large-sized substituent (**4**) at the 7-position exhibit a percentage unbound of ~70–80%. On the contrary, the other derivatives, with small-sized substituents at the 7-position, such as NA, **1**, **2**, **3**, and **5**, gave a low percentage unbound of 20–30% at 5 μg/mL. The low extent of binding of these quinolones with serum proteins may be attributed to the steric hindrance by the substituents at the 7-position of the molecule.

Nalidixic acid (NA) exhibited nonlinear binding not only with rat serum but also with RSA, although the percentage

Table II—Percentage Unbound[a] for Quinolones in the Sera of the Rat, Dog, and Human and in 4% Solution of Rat Serum Albumin (RSA)

Drug	Animal Species	Concentration Range, μg/mL[b]	Percentage Unbound[c]
Lomefloxacin	Rat	0.1–400.0	71.9 ± 0.72 (40)
	Dog	0.1–400.0	79.9 ± 0.74 (40)
	Human	0.1–400.0	79.4 ± 0.93 (40)
1	Rat	5.0	15.0 ± 0.29 (3)
2	Rat	5.0	17.6 ± 0.75 (3)
3	Rat	5.0–40.0	35.1 ± 0.68 (9)
		100.0	39.7 ± 0.37 (3)
		200.0	46.4 ± 0.88 (4)
		400.0	58.7 ± 1.05 (3)
4	Rat	5.0	78.2 ± 7.87 (3)
5	Rat	5.0	29.4 ± 1.62 (3)
Nalidixic acid	Rat	0.4–10.0	15.9 ± 0.38 (2)
		40.0	22.0 ± 0.15 (5)
		100.0	28.8 ± 0.17 (5)
		200.0	33.1 ± 0.60 (5)
		400.0	49.9 ± 0.08 (5)
	RSA	10.0–20.0	8.4 ± 0.11 (6)
		40.0	9.7 ± 0.06 (3)
		100.0	10.4 ± 0.65 (3)
		200.0	16.7 ± 0.39 (3)
		400.0	29.4 ± 0.88 (3)
Norfloxacin	Rat	0.4–100.0	85.3 ± 0.69 (35)
Ofloxacin	Rat	0.4–400.0	77.2 ± 0.46 (40)
Pipemidic acid	Rat	0.4–100.0	77.7 ± 1.20 (35)

[a] Determined by an ultrafiltration method at 37 °C. [b] Determined at drug concentrations of 0.1 (or 0.6), 0.4, 1, 4 (or 5), 10, 20 (only for nalidixic acid in RSA solution), 40, 100, and 400 μg/ml, when the concentration range was indicated; the number of determinations at each concentration was 3–5. [c] Mean ± SEM; the numbers in parentheses are the total number of determinations.

unbound was lower in albumin than in serum. Based on the results of binding of NA with RSA, it is suggested that quinolones bind mainly with albumin among the serum proteins and that they share the same binding sites on the protein molecule. However, the binding with albumin tended to be stronger than that with serum, presumably due to some influences by unknown endogenous substances in serum.

The percentages of unbound LFLX determined in the binding experiments with sera of dogs and humans are given in Table II. The percentages of unbound LFLX were 75.0 ± 0.32, 75.9 ± 0.96, 76.1 ± 2.08, 83.6 ± 0.48, 80.1 ± 1.43, 81.6 ± 1.98, 83.4 ± 1.72, and 83.2 ± 2.14% in dog serum, and 77.3 ± 0.42, 74.1 ± 0.23, 75.6 ± 0.30, 77.8 ± 1.82, 81.7 ± 2.34, 80.2 ± 0.59, 78.9 ± 0.87, and 89.7 ± 4.17% in human serum at total LFLX concentrations of 0.1, 0.4, 1, 4, 10, 40, 100, and 400 μg/mL, respectively. The extent of binding of LFLX was very similar in rats, dogs, and humans: thus, there was no species difference of serum protein binding of LFLX. There is a significant difference in the renal clearance (CL_R) among rats, dogs, and humans (rats, 9.36; dogs, 2.19; humans, 2.97 mL/min/kg).[12,13] Considering that these values are much smaller than the renal plasma flow rate (rats, 25; dogs, 15; humans, 10 mL/min/kg),[14] the renal excretion of LFLX is not limited by plasma flow. Therefore, CL_R can be expressed by the following equation:

$$CL_R = f_p(1 - R)(GFR + CL_{int,s}) \quad (1)$$

where R, GFR, and $CL_{int,s}$ represent the fraction of reabsorption, glomerular filtration rate, and secretory intrinsic clearance, respectively. Therefore, it is suggested that the difference in CL_R (per body weight) of LFLX can be attributed to the species difference of R or $CL_{int,s}$, because of no significant difference in f_p among animal species.

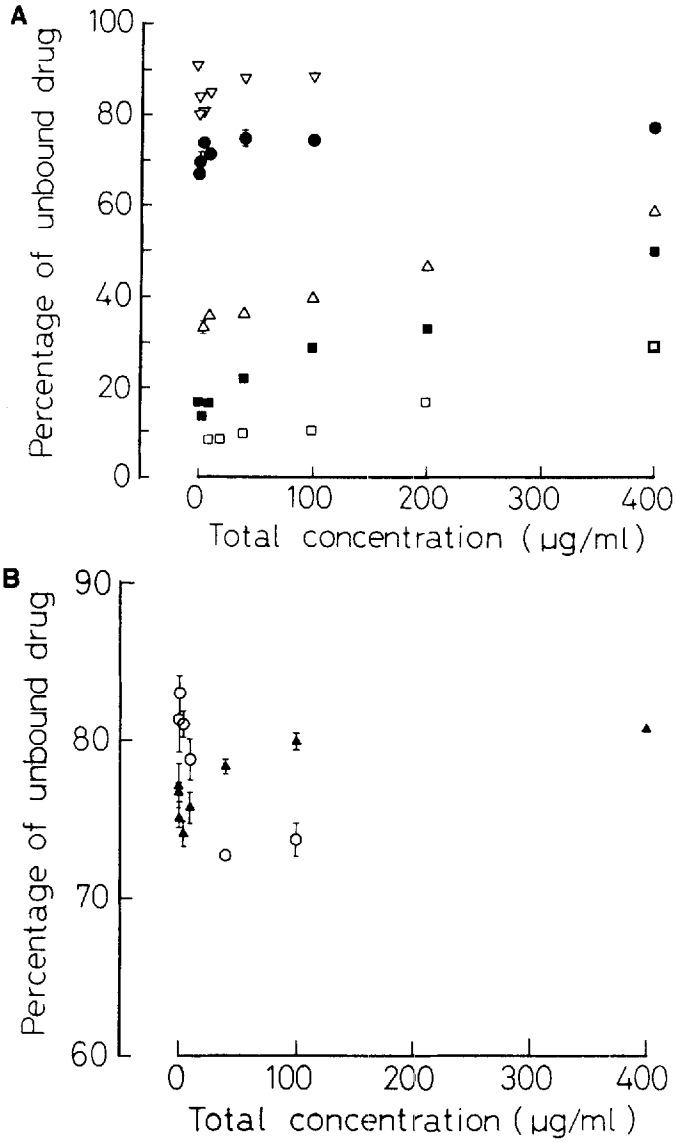


Figure 1—Effect of concentration of quinolones on the extent of binding with rat serum albumin (□) and rat serum (other symbols). Panel A presents nalidixic acid (□,■), norfloxacin (▽), lomefloxacin (●), and **3** (△). Panel B presents ofloxacin (▲) and pipemidic acid (○). Each point represents the mean ± SEM (n = 3–5).

In conclusion, quinolones were bound mainly with albumin among serum proteins, and the difference of the extent of serum protein binding of quinolone analogues is related to the size of the substituent at the 7-position of the molecule, possibly due to its steric effect.

References and Notes

1. Shimada, J. *Microbiology* **1986**, 222–225.
2. Nakamura, S.; Kurobe, N.; Kashimoto, S.; Ohue, T.; Shimizu, S. *Chemotherapy (Tokyo)* **1984**, *32(S-3)*, 86–94.
3. Montay, G.; Goueffon, Y.; Roquet, F. *Antimicrob. Agents Chemother.* **1984**, *25*, 463–472.
4. Izawa, A.; Kisaki, Y.; Irie, K.; Eda, Y.; Komatsu, T.; Namiki, H.; Mizutani, T.; Nagate, T.; Kangouri, K.; Ohmura, S. *Chemotherapy (Tokyo)* **1978**, *26(S-4)*, 48–59.
5. Hirose, T.; Okezaki, E.; Kato, H.; Ito, Y.; Inoue, M.; Mitsuhashi, S. *Antimicrob. Agents Chemother.* **1987**, *31*, 854–859.

6. Nakashima, M.; Uematsu, T.; Takiguchi, Y.; Mizuno, A.; Kanamaru, M.; Tsuji, A.; Kubo, S.; Nagata, O.; Okezaki, E; Takahara, Y. 26th Interscience Conference on Antimicrobial Agents Chemotherapy; New Orleans, LA, 1986; Abstract 430.
7. Nagatsu, Y.; Endo, K.; Irikura, T. *Chemotherapy (Tokyo)* **1981**, *29(S-4)*, 105–118.
8. Okazaki, O.; Kurata, T.; Hashimoto, K.; Sudo, K.; Tsumura, M.; Tachizawa, H. *Chemotherapy (Tokyo)* **1984**, *32(S-1)*, 1185–1202.
9. Nagata, O.; Yamada, T.; Yamaguchi, T.; Okezaki, E.; Terasaki, T.; Tsuji, A. *Chemotherapy (Tokyo)* **1988**, *36(S-2)*, 151–173.
10. Okezaki, E.; Terasaki, T.; Nakamura, M.; Nagata, O.; Kato, H.; Tsuji, A. *Drug Metab Dispos*, in press.
11. Shaw, L. M.; Fields, L.; Mayock, R. *Clin. Pharmacol. Ther.* **1982**, *32*, 490–496.
12. Okezaki, E.; Ohmichi, K.; Koike, S.; Takahashi, Y.; Makino, E.; Terasaki, T.; Tsuji, A. *Chemotherapy (Tokyo)* **1988**, *36(S-2)*, 132–137.
13. Nakashima, M.; Uematsu, T.; Takiguchi, Y.; Mizuno, A.; Kanamaru, M.; Kubo, S.; Takahara, Y.; Okezaki, E.; Nagata, O. *Chemotherapy (Tokyo)* **1988**, *36(S-2)*, 201–239.
14. Bischoff, K. B.; Dedrick, R. L.; Zaharko, D. S.; Longstreth, J. A. *J. Pharm. Sci.* **1971**, *60*, 1128–1133.

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