Old Herborn University Seminar Monograph 3:
Consequences of antimicrobial therapy for the composition of the microflora of the digestive tract.
Editors: Carl Erik Nord, Peter J. Heidt, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 55-61 (1993).

NON-SPECIFIC INACTIVATION OF ANTIMICROBIAL AGENTS

CHARLOTTA EDLUND

Department of Microbiology, Huddinge University Hospital, Karolinska Institute, S-141 86 Huddinge, Sweden

INTRODUCTION

The potential of an antimicrobial agent to disturb the normal intestinal microflora is related to its *in vitro* properties, its route of administration, dose and pharmacokinetic properties. Specific as well as non-specific inactivation of the antimicrobial agent may also affect the extent to which the intestinal microflora is altered.

The present study concerns non-specific inactivation of norfloxacin in the gastrointestinal tract. Previous studies on the new quinolones have shown that administration of these agents in therapeutic doses results in very high concentrations in faeces (Table 1). If these figures are compared with the MIC_{90} of the new quinolones against some common intestinal microorganisms, the MIC_{90} for both aerobic and anaerobic intestinal microorganisms are with few exceptions far below these levels.

Earlier studies by our research group and other groups have shown that administration of the new quinolones causes selective ecological changes in the gastrointestinal microflora. Several investigations including healthy volunteers as well as different categories of patients, show uniform results. The aerobic Gram-negative microorganisms are eliminated or strongly suppressed during administration, while the aerobic Gram-positive and the anaerobic micro-

Table 1: Concentrations of new quinolones in faeces after various doses

Quinolone	Dose (mg/day)	Faecal concentration Range respectively mean value (mg/kg)	Reference
Norfloxacin	400x2	120-1400	Meckenstock et al. 1985
Norfloxacin	400x2	2271	Pecquet et al. 1986
Norfloxacin	400x2	1756	Boerema et al. 1986
Norfloxacin	200x2	303-1906	Edlund et al. 1987
Norfloxacin	200x2	125-1000	Maschmeyer et al. 1988
Norfloxacin	400x2	250-1000	Maschmeyer et al. 1988
Ciprofloxacin	500x2	185-2220	Brumfitt et al. 1984
Ciprofloxacin	250x2	1600-6400	Maschmeyer et al. 1988
Ciprofloxacin	500x2	1200-6400	Maschmeyer et al. 1988
Enoxacin	400x2	100-500	Edlund et al. 1987
Ofloxacin	200x2	327	Pecquet et al. 1987
Pefloxacin	400x2	645	Janin et al. 1987

flora are almost unaffected (*Edlund* and *Nord*, 1988). Thus, despite high levels of new quinolones in faeces, the main part of the intestinal microflora remains unaffected.

Different theories have been raised to explain the discrepancies of the very high concentrations of quinolones in faeces and the relatively sparse effect on the gastrointestinal microflora. *Lewin* and colleagues (1989) have suggested that oxygen is required for the bactericidal activity of the new quinolones. They found that lack of oxygen results in a bacteriostatic activity of ciprofloxacin and ofloxacin against *Escherichia coli* and *Staphylococcus aureus* in contrast to the bactericidal activity exhibited under aerobic conditions. The milieu of the intestines is mainly anaerobic so this theory might partly explain the phenomenon. An inoculum effect on norfloxacin activity against anaerobic strains has been reported by *Goldstein* and colleagues (1987), while other groups have failed to find any major inoculum effects. A third theory is that quinolones have the ability to bind to microorganisms or to other faecal components, resulting in only a minor part of the agent being free in the intestines to exert the antimicrobial effect. Reversible binding to faeces has earlier been reported for other antimicrobial agents (*Hazenberg* et al., 1985; *Hazenberg* et al., 1986; *van Saene* et al., 1985). Thus gentamicin, tobramycin, polymyxin B and neomycin have been shown to bind to the solid part of faeces.

Uptake of new quinolones into bacterial cells and their interactions with cell membranes has been and is currently studied by several researchers (*Bedard* et al., 1987; *Chapman* and *Georgopapadakou*, 1988; *Cohen* et al., 1988; *Bedard* and *Bryan*, 1989). Binding of quinolones to bacterial cells might in part reflect the mechanism of action. The mode of action of the quinolones is not yet completely understood.

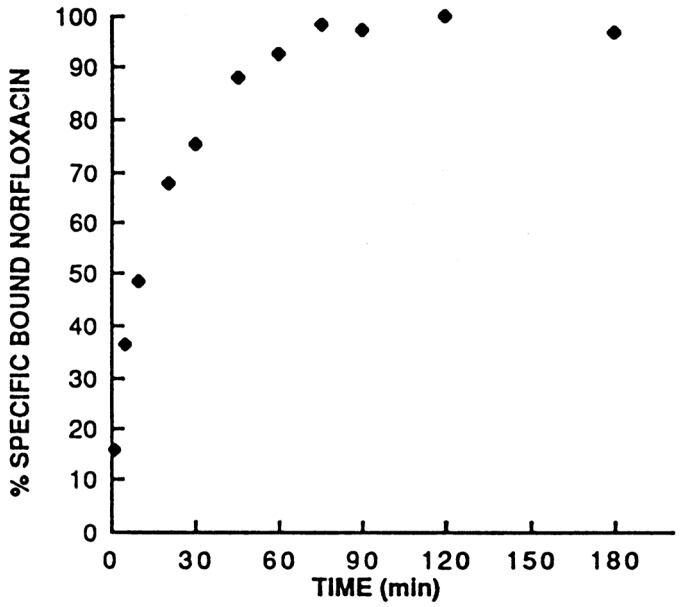

Figure 1: Association of [^{14}C]norfloxacin to faeces at 37°C. The results are expressed as percent of maximum specific binding.

The aim of the present study was to analyze the binding between quinolones such as norfloxacin and faecal material.

MATERIAL, METHODS AND RESULTS

The model used for the binding experiments was ligand-receptor interactions with radioactive labelled norfloxacin as ligand, and diluted faecal suspensions as receptor. Binding to norfloxacin to faeces was measured by using a membrane filter technique. The reaction mixture contained either a fixed concentration of [^{14}C]norfloxacin (1.5 μM) together with various concentrations of faecal suspensions, or varying concentrations of [^{14}C]norfloxacin (0.1-500 μM) and a fixed amount of faecal suspension (5.3 g/l). Specific binding was determined by the difference between total radioactivity found in the absence or presence of excess of unlabelled norfloxacin (560 μM). After binding at 37°C for various times during end over end rotation, the reaction mixtures were filtered under vacuum. The filters were washed and then counted for radioactivity in a liquid scintillation spectrometer.

The association of norfloxacin to faeces is shown in Figure 1. The specific binding of norfloxacin increases with time and reaches a plateau after 90 min incubation at 37°C. According to these results, 120 min incubation at 37°C was used to reach a maximum binding in all subsequent experiments.

Binding of norfloxacin was shown to be reversible and temperature dependent. Norfloxacin and faeces were incubated as described earlier to reach a maximum binding. The samples were then centrifuged and washed three times at 4°C, and the resuspended samples were further incubated for up to 240 min. Figure 2 shows dissociation of norfloxacin from faeces at different

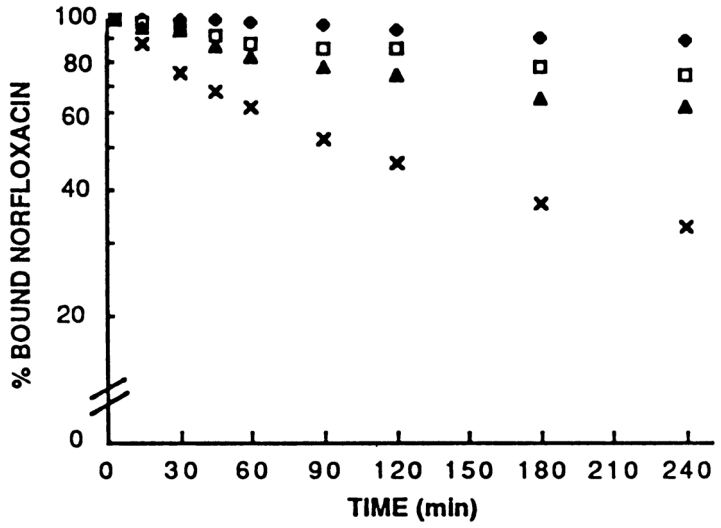

Figure 2: Dissociation of 1.5 μM [^{14}C]norfloxacin bound to faeces at various temperatures. The results are expressed as the percentage of the specific binding found at the time zero (logarithmic scale). Symbols: u, 4°C; o, 20°C; s, 37°C; x, 37°C + 560 μM norfloxacin.

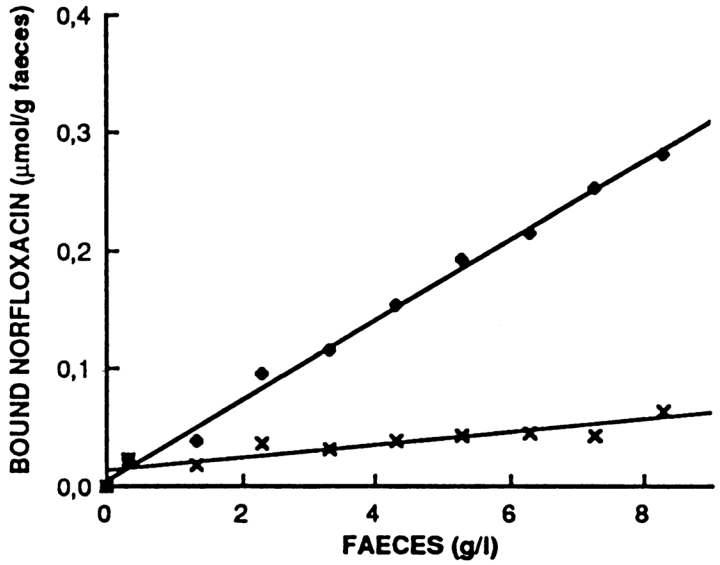

Figure 3: Binding of 1.5μM [^{14}C]norfloxacin to an increasing concentration of faeces. Symbols: u, specific binding; x, non-specific binding.

temperatures. After 240 min incubation at 37°C, 60% and 35% were dissociated in the presence and absence, respectively, of unlabelled norfloxacin. At

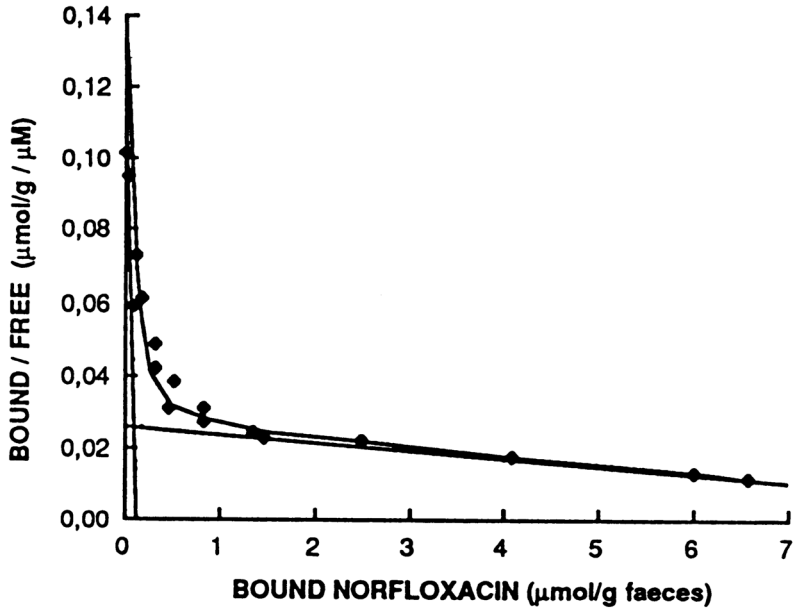

Figure 4: Scatchard plot for [^{14}C]norfloxacin binding to faeces (F1). The asymptotes as well as the curve combining the experimental data points were drawn by the computer program SAS PROC NLIN and represent the best fit.

Table 2: Numerical estimation of dissociation constants, K_D, and maximum binding capacities, B_{max}, for binding of quinolones to different faecal samples and to a suspension of *B. fragilis*. All assays were made in triplicate

Antimicrobial agent	Faeces/ bacterial suspension	K_{D1} (μM)	K_{D2}	B_{max1} (μmol/g dry weight)	B_{max2}
[^{14}C]norfloxacin	F1	1.0	450	0.74	72.4
[^{14}C]norfloxacin	F2	0.6	830	0.27	153.0
[^{14}C]norfloxacin	F3	1.4	450	0.59	63.3
ciprofloxacin	F1	3.4	860	-*	-
enoxacin	F1	2.4	920	-	-
ofloxacin	F1	2.3	860	-	-
pefloxacin	F1	2.9	530	-	-
norfloxacin	F1	1.4	550	-	-
[^{14}C]norfloxacin	*B. fragilis*	2.4	1100	0.96	121

*B_{max}-values for unlabelled quinolones could not be determined due to assay by an indirect competitive method

4°C, only 10% of the bound norfloxacin was dissociated after 240 min.

The binding of norfloxacin to faeces was found to be a linear function of faeces concentration. Figure 3 shows binding of labelled norfloxacin to increasing concentrations of faecal suspensions after 120 min incubation at 37°C.

The ability of increasing concentrations of [^{14}C]norfloxacin (0.1-500 μM) to bind to faeces (5.3 g/l) was also assayed. Scatchard plot of the data (Figure 4) was non-linear, which means that more than one binding class is involved. According to this Scatchard plot and by calculating with a non-linear regression computer program, two different binding classes were found, one with high affinity and low capacity (= class 1) and one more unspecific binding class with low affinity and high capacity (= class 2).

The equilibrium dissociation constants K_D and the total number of binding sites B_{max} for each of the binding classes were determined by the computer program. This assay was also performed for two additional faecal samples, F 2 and F 3. Table 2 shows the numerical estimates of the parameters K_{D1}, K_{D2}, B_{max1} and B_{max2}. Binding of unlabelled ciprofloxacin, enoxacin, pefloxacin and norfloxacin to faeces was determined by an indirect method using their ability to compete with 1.5 μM [^{14}C]norfloxacin binding to faeces. The K_D values for these quinolones are in the same range as those for labelled norfloxacin obtained by the direct method described earlier, which supports the accuracy of the methods used (Table 2). In order to find out if the quinolones bind to the bacterial or non-bacterial fraction of faeces, the binding between labelled norfloxacin and a suspension of *Bacteroides fragilis* was studied. Two classes of binding were found in accordance with

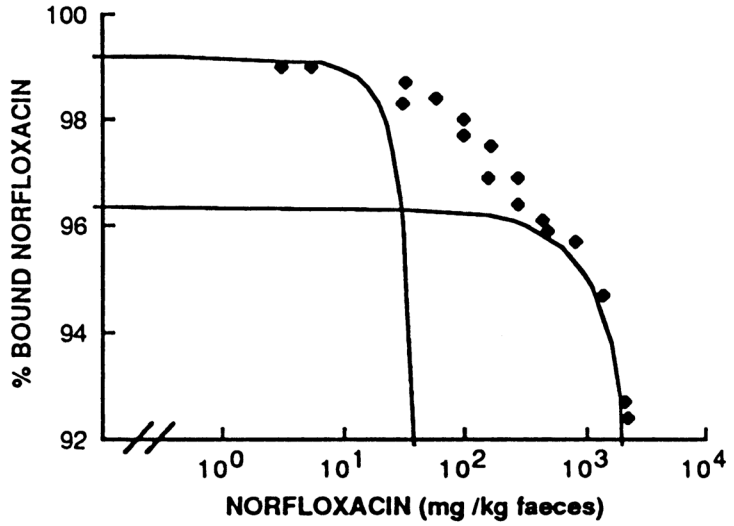

Figure 5: Fraction of bound norfloxacin related to total norfloxacin concentration in 100% faeces (extrapolated values). The experimental data points and the predicted curves for each binding class derive from studies on F1.

the findings of norfloxacin binding to the whole faecal fraction (Table 2). These results imply that norfloxacin binds mainly to the bacterial fraction of faeces.

The K_D and B_{max} values from the assay of F 1 were used to generate a curve relating fraction of bound drug to total norfloxacin concentration extrapolating to undiluted faeces. This may give a hint of the clinical situation. Figure 5 shows the predicted curves for the two binding classes and the experimental data points. At a norfloxacin concentration of 1000 mg/kg faeces, which is common in clinical situations, more than 95% of the drug is bound to faeces, and thus only 50 mg/kg is available as free drug. Furthermore, with such a high K_{D2} value the low affinity binding may be underestimated because of fast dissociation which may occur during filtration and washing procedures.

The MBC values of norfloxacin against *Enterococcus faecium* were studied in the presence of increasing concentrations of *B. fragilis* cells. *B.*

Table 3: Effect of various concentrations of *B. fragilis* on minimum bactericidal concentration of norfloxacin against *E. faecium.*

Bacteroides fragilis dry weight (g/l)	MBC (mg/l)
0	8
1.0	8
2.0	16
4.0	64
8.0	256

fragilis was used in this assay since it is one of the predominating microorganisms in the normal intestinal microflora. The assay was performed under aerobic conditions so that *B. fragilis* cells were unable to multiply but were present for binding to norfloxacin. The MBC values were strongly affected by the presence of *B. fragilis* cells as shown in Table 3. These results imply that norfloxacin binds to the *B. fragilis* cells and that only a minor fraction is free to exhibit bactericidal effect on *E. faecium*. In 100% faeces, the dry weight of bacteria is approximately 10-fold greater than the dry weight of *B. fragilis* used in this assay.

CONCLUSIONS

The present study shows that binding to faeces may act as a non-specific inactivation of norfloxacin. The results can be summarized as follows:

The specific binding of norfloxacin is reversible, saturated after 90 min incubation at 37°C and increases linear with faecal concentrations. Scatchard plots and non-linear regression computer analyses revealed two different binding classes; one primary specific binding and one secondary more unspecific binding. These results suggest that binding of norfloxacin to faeces, preferably to the bacterial fraction, may explain the paradox of the high faecal concentrations of norfloxacin versus the actual effect on the normal intestinal microflora.

LITERATURE

Bedard, J., and Bryan, L.E.: Interaction of the fluoroquinolone antibacterial agents ciprofloxacin and enoxacin with liposomes. Antimicrob. Agents Chemother. 33, 1379-1382 (1989).

Bedard, J., Wong, S., and Bryan, L.E.: Accumulation of enoxacin by *Escherichia coli* and *Bacillus subtilis*. Antimicrob. Agents Chemother. 31, 1348-1354 (1987).

Chapman, J., and Georgopapadakou, N.: Routes of quinolone permeation in *Escherichia coli*. Antimicrob. Agents Chemother. 32, 438-442 (1988).

Cohen, S., Hooper, D., Wolfson, J., Souza, K., McMurry, L., and Levy, S.: Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. 32, 1187-1191 (1988).

Edlund, C., and Nord, C.E.: A review on the impact of 4-quinolones on the normal oropharyngeal and intestinal human microflora. Infection 16, 8-12 (1988).

Goldstein, E., Citron, D., and Corrado, M.: Effect of inoculum size on in vitro activity of norfloxacin against fecal anaerobic bacteria. Am. J. Med. 82, 84-87 (1987).

Hazenberg, M.P., Pennock-Schröder, A.M., and van de Merwe, J.P.: Binding to and antibacterial effect of aztreonam, temocillin, gentamicin and tobramycin on human faeces. J. Hyg. 95, 255-263 (1985).

Hazenberg, M.P., Pennock-Schröder, A,M,, and van de Merwe, J.P.: Reversible binding of polymyxin B and neomycin to the solid part of faeces. J. Antimicrob. Chemother. 17, 333-339 (1986).

Lewin, C.S., Morrissey, I., and Smith, J.T.: Role of oxygen in the bactericidal action of the 4-quinolones. Rev. Infect. Dis. 11 (Suppl. 5), S913-S914 (1989).

van Saene, J.J.M., van Saene, H.K.F., Stoutenbeek, C.P., and Lerk, C.F.: Influence of faeces on the activity of antimicrobial agents used for decontamination of the alimentary canal. Scand. J. Infect. Dis. 17, 295-300 (1985).