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Impact of pH on the antibacterial activity of Norfloxacin in its combined use with Oxalic Acid against *Escherichia coli* ATCC 25922

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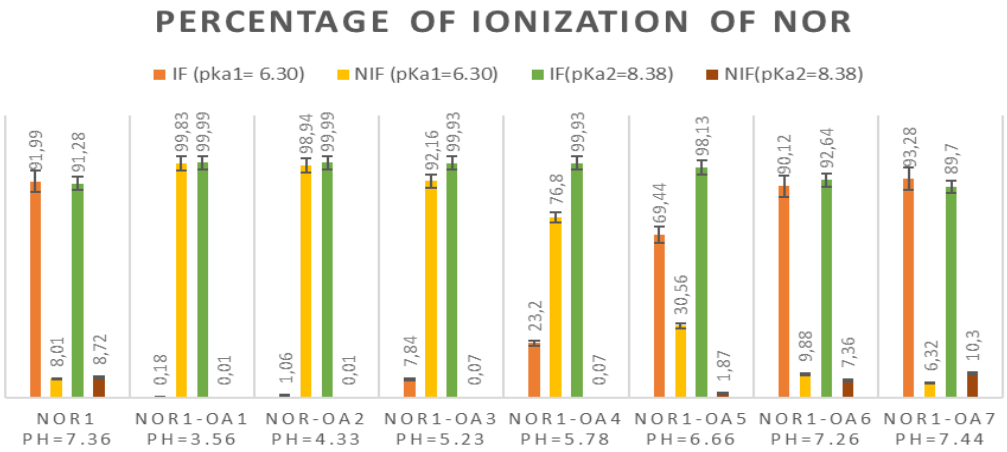
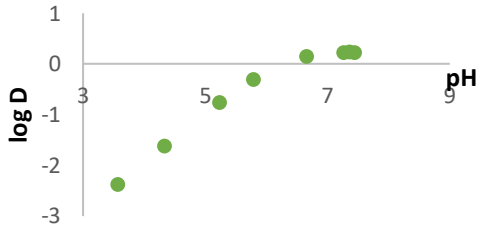
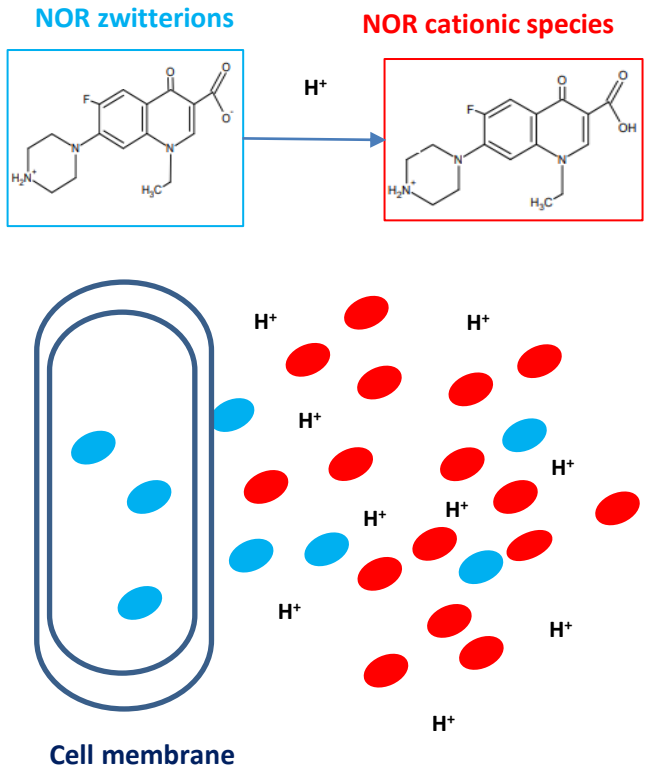
Graphical Abstract







Abstract: The bacterial susceptibility and the translocation of fluoroquinolones (FQs) are influenced by pH, since it determines the proportion of micro species of the drug. Norfloxacin (NOR) and Oxalic Acid (AO) are antibacterial compounds. In this work, we evaluated the antibacterial activity of the NOR-AO combination on the *Escherichia coli* ATCC 25922 strain using the checkerboard method. Besides, we analyzed the pH effect on the NOR-AO combination. We determined the extent of NOR ionization equilibrium and calculated the apparent log P to establish the lipophilicity of NOR at the different pHs assayed. The minimum inhibitory concentration (MIC) obtained for AO and NOR was 1250 µg/mL and 0.25 µg/mL, respectively. The interaction of NOR-AO was indifferent in the concentrations worked (CIF 1.12). However, an atypical behavior was observed in *E.coli* growth. We observed that at pH values <5.8 and log D < -0.3, the cationic species of NOR predominates, decreasing its activity. As pH increases, the predominant species is zwitterionic with increased lipophilicity and restoration of NOR activity. Therefore, the acid conditions given by the presence of AO decrease the concentration of the neutral species of NOR and therefore the amount of drug capable of diffusing directly through the membrane. There is controversy in the literature regarding the mechanism of FQs translocation through the bacterial membrane, however, our results show that the pH of the medium is a determining factor that directly impacts the antibacterial activity. To deepen this study, we will continue testing new concentrations and combinations with other organic acids.

Keywords: Ionization; Norfloxacin; Oxalic Acid; pH; translocation.

Introduction

- One of the strategies employed to combat antimicrobial resistance is the use of combinations of antibacterial agents that generate synergism. NOR (an FQ antibiotic) is effectively used to treat bacterial infections caused by Gram-positive and Gram-negative bacteria. These antibacterial agents act by inhibiting bacterial enzymes topoisomerase IV and DNA gyrase. OA is an organic acid with reports of antibacterial activity (Towle et al; 2018, Kumar et al; 2018).

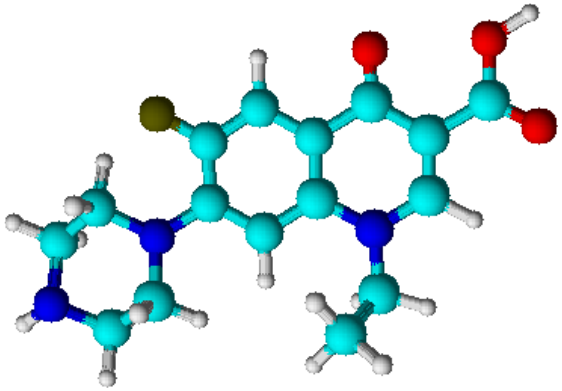


Figure 1. NOR

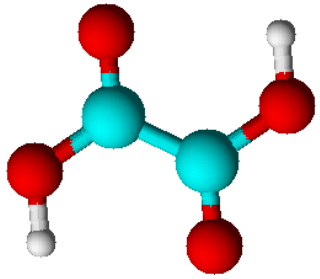


Figure 2. OA

Introduction

- FQs translocate across the bacterial membrane by active transport and to a lesser extent by passive transport. Gram-negative bacteria, such as *E. coli*, have a highly hydrophobic outer membrane, and translocation of FQs through it is mainly governed by the presence of "porins" and "self-promotion" mechanism, among others. For a given FQ, the contributions of the porin and non-porin pathways to total accumulation in the translocation process depend on their hydrophobicity (Cama et al; 2015, Cramariuc et al; 2013).

Introduction

The solubility of FQs varies depending on the medium and the solvent in which it is found. In figure 3 we can observe the pH partitioning profile of the NOR along with other FQs, they have a parabolic shape. This reflects the maximum lipophilicity of the compounds at their isoelectric points (Takács-Novák et al; 1992).

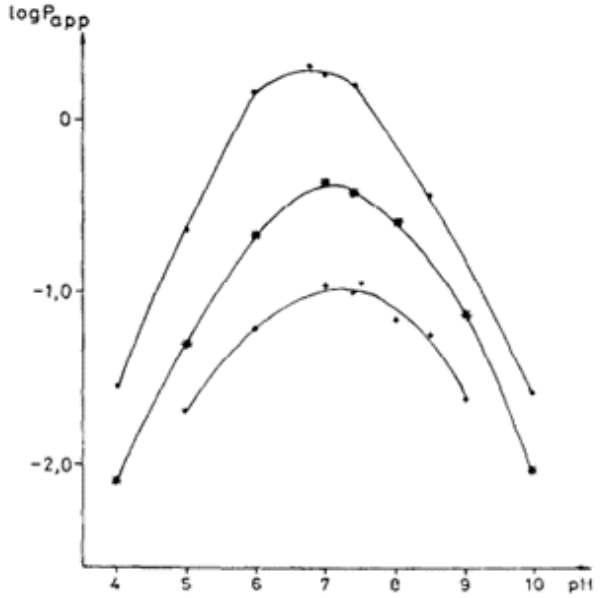
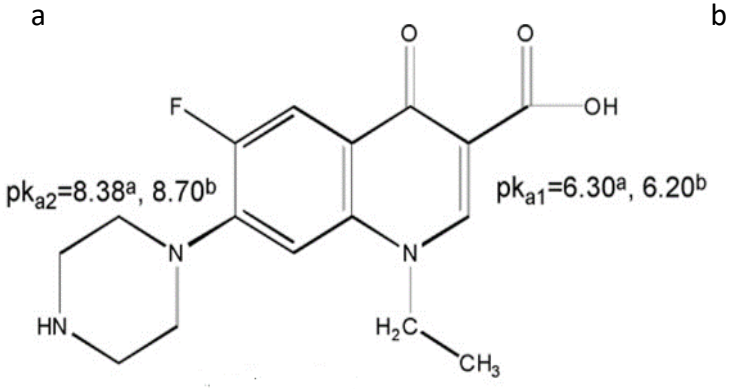


Figure 3. Fluoroquinolone pH-partition profiles: (•) pefloxacin, (*) ofloxacin, (+) norfloxacin (Takács-Novák et al; 1992).

Introduction

The changes in bacterial susceptibility to FQs due to variations in pH are related to changes in FQ uptake due to alterations in the electrical charge of the antibiotic molecule. One of the most relevant characteristics of these antibacterials is the presence of ionizable functional groups. As the balance of the species depends on the pH, the solubility of the FQs also varies according to the medium and the solvent in which it is found.

a



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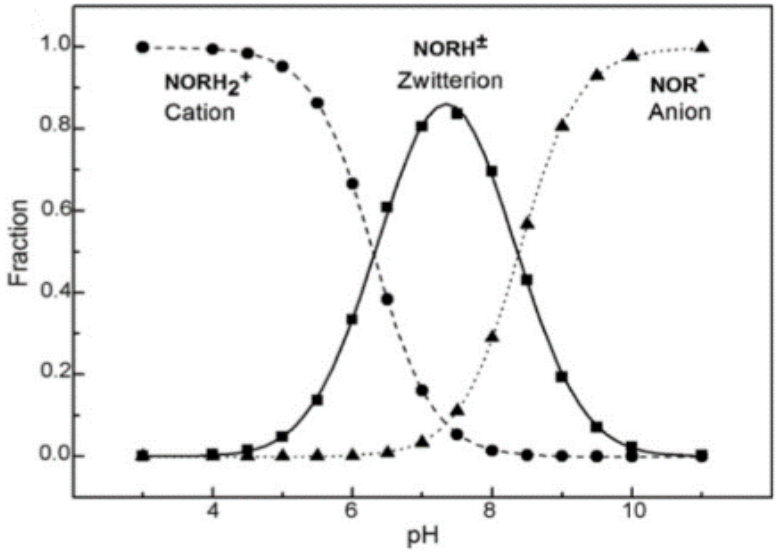


Figure 4. (a) Chemical structure of NOR, (b) pH-dependent NOR speciation (Zhang et al; 2008).

Introduction

FQs have two ionizable functional groups. (Takács-Novák et al; 1992). As shown in Figure 5, most of the molecules that belong to this family form zwitterions, including NOR; with an isoelectric point ranging between 6.8 and 7.8, therefore, at physiological pH, the predominant forms are zwitterions, followed by the neutral form (Millanao et al; 2021).

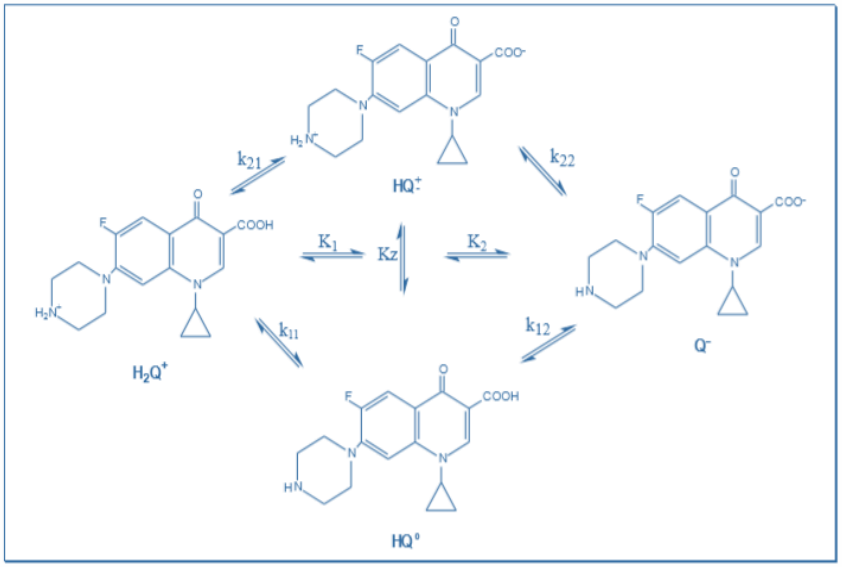


Figure 5. Interconversion scheme between the four CIP microspecies together with the macro and micro constants. The same scheme can be extrapolated to the case of NOR (Ross DL; 1992).

Objectives

- Evaluate the antibacterial activity of the NOR-OA combination in the *E. coli ATCC* 25922 strain.
- Calculate the percentages of ionization and log D of NOR to understand how the presence of OA affects its antibacterial activity in the NOR-OA combination.

Methodology

The minimal inhibitory concentration (MIC) of NOR and OA were determined, and, in turn, the antibacterial effect of the NOR-OA combination was evaluated by the checkerboard method (Pinto Vitorino et al; 2017), using the microdilution technique in a 96-well plate and macrodilution in tubes to facilitate the measurement of pH. The guidelines recommended by the CLSI were followed (M100; 2022). Assays were performed in quadruplicate. In turn, the effect of pH on the NOR activity in the NOR-AO combination was evaluated. The percentages of ionization of the NOR species in equilibrium were calculated, using the Henderson-Hasselbalch equation (Equation 1), and the apparent log P (log D) (Equation 2) to establish the lipophilicity of NOR at different pH.

$$pH - pka = log\left(\frac{x}{100-x}\right)$$

Equation 1: Henderson-Hasselbalch equation for weak acids.

$$log\,P = logD + log\left(1 + \frac{K_{21}}{K_{11}} + \frac{K_{12}}{[H^+]} + \frac{[H^+]}{K_{11}}\right)$$

Equation 2: Establishes the relationship between the true partition coefficients (log P) and log D.

Results and discussion

The MIC is defined as the minimum concentration of drug capable of inhibiting the visible growth of the microorganism. On the other hand, the FIC index evaluates the antibacterial effect of two or more drugs that are used in combination. This index allows to predict the synergistic (S), partially synergistic (PS), Indifferent (I) and Antagonistic (A) effect. Subsequently, the FIC indices were calculated using equation 3. Table 1 shows the criteria for interpreting the FIC indices.

$$CIF\ Index = \frac{MIC\ drug\ A\ in\ combination}{MIC\ drug\ A\ only} + \frac{MIC\ drug\ B\ in\ combination}{MIC\ drug\ B\ only}$$

Equation 3. Calculation of the FIC index.

Table 1. FIC indices interpretation criteria.

Antibacterial effect	FIC Index
Synergism	≤ 0,5
Partial Synergism	>0,5-1
Indifference	>1 ≤ 4
Antagonism	> 4

Results and discussion

Table 6. Checkerboard NOR-OA in *E. coli* ATCC 25922 and the respective pH of the solutions.

	N1 0.25 µg/mL pH: 7.36	N2 0.125 µg/mL pH:6.15	N3 0.06 µg/mL pH: 6.13	N4 0.003 µg/mL pH:6.86	N5 0.015 µg/mL pH:6.47
OA1 2500 µg/mL pH: 3.59	pH: 3.56	pH:3.56	pH:3.61	pH:3.62	pH:3.66
OA2 1250 µg/mL pH: 4.34	pH: 4.33	pH:4.35	pH: 4.33	pH:4.35	pH: 4.38
OA3 625 µg/mL pH: 5.27	pH: 5.23	pH: 5.28	pH: 5.45	pH: 5.50	pH: 5.33
OA4 312.15 µg/mL pH: 5.28	pH: 5.78	pH: 6.16	pH:7.21	pH: 7.58	pH: 7.27
OA5 156.15 µg/mL pH: 6.57	pH: 6.66	pH: 6.37	pH:7.53	pH:7.75	pH: 6.67
OA6 78.12 µg/mL pH: 6.55	pH: 7.26	pH: 5.81	pH:7.40	pH: 8.03	pH: 8.18
OA7 39.06 µg/mL pH: 6.64	pH: 7.44	pH: 5.77	pH: 7.40	pH: 8.16	pH: 8.26

Pink squares: indicate inhibition of bacterial growth. **Green squares:** indicate bacterial growth.

Results and discussion

- The MIC obtained for OA and NOR was 1250 µg/mL and 0.25 µg/mL respectively.

- Interaction of NOR-OA: The FIC index indicates that no improvement in antibacterial activity is observed in the combination concerning the individual drugs, presenting an indifferent effect in the range of concentrations tested in the checkerboard.

- Atypical behavior: In column 2, which corresponds to NOR1 in the checkerboard, we could appreciate that in the combinations N1/OA3 and N1/OA4 there was bacterial development, even though in both combinations the concentration of NOR corresponded to the MIC of the drug in the checkerboard. We attribute this to the effect of the pH on NOR activity.

Results and discussion

- The acidity that OA confers to the medium (pH of 5.23 in OA3/N1 and pH 5.78 in OA4/N1) affects the activity of NOR due to the physicochemical properties of this drug and its mechanism of translocation through the membrane (Millanao et al; 2021).

- This is possibly due to reduced uptake of fluoroquinolones in bacterial cells, as has been observed in some studies on bacterial uptake of ciprofloxacin and reversibility of the effect when the pH is restored (Erdogan et al, 2011). This is consistent with the effect observed in the combinations OA5/N1, OA6/N1, and OA7/N1, where we can show that NOR activity is restored as the pH increases.

Results and discussion

Calculation of the ionized and non-ionized fraction of NOR at pka_1 6.30 and pka_2 8.38

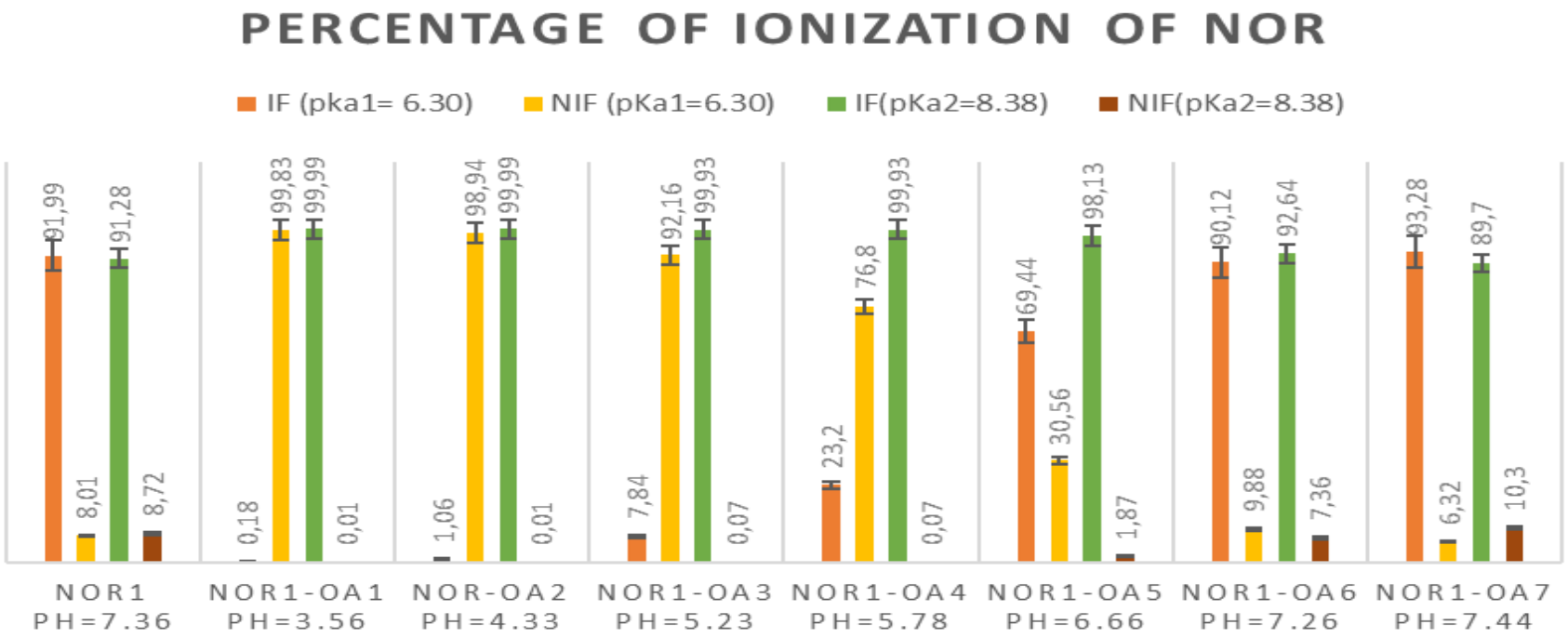


Figure 7. Percentage of NOR ionization depending on the pH of the medium.

Results and discussion

Calculation of the ionized and non-ionized fraction of NOR at pka_1 6.30 and pka_2 8.38

Table 2. Predominant NOR species at each pH.

Drug / Combination	pH	Predominant species
NOR1	7.36	Zwitterion
NOR1/OA1	3.56	cationic species (NH_2^+)
NOR1/OA2	4.33	cationic species (NH_2^+)
NOR1/OA3	5.23	cationic species (NH_2^+)
NOR1/OA4	5.78	cationic species (NH_2^+)
NOR1/OA5	6.66	Zwitterion
NOR1/OA6	7.26	Zwitterion
NOR1/OA7	7.44	Zwitterion

Results and discussion

- Analyzing the percentages of ionized species of NOR in relation to the pH of the medium, we could appreciate that, at acidic pH values, where the NOR presents a decrease in its activity (N1/OA4 and N1/OA5), the predominant species is the cationic species, which hinders its passage through the membrane.

- We could also observe that as the pH increases, the NOR activity is restored. This is probably because at these pH values the zwitterionic species predominates with the highest solubility in a polar environment. It would facilitate its passage through the pores of the bacterial membranes (Kłosinska-Szmurło et al; 2014, Millanao et al; 2021).

Results and discussion

Lipophilicity: Calculation of distribution coefficient or Log D

Table 3 shows the microconstants, H^+ concentration, and log D for the NOR alone and in each combination.

Table 3. Microconstants of NOR, concentration of H^+ for the calculation of log D.

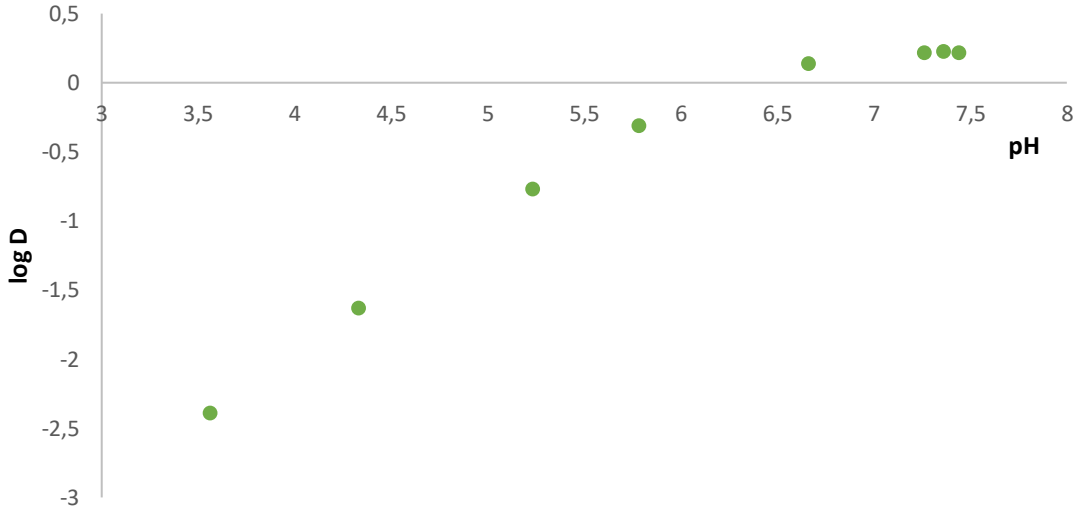
NOR microconstants*	DRUG/ Combination	pH	CC de H^+	Log D
	N1	7,36	$4{,}37\times10^{-8}$ M	0,23
Pka21= 6,28	NOR1/OA1	3,56	$2{,}75\times10^{-4}$M	-2,39
	NOR1/OA2	4,33	$4{,}68\times10^{-5}$M	-1,63
Pk11= 7,43	NOR1/OA3	5,23	$5{,}89\times10^{-6}$M	-0,77
	NOR1/OA4	5,78	$1{,}66\times10^{-6}$M	-0,31
	NOR1/OA5	6,66	$2{,}19\times10^{-7}$M	0,14
Pk12= 7,32	NOR1/OA6	7,26	$2{,}8\times10^{-7}$ M	0,22
	NOR1/OA7	7,44	$3{,}63\times10^{-08}$M	0,22

*Takács-Novák et al; 2000

Results and discussion

Figure 8 shows the Distribution coefficient (log D) of NOR, calculated as a function of the pH of the medium in the NOR-OA combinations.

Figure 8. Distribution coefficient (log D) vs. pH of the medium in the combinations of NOR-AO.



Results and discussion

- Figure 8 shows that under acid conditions log D decreases. The concentration of the liposoluble NOR species (the neutral species) decreases and therefore the amount of drug capable of diffusing directly through the membrane.

- There is an increase in the concentration of hydrosoluble cationic species but unable to translocate through the lipid bilayer, either by diffusion through the lipid membranes or the pores.

- These results reinforce the idea that only the forms without net charge are the ones that can translocate through the bacterial cell membrane.

- Studies of the translocation kinetics of NOR indicate that it depends on the concentration of divalent cations (Wang et al; 2020). Considering that OA is a chelating agent and that it provides acidity to the medium, we could say that its presence in the combination would not only affect the activity of NOR by modifying the balance of its species, but also by the chelation of divalent ions.

Conclusions

- The MIC obtained for OA and NOR was 1250 µg/mL and 0.25 µg/mL, respectively. The interaction of NOR-OA was indifferent in the concentrations worked (CIF 1.12). The atypical behavior observed in *E.coli* growth is due to the acid conditions given by the presence of AO. This decrease the concentration of the neutral species of NOR and therefore the amount of drug capable of diffusing directly through the membrane.

- The mechanism of entry at the molecular level of these drugs through membranes is still under debate. The susceptibility of bacteria to FQs in response to pH changes is a complicated process, which could involve both changes in porin expression and changes in drug transport through porins in the membrane, as well as through the inner phospholipid membrane. However, our results show that the pH of the medium is a determining factor that directly impacts the antibacterial activity. To deepen this study, we will continue testing new concentrations and combinations with other organic acids.

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Acknowledgments

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