Activity of marbofloxacin against *Escherichia coli* in the presence of serum of water buffalo (*Bubalus bubalis*)

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### SUMMARY

The *in vitro* activity of marbofloxacin against *Escherichia coli* in the presence of buffalo serum was studied. The estimated MIC value of marbofloxacin for the tested strain was 0.0312 µg/mL, and two assays of time-kill curves were performed: the first one in a standard culture medium and the second one in the presence of buffalo serum. Marbofloxacin presented a biphasic bactericidal kinetics; the first phase characterized by a concentration-dependent mode of action, and a second phase in which the efficacy was determined by the length of exposure. This phenomenon could be explained by the selection of a sub-population of persister cells. The slow elimination rate of cells observed at high concentrations of marbofloxacin even in the presence of buffalo serum should be taken into account for the design of dosing regimens in this species. Appropriate dosing regimens should be designed for eradicating the surviving cells, because this persistence property could ensure the survival of this sub-population, which can be associated with the recalcitrant nature of chronic infections.

**Keywords:** Marbofloxacin, *Escherichia coli*, buffalo serum, *in vitro* activity, persisters

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### Introduction

The quantification of activity of antibacterial agents is the basis for their rational and safe use in the treatment of bacterial infectious diseases. Since the discovery of them, the minimum inhibitory concentration (MIC) is the main pharmacodynamic parameter used for the design of antibacterial regimens [33]. Nonetheless, in the last three decades, certain *in vitro* properties of these agents as post-antibiotic effect (PAE) and the activity of sub-inhibitory concentrations (sub-MIC effect) [35], have been reconsidered and have provided more information about the PD of antibiotics. Similarly, *in vitro* time-kill curves became a useful tool for evaluating the efficacy in function of time of different concentrations of an antibiotic [25].

However, all the *in vitro* assays above mentioned only assess the activity of fixed concentrations of an antibiotic, whereas in a living organism, the total effect of certain dose depends on the large range of concentrations encountered in the patient due the pharmacokinetic processes of absorption, distribution and elimination [38, 45].

An alternative *in vitro* model that introduces the fluctuating concentration of the antimicrobial agent is the assay to determine the postantibiotic sub-CIM effect (PAE sub-CIM). This model simulates the brief exposure of bacteria to suprainhibitory concentrations (>MIC) observed during the peak plasma concentration followed by a more or less prolonged subinhibitory concentrations (<MIC) similar to what happens during the elimination phase in the *in vivo* scenario [33, 35].

In this sense, several dynamics *in vitro* models have been developed for mimicking the changes in the antibiotic concentrations in body fluids, allowing evaluate the relationship between concentrations, and bacterial population versus time [7, 11].

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However, all these experimental conditions differ from what happens in a living organism, due to absence of components of the host immune system [15]. By far the most important factor for the outcome of an infection is the interaction between patient and causative microorganism, since antibiotic treatment only helps indirectly by combating the microorganism [9, 28]. This is because the clinical experience indicates that the success of antimicrobial therapy depends on the normal function of the patient’s defense system [23, 31]. Therefore, to establish optimum therapeutic regimens a thorough knowledge of the interaction between antibiotic and microorganism is not sufficient [28].

The addition of serum to the culture media, creates an in vitro environment that is intermediate between a standard in vitro assay and a clinical assay, and allows simulate conditions present in the in vivo scenario for evaluating the interaction between antibiotic, bacteria and host.

Susceptibility to the serum bactericidal system is a widespread characteristic of gram-negative bacteria. This phenomenon has been widely noted and studied since the late 1800s [44] and has been shown to be complement mediated [20, 39].

In addition to complement, lysozyme, calcium (Ca2+) and magnesium (Mg2+) are important components of the extracellular fluids that are required for killing Gram-negative bacteria. Calcium and magnesium ions are essential for initiation of classical complement sequence [32]. It has been established that immunoglobulins (IgG, IgM) antibodies were able to promote killing of some Gram-negative bacteria in vitro in the presence of complement [34] but immunoglobulin-M fraction was proved to be bactericidal [42].

In view of that, the assays of in vitro time-kill curves performed by incorporation of serum of the host to the culture medium, allows us to simulate environmental conditions that resemble those found in an in vivo assay.

The water buffalo (Bubalus bubalis) occupies a place of economic importance in the livestock industry of many parts of the world. In Argentina, in the last 50 years, some livestock producers have begun to evaluate the use of buffalo due to fertility, longevity, efficiency of feed conversion, production of meat and milk, and fundamentally for their ability to adapt to environmental conditions difficult or impossible for cattle production.

In the dairy region of province of Santa Fe, Argentina, there are dairy cattle farms that are no longer profitable, and the incorporation of water buffalo herds to these farms could be an alternative for the maintenance of these production systems. Because of dairy cattle are raised together with buffalo in some areas, similar diseases affect both species, accordingly the implementation of the same therapy is a common situation [6].

Marbofloxacin is a fluoroquinolone antibacterial drug developed for use in veterinary medicine [41], presenting a broad-spectrum activity against many pathogens of veterinary importance and is indicated for the treatment of gastroenteritis and mastitis caused by sensitive strains of *E. coli* in neonatal calves and dairy cows respectively [45].

This drug presents a pattern of bactericidal activity characterized by concentration-dependent killing [1, 27, 36], that’s means the higher the drug concentration, the greater the rate and extend of bactericidal activity [14].

Nevertheless Blot and colleagues [10] classified fluoroquinolones agents as concentration-dependent with time-dependence mode of action, in which the antimicrobial effect is defined by the area under the time-concentration curve over a 24-hour period (AUC0-24h) divided by the MIC (AUC0-24h/MIC)

Marbofloxacin has been authorized for the treatment of bovine infections at a daily dosage of 2 mg/kg for 3-5 days administered by intravenous, subcutaneous or intramuscular routes.

Although the PK behavior of marbofloxacin in buffalo and PK/PD indices for many bacteria of veterinary interest have been reported [26] there are no studies of in vitro activity of marbofloxacin on *E. coli* in the presence of buffalo serum (BS).

Many methodologies to quantify and evaluate the in vitro antibacterial effects of bacterial killing curves have been described [3, 13, 17, 19, 29, 33], but the treatment and interpretation of the obtained results remain a problem to be solved. Many results of such trials are lost because their interpretation is performed by a single method or using a single efficiency parameter type.

In our case, it is important to know if the BS can enhance the effectiveness of an antibiotic with concentration activity as marbofloxacin.

In view of that, the purpose of this study was to investigate and quantify the in vitro bactericidal activity of marbofloxacin against *E. coli* (ATCC 25922) in the presence of BS by in vitro time-kill curves assays, expressing the antibacterial effect in two ways: estimation of bacterial elimination rate by pharmacokinetic-pharmacodynamic modeling, and to quantify the antimicrobial effect by the use of descriptive and integral parameters.

**Materials and methods**

**MICROORGANISM AND ANTIBIOTIC**

A strain of *E. coli* (ATCC 25922) and marbofloxacin (Sigma-Aldrich, Chemical Company, St. Louis, USA) was selected to perform this assay.

**References**
WATER BUFFALO (BUBALUS BUBALIS) SERUM

Normal buffalo serum (BS) samples were obtained from six healthy adult water buffaloes with no history of antimicrobial therapy within the previous 3 months. The serum was pooled and stored at -20°C until use.

BACTERIAL INOCULUM

The bacterial inoculum was prepared from colonies incubated on appropriate agar plates overnight. The microorganisms were suspended in sterile isotonic saline solution to a concentration equivalent to a 0.5 value in the McFarland scale (1 x 10⁸ colony forming unit [cfu]/mL) measured with a turbidimeter. After that, serial dilutions were performed to obtain a final concentration of 0.5 x 10⁶ cfu/mL.

DETERMINATION OF MIC VALUES

The MIC of marbofloxacin on E. coli (ATCC 25922) was determined by macrodilution method [12] in Muller-Hinton broth (MHB) (Britania, Buenos Aires, Argentina). The growth media was prepared according to the manufacturer’s instructions and autoclaved prior to use at 121°C (15 min/1L). Broth was prepared the day before of the assay, so that it was stored in a refrigerator at approximately 7°C until use. The MIC was determined in the range of concentrations of 0.002 µg/mL to 1 µg/mL by tree sets of doubling dilutions. The value of the MIC considered was the largest value observed in the three sets of dilutions.

TIME-GROWTH CURVES

Bacterial cultures in MHB and MHB/BS 50:50 (MHB-BS) adjusted to 0.5 x 10⁶ cfu/mL were incubated at 35°C during 5 h. Aliquots (100 µl) were removed from the incubation cultures at 0, 0.5, 1, 2, 3.5 and 5 h and them were serially diluted in sterile isotonic saline solution, and colonies were counted after overnight incubation at 35°C.

TIME-KILL CURVES

The time-kill curves were performed taking as a reference the MIC determined in MHB. Bacterial cultures in MHB and MHB-BS adjusted to 0.5 x 10⁶ cfu/mL, were exposed to constant concentrations of marbofloxacin equivalent to 0.25 x MIC, 0.5 x MIC, 1 x MIC, 4 x MIC, 8 x MIC, 16 x MIC and 64 x MIC, and then incubated at 35°C during 5 h. Aliquots (100 µl) were removed from the incubation cultures at 0, 0.5, 1, 2, 3.5 and 5 h and then were serially diluted in sterile isotonic saline solution. Agar plates were inoculated with 100 µl of the final dilutions by duplicate, and the colonies were counted after overnight incubation at 35°C.

CONSTRUCTION OF TIME-GROWTH AND TIME-KILL CURVES

The values of cfu/mL at each sampling time were estimated by multiplying the number of cfu/plate by the correction factor derived from the serial dilution for each particular sample. The limit of detection was 10 cfu/mL. In each time-growth and time-kill curves, the number of bacteria was expressed as the average of counts made in duplicate. In order to facilitate the construction and interpretation of the curves, the bacterial counts were expressed as log₁₀ cfu/mL.

PHARMACOKINETIC-PHARMACODYNAMIC MODELING

The analysis and mathematical modeling of the time-growth and time-kill curves data were performed with the non-linear regression software ADAPT II (BMSR, University of Southern California, USA).

The parameters were calculated directly from viable counts (cfu/mL) without log transformation. Weightings used for the xth measured viable count were 1, 1/√x, 1/x and 1/x². Plots of weighted residuals were inspected and the best weighting scheme was selected based on the scatter and random distribution of residuals about the abscissa.

The data of bacterial count obtained in the time-growth curves in MHB and MHB-BS were fitted with the equation 1, which considers the limited available nutrients and space of the in vitro system.

\[
\frac{dN}{dt} = k_g \cdot \left(1 - e^{-z.t}\right) \cdot N
\]

Where \(dN/dt\) is the change in the number of bacteria as a function of time, \(k_g\) (h⁻¹) is the bacterial growth rate constant in the absence of marbofloxacin in MHB, \((z)\) is an adaptation rate constant for describing a delayed effect in growth and \(N\) (cfu/mL) is the number of viable bacteria.

The value of the bacterial kill rate constant \(k_{elb}\) resulting from the activity of BS was estimated with the equation 2.

\[
k_{elb} = k_g^{MHB} - k_g^{MHB-BS}
\]

Where \(k_g^{MHB}\) (h⁻¹) and \(k_g^{MHB-BS}\) (h⁻¹) are the bacterial growth rate constant in the absence of marbofloxacin in MHB and MHB-BS respectively.

Data obtained in time-kill curves in MHB and MHB-BS were fitted with the equation 3, which describes a biphasic killing pattern of marbofloxacin activity.

\[
\frac{dN}{dt} = \left[(k_g - k_{elb}) \cdot \left(1 - e^{-z.t}\right)\right] \cdot N
\]

Where \(k_g\) (h⁻¹) is the bacterial growth rate constant estimated from the fitting of the time-growth curves in MHB.
which was considered as reference, and \(k_{\text{elb}}\, (\text{h}^{-1})\) is the kill rate constant associated to an initial phase of rapid killing of the bulk bacterial population, resulting from the concentration-dependent activity of marbofloxacin in MHB and in the presence of BS, and (a) is a dimensionless factor used to fit the decrease of kill rate associated with the slow elimination of a tiny fraction of the initial count which survives after a few hours of antibiotic exposition. Other symbols were explained previously. In order to evaluate the effect of the BS on the bacterial elimination rate of \(E.\, coli\), the time required for the bacterial viable count to reduce by 50\% \((t_{50})\) at several concentrations in MHB and MHB-BS was estimated as: \(\ln2/ k_{\text{elb}}\). The relationship between marbofloxacin concentrations and \(k_{\text{elb}}\) in MHB and MHB-BS was fitted with the commonly used \(E_{\text{max}}\) model:

\[
k_{\text{elb}} = \frac{k_{\text{max}} \cdot C^n}{C_{50}^n + C^n}
\]

where \(k_{\text{max}}\, (\text{h}^{-1})\) is the maximum killing rate constant; \(C\, (\mu\text{g/mL})\) is the fixed marbofloxacin concentration, \(C_{50}\, (\mu\text{g/mL})\) is the concentration of marbofloxacin necessary to produce 50\% of the maximum effect and \(n\) is the Hill coefficient or shape factor. Other symbols were explained previously.

**QUANTIFICATION OF TIME-KILL CURVES AND ANTIMICROBIAL EFFECT**

The antimicrobial effect of marbofloxacin in MHB and MHB-BS against \(E.\, coli\, (\text{ATCC 25922})\) was quantified by different parameters:

**DESCRIPTIVE PARAMETERS**

Descriptive parameters are depicted in figure 1, where \(N_0\) is the initial inoculum count expressed as \(\log_{10}\, \text{cfu/mL}\), \(N_n\) is the \(\log_{10}\) of the bacterial count (cfu/mL) resulting from the exposure to antibiotic at the end of the experiment, \(t_0\) and \(t_n\) are the start time (0 h) and the end time (5 h) respectively. In this study the measurement of bacterial elimination was determined by the actual reduction in viable counts at \(t_n\).

**INTEGRAL PARAMETERS**

Some previously reported integral parameters [17] were used for quantifying the antibacterial efficacy of marbofloxacin against \(E.\, coli\, (\text{ATCC 25922})\) (Figure 1); AUC\(_N\), is the algebraic sum of the areas around the \(N_n\) level from \(t_0\) to \(t_n\) and represent an equilibrium of the bacteria growth and death over time; AUBC is the area under the viable bacterial count resulted from the antibiotic exposure from \(t_0\) to \(t_n\) and AAC represents the theoretical bacterial biomass eliminated from \(t_0\) to \(t_n\) calculated as AUC\(_N\) - AUBC. The areas under the curves were calculated by the linear trapezoidal rule method from the experimental data (cfu/mL) without \(\log_{10}\) transformation [4].

**EFFICACY BREAKPOINTS**

Three breakpoints for descriptive parameters were considered for evaluating the efficacy (E) of marbofloxacin concentrations against \(E.\, coli\, (\text{ATCC 25922})\): (i) bacteriostasis was defined as no change in the initial bacterial count (E = 0) after 5 h incubation, (ii) bactericidal action was defined as 3 \(\log_{10}\) reduction of the initial bacterial count (E = 99.9\%) after 5 h incubation and (iii) virtual eradication of organisms was defined as 4 \(\log_{10}\) reduction of the initial bacterial count (E = 99.99\%) after 5 h incubation [43].

In the case of integral parameters, the percent of reduction of bacteria biomass of \(E.\, coli\) obtained by each concentration of marbofloxacin in MHB and MHB-BS was estimated using the integral endpoints of the antimicrobial effect by the following equation:

\[
\text{Reduction of bacteria biomass} = (\text{AAC/AUCN}_N) \times 100
\]

**Results**

The estimated MIC value of marbofloxacin against \(E.\, coli\, (\text{ATCC 25922})\) in MHB was 0.0312 \(\mu\text{g/mL}\), and the time evolution of viable count in function of several concentrations of marbofloxacin in MHB and MHB-BS are presented in figure 2.

The viable bacterial count observed at the end of the assay in the time-growth curve in the presence of BS expressed as \(\log_{10}\, \text{cfu/mL}\) was low (7.05) than that observed in an standard culture medium (7.44).
In time-kill curves, the efficacy of marbofloxacin at the range of concentrations of 0.25 x MIC to 1 x MIC was greater in the presence of BS than those observed in MHB. However efficacies of 99.9% - 99.99% were observed for concentrations ranged from 4 x MIC to 64 x MIC.

The visual inspection of semilogaritmic plot of time-kill curves showed that bulk _E. coli_ population presented a biphasic killing pattern. In the first phase, a concentration dependent mode of action was observed. However, when viable bacteria count decreased below a certain threshold a slow killing rate was observed.

This phenomenon allowed us to distinguish two theoretical areas. The first was termed concentration-dependent area, in which the increased marbofloxacin concentration is the increased bactericidal elimination rate. This area is limited from \( N_0 \) until a viable count between 0.5 % - 0.04 % of \( N_0 \).

The second theoretical area is related with the slow elimination of a tiny fraction of the initial count which survives after a few hours of antibiotic exposition, and is comprised by all bacterial counts below 0.5 % - 0.04 % of \( N_0 \). In this area the bacterial elimination rate was not modified by the increasing concentrations of marbofloxacin (Figure 3).

The efficacy of marbofloxacin on _E. coli_ (ATCC 25922) expressed as percent of reduction of bacteria biomass presented similar values in MHB than MHB-BS at concentrations ranged from 1 x MIC to 64 x MIC. However at sub-inhibitory concentrations, the percent of reduction of bacterial biomass was greater in the presence of BS than those observed in MHB (Table I).

The fitted curves of viable bacterial count of _E. coli_ (ATCC 25922) in the presence of constant concentrations of marbofloxacin in MHB and MHB-BS are presented in figure Figure 2:

**Figure 2:** Time-kill curves of _E. coli_ (ATCC 25922) in (A) Muller Hinton broth (MHB) and (B) Muller Hinton broth/serum of buffalo 50:50 (MHB-BS) in the absence of antibiotic (control) or in the presence of constant concentrations of marbofloxacin; 0.0078 µg/ml (0.25 x MIC), 0.0156 µg/ml (0.5 x MIC), 0.0312 µg/ml (1 x MIC), 0.124 µg/ml (4 x MIC), 0.25 µg/ml (8 x MIC), 0.5 µg/ml (16 x MIC) and 2 µg/ml (64 x MIC). The dotted lines represent the baseline that indicates (a) bacteriostasis, (b) the 99.9% (antibacterial activity) and (c) the 99.99% (virtual bacterial eradication) reduction of the original inoculum count after 5 h incubation. Each datum point represents the mean (n = 2) of colony forming units per milliliter (cfu/mL).

**Figure 3:** Schematic representation of the biphasic killing pattern of marbofloxacin against _E. coli_ (ATCC 25922) in Muller Hinton broth (MHB) and Muller Hinton broth/serum of buffalo 50:50 (MHB-BS). The dotted line represents the limit (0.5 % to 0.04 % of \( N_0 \)) between (A) theoretical concentration-dependent area and (B) area associated with the slow elimination of a tiny fraction of the initial count which survives after a few hours of antibiotic exposition; \( N_0 \) is the initial inoculum count expressed as log\(_{10}\) ufc/mL, \( N_{tn1} \) to \( N_{tn2} \) to are the log\(_{10}\) of the bacterial count (cfu/mL) resulting from the exposure to increased marbofloxacin concentrations at the end of the experiment; \( t_0 \) and \( t_n \) are the start time (0 h) and the end time (5 h) respectively.

**Table I:** Reduction of bacteria biomass of an inoculum of _E. coli_ (ATCC 25922) exposed at constant concentrations of marbofloxacin during five hours in Muller Hinton broth (MHB) and Muller Hinton broth/serum of buffalo 50:50 (MHB-BS). The percent of reduction was estimated using the integral endpoint of the antimicrobial effect.

<table>
<thead>
<tr>
<th>Marbofloxacin (µg/mL)</th>
<th>Culture medium</th>
<th>diff</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHB</td>
<td>MHB-BS</td>
</tr>
<tr>
<td>0.0078 (0.25 x MIC)</td>
<td>NC</td>
<td>56.81</td>
</tr>
<tr>
<td>0.0156 (0.5 x MIC)</td>
<td>33.17</td>
<td>68.54</td>
</tr>
<tr>
<td>0.0312 (1 x MIC)</td>
<td>80.79</td>
<td>86.82</td>
</tr>
<tr>
<td>0.125 (4 x MIC)</td>
<td>85.37</td>
<td>90.15</td>
</tr>
<tr>
<td>0.25 (8 x MIC)</td>
<td>94.41</td>
<td>94.30</td>
</tr>
<tr>
<td>0.5 (16 x MIC)</td>
<td>94.18</td>
<td>95.35</td>
</tr>
<tr>
<td>2 (64 x MIC)</td>
<td>95.30</td>
<td>95.40</td>
</tr>
</tbody>
</table>

NC is a value not calculable because an increase in biomass was observed, diff is the difference between the percent of reduction of bacteria biomass estimated as % eliminated in MHB-BS minus % eliminated in MHB.
4, and the values of $k_g$ in MHB and MHB-BS and the values of $k_{elb}$ and $t_r$ of each marbofloxacin concentration in MHB and MHB-BS are listed in table II.

The estimated bacterial kill rate constant ($k_{elb}$) resulting from the activity of BS was 0.099 h\(^{-1}\). The bacterial elimination rate values ($k_{elb}$) estimated from the viable counts observed at the concentration dependent phase were high in the presence of BS than those observed in MHB consequently a faster decrease ($t_r$) of viable cells was observed in MHB-BS than that in MHB (Table II).

The fitting of the concentration-response relationship between marbofloxacin concentrations and $k_{elb}$ values in MHB and MHB-BS are depicted in figure 5, and the estimated pharmacodynamic parameters are presented in Table III.

Similar values for the maximum killing rate ($k_{max}$) of marbofloxacin were estimated in MHB and MHB-BS. However in the presence of BS the values of $k_{elb}$ obtained at concentrations in the range of 0.0078 µg/mL and 2 µg/mL were higher than those observed in standard culture medium.

### Table II: Pharmacodynamic parameters of marbofloxacin against E. coli (ATCC 25922) in Muller Hinton broth (MHB) and Muller Hinton broth/serum of buffalo 50:50 (MHB-BS).

<table>
<thead>
<tr>
<th>Marbofloxacin (µg/mL)</th>
<th>Culture medium</th>
<th>MHB</th>
<th>MHB-BS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_g$ (h(^{-1}))</td>
<td>$k_{elb}$ (h(^{-1}))</td>
<td>$t_r$ (min)</td>
</tr>
<tr>
<td>Control</td>
<td>1.527</td>
<td>0.113</td>
<td>368.0</td>
</tr>
<tr>
<td>0.0078 (0.25 x MIC)</td>
<td></td>
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<tr>
<td>0.0156 (0.5 x MIC)</td>
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<td></td>
<td></td>
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<tr>
<td>0.0312 (1 x MIC)</td>
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<td></td>
<td></td>
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<tr>
<td>0.125 (4 x MIC)</td>
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<tr>
<td>0.25 (8 x MIC)</td>
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<tr>
<td>0.5 (16 x MIC)</td>
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<td></td>
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<tr>
<td>2 (64 x MIC)</td>
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</table>

$k_g$ is the bacterial growth rate constant; $k_{elb}$ is the bacterial killing rate constant, and $t_r$ is the time required for the bacterial viable count to decrease by half.\(^*\) is the estimated value of $k_{elb}$ resulting from the activity of BS calculated as $k_{elb}$ estimated in MHB minus $k_g$ estimated in MHB-BS.
ACTIVITY OF MARBOFLOXACIN AGAINST ESCHERICHIA COLI IN THE PRESENCE OF BUFFALO SERUM

Discussion

The concentration-effect relationship of antibiotics is often not well known, because the antibiotic effect observed in vitro does not consider other factors present in the host which determine the real efficacy in vivo.

In view of that, the study of the in vitro activity of antibiotics should simulate the conditions present in the in vivo scenario to evaluate the interaction between the bacteria, antibiotics and immune factors present in the living organism and thus generate information to be used for the design and optimization of dosage regimens. In the present study, the experimental design was based on: (i) the intrinsic antibacterial activity of BS and (ii) the assumption that marbofloxacin is an antibacterial drug with a concentration-dependent mode of action and that five hours of exposure to high levels of the drug would be enough to attain a virtual eradication of the bacterium (4 log₁₀ reduction of log₁₀ N₀).

Our results showed that the growth of E. coli (ATCC 25922) was negatively affected by the presence of BS. Accordingly, growth rates and viable counts after five hours of incubation were lower in the presence of BS than in MHB (Table II). The fitting of the growth curves allowed calculating an elimination rate (0.099 h⁻¹). This finding could be associated with the antibacterial activity of BS components.

The presence of BS increased the efficacy of sub-inhibitory concentrations of marbofloxacin, which was evidenced by the lower viable counts at the end of the assay and the higher values of the percent of bacterial biomass eliminated at concentrations equivalent to 0.25 x MIC and 0.5 x MIC (Table I). On the other hand, the dose-response relationship between marbofloxacin concentrations and the estimated killing rate constant (kₘₐₓ) showed that the estimated maximum killing rate constant (kₘₐₓ) in MHB and MHB-BS were similar. However, the estimated values of kₘₐₓ were higher in the presence of BS than in MHB (Table III).

All the aforementioned findings could be explained by the presence of the components of BS, which, by interacting with marbofloxacin, could have improved its antibacterial efficacy against E. coli (ATCC 25922), particularly at sub-inhibitory concentrations. Another interesting finding was that the concentration-dependent activity of marbofloxacin finished when the viable counts decreased below 0.5% - 0.04% of N₀, which represent a log₁₀ reduction of 2.30 and 3.40 of log₁₀ N₀ respectively. The significance of this finding resides on the fact that the fast decay of viable counts due to the concentration-dependent mode of action was not enough to guarantee the virtual eradication of the bacterium (≥4 log₁₀ reduction of log₁₀ N₀).

Taking into account the schematic representation of the antibacterial activity of marbofloxacin depicted in figure 3, the limit between the concentration-dependent area and the slow elimination area could be determined by the presence of a small number of viable bacteria which survived to a marbofloxacin concentration that exceeded a certain threshold, which in our case was 1 x MIC (0.0312 µg/mL). Thus, all the concentrations above 1 x MIC exhibited similar profiles of bacterial elimination, being the time of exposure to constant concentrations of marbofloxacin an important factor which determined the final efficacy.

These 'survivors' are cells that neither grow nor die in the presence of antibiotic concentrations and are so-called 'persister cells' [2, 8, 24]. Persister cells are highly multidrug-tolerant cells which are transiently refractory to killing, without having acquired resistance through genetic modification and thus being phenotypic variants of regular bacteria [8, 21, 22, 24]. When the antibiotic pressure drops, persister cells will give rise to a population that is as susceptible as the original one, and that again possesses a similarly small proportion of them.

Persisters have a significantly reduced growth rate [5], and their indifference to the presence of antibiotic concentrations can be explained by a global shutdown of the processes essential for active growth, as the very processes that are targeted by antibiotics are no longer operational and hence no longer subject to inhibition. This phenomenon corroborates the non-specific nature of persister cell drug tolerance [16].

Compared with other antibacterial agents after intramuscular administration, marbofloxacin, as other fluoroquinolones, has a flatter time-concentration curve,

<table>
<thead>
<tr>
<th>Pharmacodynamic Parameters</th>
<th>MHB</th>
<th>MHB-BS</th>
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<tbody>
<tr>
<td>k₀ (h⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kₘₐₓ (h⁻¹)</td>
<td>30.14</td>
<td>1.557</td>
</tr>
<tr>
<td>Cₘₐₓ (µg/mL)</td>
<td>0.762</td>
<td>29.40</td>
</tr>
<tr>
<td>n</td>
<td>0.809</td>
<td>0.472</td>
</tr>
</tbody>
</table>

k₀ is the estimated basal bacterial killing rate constant observed in MHB-BS; kₘₐₓ is the maximum bacterial killing rate constant; Cₘₐₓ is the concentration of marbofloxacin necessary to produce 50% of the maximum effect and n is the Hill coefficient.

Table III: Pharmacodynamic parameters estimated by fitting of the concentration-response relationship between marbofloxacin concentrations and the estimated bacterial killing rate constant (kₘₐₓ), against E. coli (ATCC 25922) in Muller Hinton broth (MHB) and Muller Hinton broth/serum of buffalo 50:50 (MHB-BS).
lower peak concentration ($C_{\text{max}}$) and less distinction between $C_{\text{max}}$ and trough plasma concentrations. In addition, its high volume of distribution contributes to a lower renal excretion and a longer half-life [40]. After intramuscular administration to buffalos at a dose of 2 mg/kg, marbofloxacin exhibits $C_{\text{max}}$ values of $1.11 \pm 0.19 \mu g/mL$, reaching this peak concentration at $0.78 \pm 0.21$ h and presenting a half-life of $5.75 \pm 1.59$ h [18].

For antibiotics with concentration-dependent activity as marbofloxacin, the in vivo effect depends on the possibility of achieving a concentration above the MIC for the infecting pathogen as high as possible to ensure maximal killing. So, the PK-PD predictive indices of in vivo efficacy could be $C_{\text{max}}$/MIC ($\geq 10$) and AUC/MIC ($\geq 125$) [18, 37].

In the case of marbofloxacin, a peak concentration of $1.11 \pm 0.19 \mu g/mL$ [18] would represent a $C_{\text{max}}$/MIC of $35.5$ for the tested strain, which widely exceeds the breakpoint of $10$. Moreover, after reaching the peak concentration, the levels of drug in plasma decreased gradually. This suggests that high levels of the antibiotic are not operative for a long time.

On the other hand, the results obtained in vitro showed that the fast reduction of bacterial count that occurred between concentrations of $2 \mu g/mL$ ($64 \times$ MIC) and $0.125 \mu g/mL$ ($4 \times$ MIC) disappeared after a brief time of exposure ($4$ to $5$ h) due to the selection of a sub-population of persister cells (Figure 1). This finding allows us to infer a limited impact of high $C_{\text{max}}$/MIC ratios on the in vivo efficacy of marbofloxacin against *E. coli*. [30]. This phenomenon could impair the efficacy of other classes of concentration-dependent antibiotics as aminoglycosides, and the prolonged half-life would be useful to increase the length of exposure to eradicate persister cells.

In conclusion, the results obtained in this study show that the limit between the concentration-dependent and time-dependent antibacterial mode of action of marbofloxacin is not yet well defined. On the other hand, the slow elimination rate of persister cells observed at high concentrations of marbofloxacin even in the presence of BS should be taken into account for the design of dosing regimens in this species. Appropriate dosing regimens should eradicate the surviving cells, because this persistence property could ensure the survival of this sub-population, which can be associated with the recalcitrant nature of chronic infections.

The results obtained allow us to make some considerations about the impact of the pharmacokinetic changes caused by the illness on the in vivo efficacy of marbofloxacin. As a general rule, the volume of distribution ($V_d$) in critically ill patients is greater than normal [10]. Therefore, for a given patient and dose, the peak concentration ($C_{\text{max}}$) is lower. If the antibiotic clearance remains unchanged, the increased $V_d$ proportionally decreases the elimination rate constant ($k_e$), which prolongs the half-life.

Assuming linear pharmacokinetics, the increased dose is the increased $C_{\text{max}}$ or AUC. Therefore, based on our results, the clinical success of the therapy will depend on the length of exposure, even in the case of a concentration-dependent antibiotic as marbofloxacin.

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**References**

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