

Influence of the Sodium Salt of 3 α ,7 α -Dihydroxy-12-Oxo-5 β -Cholanate on Antimicrobial Activity of Ampicillin *In Vitro*

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ABSTRACT

Background: Multiple resistances to antibiotics are an emergent problem worldwide. Scientists intensively search for new substances with the antimicrobial potential or the mode to restore the activity of old-generation antibiotics. Ampicillin is the antibiotic with the expanded range of antimicrobial activity, but its use has decreased due to the poor absorption and highly developed resistance. *In vivo* studies showed that ampicillin has better absorption and bioavailability if combined with bile acid salts. The aim of this study was to examine antimicrobial effects of ampicillin alone and its combination with semisynthetic monoketocholeic acid salt (MKH) *in vitro*.

Materials, Methods & Results: In this study, commercial preparation of ampicillin and sodium salt of 3 α ,7 α -dihydroxy-12-oxo-5 β -cholanate were used. Their effects were evaluated on *Escherichia coli* (*E. coli*), *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*), obtained from urine specimens of dogs with clinically manifested cystitis. The first two investigated strains were ampicillin-sensitive, while *E. faecium* was resistant to ampicillin. Modified macrodilution method according to Clinical and Laboratory Standards Institute Guidelines (M7-A8) was performed. Bacterial suspension equivalent to 0.5 McFarland was prepared in saline, compared to the standard (Biomérieux) *ad oculi*. The density was checked spectrophotometrically at a wavelength of 625 nm and adjusted if necessary to the desired absorbance from 0.08 to 0.1. The resultant suspension was diluted 1:100 and inoculated in test tubes. Number of bacteria was counted on Petri plates using dilutions from 10⁻³ to 10⁻⁷ in order to obtain valid and countable plates. One hundred microliters of appropriate dilutions were aseptically plated in triplicate onto nutrient agar. Plates were incubated on 37°C for 72 h, under aerobic conditions. The number of colony forming units (CFU) was determined by direct counting. As a valid for enumeration, we took plates with 30 to 300 CFU. Percentage of killed bacteria for ampicillin was from 69.33-95.19% for *E. coli*, 87.12-96.92% for *E. faecalis* and 7.20-33.30% for *E. faecium*. Ampicillin applied in the combination with MKH killed 99.99% to 100% of *E. coli*, 94.59% to 99.91% of *E. faecalis* and 31.73% to 64.76% of *E. faecium*. Mean percentage of killed bacteria for ampicillin was 81.93% for *E. coli*, 91.64% for *E. faecalis*, and 18.13% for *E. faecium*, while in combination with MKH percentage was 99.96% for *E. coli*, 98.23% for *E. faecalis* and 47.54% for *E. faecium*.

Discussion: Results are presented as pharmacological minimal inhibitory concentration (MIC) values. Ampicillin was applied at the concentration higher than the therapeutic one, which could explain high MIC values for *E. coli* and *E. faecalis*. The combination of ampicillin with MKH showed the best improvement of antimicrobial effect on *E. faecium* ($\Delta = 29.41\%$), isolate that was resistant to ampicillin when applied alone. In all the investigated isolates, the combinations with MKH were more effective than ampicillin administered alone. It seems that MKH demonstrates a synergistic antimicrobial activity with ampicillin *in vitro*, which considerably decreases MIC values for all investigated isolates. These results implicate that MKH could restore the previous activity of ampicillin against some bacteria, which could be a significant benefit for clinical practice.

Keywords: antimicrobial effect, ampicillin, resistance, bile acid, *in vitro*.

INTRODUCTION

Multiple resistances to antibiotics are fast growing problem in human and veterinary medicine [3-5,23,25]. Scientists search both for new substances with an antimicrobial activity [8-10,21] and for new ways to restore the previous activity of older antibiotics.

Ampicillin is a cheap and safe drug with high therapeutic index. It had the expanded range of antimicrobial activity, but its use has decreased due to developing resistance. Unabsorbed part of the drug (\approx 50%) causes reduction of gut microflora, which leads to diarrhea.

Bile acids promote transmembrane transport and increase absorption of drugs, which affects their pharmacokinetics [13,20]. Synthetic derivatives of bile acids enhance a drug transport across the blood-brain barrier [11,12,24]. Co-administration of ampicillin with bile acids protects normal intestinal flora [22]. Several *in vivo* studies showed better absorption and higher bioavailability of antibiotics if applied in the combination with bile acid salts [6,16,22,27].

There is a question how this combination affects bacteria *in vitro*. Specific structure of bacterial cell wall (peptidoglycan) could be a reason for differences between the penetration through bacterial and mammal membranes. On the other hand, it is expected that there is analogy between them, and that bile acids decrease the surface tension of bacterial cell membranes [1,7,18,26]. This experiment was performed due to lack of *in vitro* studies on this subject available in literature.

The aim of this study was to evaluate differences between the antimicrobial effects of ampicillin alone and its combination with the sodium salt of 3 α ,7 α -dihydroxy-12-oxo-5 β -cholanate (MKH) *in vitro*.

MATERIALS AND METHODS

Investigated substances

Ampicillin¹ commercial preparation for oral application in the form of a capsule by ICN Galenika, Serbia.

Cholic acid² (98%) was used for the synthesis and purification of the sodium salt of 3 α ,7 α -dihydroxy-12-oxo-5 β -cholanate (MKH)³ according to the procedure by Miljković *et al.* [15].

Investigated microorganisms

The investigated species included *Escherichia coli* (*E. coli*), *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*). The first two strains

were sensitive to ampicillin, while *E. faecium* was ampicillin-resistant. Isolates were obtained from the urine of golden retrievers with clinically manifested cystitis.

Design of experiment

An antimicrobial activity was investigated by a modified macrodilution method according to Clinical and Laboratory Standards Institute (CLSI) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically [2].

Preparation of the bacterial suspensions (McFarland 0.5)

A bacterial suspension equivalent to 0.5 McFarland was prepared in saline, compared to the standard (Biomérieux)⁴ *ad oculi*. The density was checked spectrophotometrically at a wavelength of 625 nm and adjusted if necessary to the desired absorbance from 0.08 to 0.1 (Agilent 8453 UV-Visible Spectrophotometer)⁵. The resultant suspension was diluted 1:100 and inoculated in test tubes. In this way, the inoculum density ranging from 1 to 5x10⁵ organisms/mL of the suspension was provided.

Evaluation of the number of formed colonies (colony forming units = CFU) from such a suspension was done by counting colonies on the agar surface at dilutions from 10⁻³ to 10⁻⁷. One hundred microliters of appropriate dilutions were aseptically plated in triplicate onto the nutrient agar. Plates were incubated on 37°C for 72 h, under aerobic conditions. The number of CFU was determined by direct counting. As a valid for enumeration, we took plates with 30 to 300 CFU [2].

Ampicillin was applied at the concentration 10 mg/L due to the analogy with commercial antibiogram disks for the routine identification of the antimicrobial susceptibility of bacteria. MKH was applied at the concentration 10 mmol/L. Each assay was repeated four times (n = 4). In this study, a pharmacological minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), were determined. These values represent the percentages of killed bacteria in comparison to the number of bacteria that were brought into the experiment. MIC_n is the lowest concentration that kills n percents of microorganisms inoculated in the experiment, for instance, MIC₉₉, MIC₉₀ and MIC₈₀ are the lowest concentrations that kill 99%, 90% and 80% of microorganisms, respectively. MBC is the lowest concentration of the investigated substances that kills at least 99.9% of microorganisms inoculated in the experiment [21].

Statistical analysis

The level of significance between the groups was assessed with the Student's *t*-test for small independent samples using Excel software package (Microsoft Office 2007)⁶. All data are expressed as a mean \pm standard deviation (SD). A value of *P* < 0.05 was considered to be statistically significant.

RESULTS

Ampicillin killed 69.33-95.19% of *E. coli* when applied alone, and from 99.99% to 100% of the same strain when applied in the combination with MKH (Figure 1).

Similarly, an antibiotic applied alone eradicated 87.12-96.92% of *E. faecalis* while its combination

with MKH killed from 94.59% to 99.91% of the same strain (Figure 2).

The biggest difference in the percentage of killed bacteria was shown for *E. faecium*: from 7.20% to 33.30% when ampicillin was applied alone, and from 31.73% to 64.76% when applied in the combination with MKH (Figure 3).

Mean percentages of killed bacteria for ampicillin were 81.93% for *E. coli* (MIC_{81.93}), 91.64% for *E. faecalis* (MIC_{91.64}), and 18.13% for *E. faecium* (MIC_{18.13}). When ampicillin was administered with MKH, mean percentages of killed bacteria were 99.96% for *E. coli* (MBC), 98.23% for *E. faecalis* (MIC_{98.23}), 47.54% for *E. faecium* (MIC_{47.54}) [Table 1].

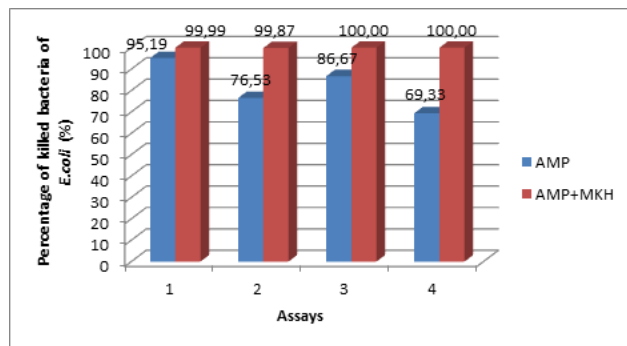


Figure 1. Comparison of antimicrobial effect of ampicillin and its combination with MKH on *Escherichia coli*.

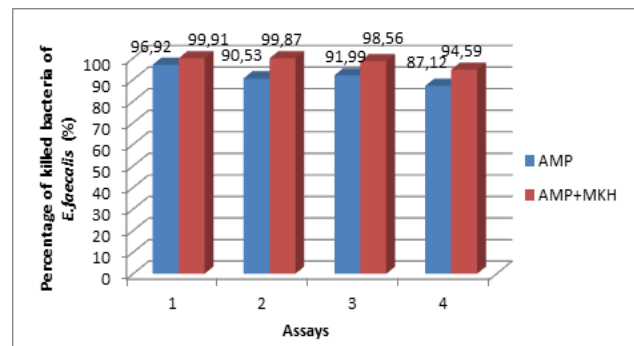


Figure 2. Comparison of antimicrobial effect of ampicillin and its combination with MKH on *Enterococcus faecalis*.

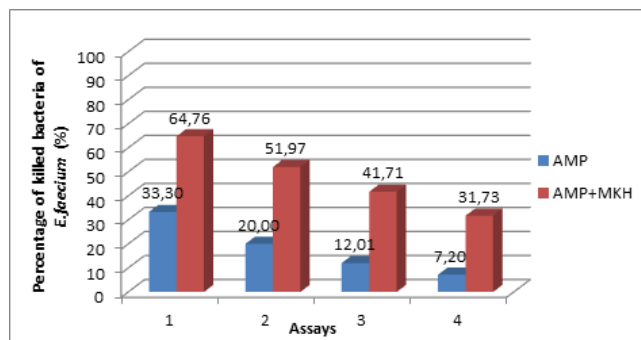


Figure 3. Comparison of antimicrobial effect of ampicillin and its combination with MKH on *Enterococcus faecium*.

Table 1. Percentage of killed bacteria for ampicillin and its combination with MKH.

Investigated strain	Percentage of killed bacteria(MIC)	
	Ampicillin (n = 4) [†]	Ampicillin + MKH (n = 4) [†]
<i>Escherichia coli</i>	81.93 \pm 9.82	99.96 \pm 0.06*
<i>Enterococcus faecalis</i>	91.64 \pm 3.52	98.23 \pm 2.17*
<i>Enterococcus faecium</i>	18.13 \pm 9.88	47.54 \pm 12.25*

[†]Four assays were conducted. *Significantly different from group treated with ampicillin alone (*P* < 0.05). All values are expressed as mean \pm standard deviation.

DISCUSSION

The authors apply different interpretations of the term MIC (microbiological, epidemiological, and pharmacological) in the literature [21]. In this paper, pharmacological MIC was used. Ampicillin killed 69.33-95.19% of *E. coli*, 87.12-96.92% of *E. faecalis* and 7.20-33.30% of *E. faecium* organisms. When the same concentration of ampicillin was applied in the combination with MKH, antimicrobial effects were significantly different: percentages of killed bacteria were in the following ranges: *E. coli* from 99.99% to 100%, *E. faecalis* from 94.59% to 99.91% and *E. faecium* from 31.73% to 64.76% (Figures 1, 2 & 3).

Ampicillin applied alone killed high percentage of bacteria, over 80% for *E. coli* and over 90% for *E. faecalis* (Table 1). Such high rate of killed bacteria can be explained by the fact that ampicillin was applied in the concentration of 10 mg/L, which is more than the therapeutic one. After common oral dose of 500 mg, the expected blood concentration of ampicillin is 3 mg/L, while the concentration after intravenous application is 7 mg/L [19]. Ampicillin applied alone had a much weaker antimicrobial effect on *E. faecium* (mean value for MIC was 18.13%) in comparison to *E. coli* and *E. faecalis*, despite its high concentration. This was rather expected, since the investigated isolate of *E. faecium* was resistant to ampicillin. Combinations with MKH had better effects on all investigated isolates than ampicillin applied alone. MIC values for *E. coli* and *E. faecalis* were raised from 81.93% to 99.96% ($\Delta = 18.03\%$) and from 91.64% to 98.23% ($\Delta = 6.59\%$), respectively. The most significant difference between antimicrobial effects of ampicillin and its combination with MKH was demonstrated in *E. faecium* - the efficiency increased from 18.13% to 47.54% ($\Delta = 29.41\%$). The combination of ampicillin with MKH averagely showed 3.11 times (from 1.94 to 4.41 times) better antimicrobial effect on *E. faecium* than ampicillin applied alone (Figure 3).

Such an increase of antibacterial effect is most likely the result of *E. faecium*'s resistance to ampicillin. The co-administration of ampicillin with MKH significantly increased MIC values, which provides

new opportunities for the treatment of causative agents resistant to ampicillin.

Previous *in vivo* studies evaluated the benefit of the concomitant use of ampicillin and MKH due to the better absorption and bioavailability [14,17] and the better preservation of normal gut flora [22]. This investigation demonstrated that the combination of ampicillin with MKH improves an antimicrobial activity *in vitro*, for which we have not found the comparable data in the available literature.

CONCLUSIONS

In vitro combinations of ampicillin and MKH manifested significantly better antimicrobial effects on all the investigated isolates than ampicillin alone. Furthermore, the concomitant use of ampicillin and MKH could be useful both for reducing harmful effects of ampicillin on the intestinal flora and for increasing of an oral bioavailability of ampicillin. The co-administration of MKH with ampicillin showed the most significant improvement of the antimicrobial effect on ampicillin resistant *E. faecium*, which suggests that MKH could block mechanisms of bacterial resistance to ampicillin.

These results implicate that MKH could restore the previous activity of ampicillin to some resistant bacterial strains.

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Declaration of interest. The authors declare that they have no conflict of interests.

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