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Preliminary Testing Of Microbiological Efficacy And Quality Of Ethacridine Lactate Solution During The Period Of Use.

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ABSTRACT

Ethacridine lactate, also known as rivanol, is anantiseptic effective against many Gram positive bacteria, but weakly effective against Gram negative bacteria and bacterial spores. It is used as dermoantiseptic as 0.05% or 0,1% solution, and 0.1% ointment. A solution of ethacridine lactate is very often compounded in a pharmacy as extemporaneous medicine and is not subject to quality control or registration as a conventional medicine. In this study Kelsey-Sykes test has been used for efficacy testing of extemporaneous ethacridin lactate solution during and after the recommended period of use. Microbiological quality was also tested according to procedure for microbiological examination of non-sterile product. *Ex tempore* prepared sample showed microbiological efficacy. Thirty days after the preparation the efficacy was reduced but acceptable, and it was insufficient ninety days after preparation. There was no observed microorganism growth in microbiological quality assays. A period of thirty days after solution preparation can be considered as a limitation period, the relevant timeframe in which ethacridine lactate still shows a satisfactory efficacy. Based on specific tests and tests on the total number of aerobic microorganisms, it can be concluded that all tested samples complied with specifications of microbiological quality.

Keywords: ethacridine lactate solution, microbiological efficacy, microbiological quality

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INTRODUCTION

Infectious diseases have a significant share in the morbidity and mortality of human population and therefore their prevention is very important. The local antiinfective agents, disinfectants and antiseptics are used in the treatment of local infections, removing pathogens from different surfaces, accessories and equipment used in diagnostics, etc. Unlike disinfectants, which are chemically corrosive and show high non-selective toxicity during use, antiseptics have a certain selective local toxicity towards targeted pathogens. Three steps are required for their binding to the cell membrane of the microorganism, penetration into the microbial cell and reaction with one or more cell components. The basic antiseptic deficiency is insufficient selectivity, such as tissue damage. Therefore, they are usually used topical [1]. Acridine derivatives as antimicrobial agents were first introduced in clinical use in 1917. They are slow acting antiseptic, effective against many Gram positive bacteria, but weakly effective against Gram negative bacteria and bacterial spores [2]. They can be used topically in the treatment of eye infections, throat or urogenital tract infections. Ethacridine lactate (Figure 1), salt with lactic acid, is used as dermoantiseptic as 0.05% or 0,1% solution and 0.1% ointment [3,4].

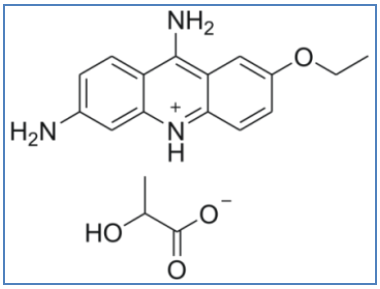

Fig 1: Chemical structure of ethacridine lactate (5)

Applied to the skin, ethacridine lactate penetrates the tissue and is associated with proteins and thereby exhibits antimicrobial activity. In the presence of organic substances, antimicrobial activity is increased. Like other derivatives of acridine, ethacridine lactate is slow acting disinfectants effecive on Gram-positive bacteria [3,5]. A solution of ethacridine lactate is very often compounded in a pharmacy as a extemporaneous medicine and is not subject toquality control or registration as a conventional medicine. The European Pharmacopoeia (Ph. Eur.), United States Pharmacopoeia (USP) and various monographs in national Pharmacopoeias provide general information on the requirements and validation of the process of preparation of extemporaneous preparations. Similar guidelines are also provided by the Committee of Ministers of the Council of Europe [3,6,7].

The aim of this study was to determine microbiological efficacy and quality of the 0,1 % extemporaneous ethacridine lactate solution, freshly prepared and after 30, 60 and 90 days after preparation, stored at room temperature. The examination was conducted with the aim of determining the efficacy and microbiological quality during and after the recommended use.

EXPERIMENTAL

Three samples of ethacridine lactate were used for determination of microbiological efficacy and quality, Sample A – ex tempore prepared 0,1% ethacridine lactate solution, Sample B – 0,1% ethacridine lactate solution 30 days after preparation, stored at room temperature, Sample C – 0,1% ethacridine lactate solution after 90 days, unopened, stored at room temperature.

For determination of ethacridine lactate microbiological efficacy Kelsey-Sykes test was used, which is commonly used in routine efficacy control of disinfectants and antiseptics. Microbiological quality was tested according to Ph Eur. 2.6.13. Monography - Microbiological examination of non-sterile products: Test to specified microorganisms. (Ph. Eur.9). In the microbiological quality control assay, opened, 90 days old, ethacridine lactate solution, was also used.

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Kelsey-Sykes test

Bacterial stock suspension

For the preparation of bacterial stock suspension Staphylococcus aureus (ATCC, 6538) at the concentration of 10^5 CFU/mL was used. The concentration adjustment was carried out by spectrophotometry at 640 nm, wherein transmittance for concetration of 10^5-10^7 CFU/mL were 66.44% -81.2%. As a positive control sterile purified water was used, and negative control was tube of 5 mL Soybean casein digest agar (CB). In 3 mL of each tested solution, every 10 minutes (for 30 minutes), 1 mL of stock bacterial suspension was added. 20 µL of each inoculated sample was transferred to each to 5 tubes containing 5 mL of CB. The same procedure was repeated twice for all tested samples. For the positive control test 3 mL sterile purified water was used. Negative control was tube with pure CB, incubated 48h at 37^0C. Inoculation procedure and test times are given in Table 1.

Table 1: Inoculation procedure and test time

Time (min)	Sample (A)	Time (min)	Sample (B)	Time (min)	Sample (C)	Time (min)	Positive control
0	Add mL susp.	1	Add 1 mL susp.	5	Add 1 mL susp.	6	Add 1 mL susp.
8	Transfer 20 µl to 5 mL CB	9	Transfer 20 µl to 5 ml CB	-		-	
10	Add 1 mL susp.	11	Add 1 mL susp.	13	Transfer 20 µL to 5 ml CB	14	Transfer 20 µl to 5 ml CB
-		-		15		16	Add 1 mL susp.
18	Transfer 20 µL to 5 mL CB	19	Transfer 20 µL to 5 ml CB	-	Add 1 mL susp.	-	
20	Add 1 mL susp.	21	Add 1 mL susp.	23	Transfer 20 µl to 5 mL CB	24	Transfer 20 µL to 5 ml CB
-		-		25	Add 1 mL susp.	26	Add 1 mL sussp.
28	Transfer 20 µL to 5 mL CB	29	Transfer 20 µL to 5 mL CB	33	Transfer 20 µL to 5 ml CB	34	Transfer 20 µl to 5 mL CB

After the final inoculation, all inoculawere incubated at 37^0C for 48 hours. After the incubation the microorganisms growth in 5 replicates of the positive control were compared to the 5 sample's replicates. Microorganisms growth inhibition was determined. In all tubes, microorganisms growth was observed.
None of the tested samples inhibited the growth of 10^5 CFU/mL. Therefore, the initial bacterial stock suspension was diluted to the concentration of 10^3CFU/mL. Aliquote of 3 mL each tested sample was used for the analysis.

The modified test procedure is shown in Table 2. Microbiological quality of all samples were tested for the presence of E. Coli (ATCC 8739), Salmonella sp.(ATTC 700623), P. Aeruginosa (ATCC 9027), S. Aureus (ATCC 6538) and total count of microorganisms according to Ph Eur. 2.6.13. - Microbiological examination of non-sterile products: Test to specified micro – organisms. (Ph. Eur. 9).

ISSN: 0975-8585

Table 2: Modified Kelsey - Sykes Test procedure

Time (min)	Sample A	Time (min)	Sample B	Time (min)	Sample C
0	Add 1 mL susp.	1	Add 1 mL susp.	2	Add 1 mL susp.
20	Transfer 20 µl to 5 mL CB	21	Transfer 20 µl to 5 mL CB	22	Transfer 20 µl to 5 mL CB
30	Transfer 20 µl to 5 mL CB	31	Transfer 20 µl to 5 mL CB	32	Transfer 20 µl to 5 mL CB
40	Transfer 20 µl to 5 mL CB	41	Transfer 20 µl to 5 mL CB	42	Transfer 20 µl to 5 mL CB

RESULTS AND DISSCUSION

According to the results shown in Tables 3 and 4, Sample A has been shown to be sufficiently effective, even after a 20 minute period. Sample B showed different results in two replicates. After 24 hours tested solution showed antimicrobial efficacy after 20, 30 and 40 minutes. After 48 hours, turbidity CB after 20 and 40 minutes was observed, but after 30 minutesturbidity was not observed. Sample C, unopened sample 90 days old, showed insufficient efficacy. Antimicrobial efficacy was observed after 30 minutes, however, it was not confirmed 40 minutes after inoculation.

Table 3: Antimicrobial efficacy of the tested samples after incubation (24 hours)

	Time (min)	Soybean casein digest agar (CB) after 24 hours					Antimicrobial efficacy
Sample A	20	-	-	+	+	-	Yes
	30	+	-	-	-	-	Yes
	40	-	-	-	-	-	Yes
Sample B	20	-	-	+	+	+	Yes
	30	-	-	-	-	-	Yes
	40	-	-	-	-	-	Yes
Sample C	20	-	+	+	+	+	No
	30	-	+	+	-	+	Yes
	40	+	+	+	+	-	No

(-) no turbidity, no growth of microorganisms, (+) turbidity as a consequence of growth of microorganisms; turbidity should not be present in at least two of the five tubes

Table 4: Antimicrobial efficacy of the tested samples after incubation (48 hours)

	Time (minute)	Soybean casein digest agar (CB) after 48 hours					Antimicrobial efficacy
Sample A	20	-	-	+	+	-	Yes
	30	-	+	-	-	-	Yes
	40	+	+	-	-	-	Yes
Sample B	20	+	+	+	+	+	No
	30	+	+	+	-	-	Yes
	40	+	+	-	+	+	No
Sample C	20	+	+	+	+	+	No
	30	+	+	-	+	-	Yes
	40	+	-	+	+	+	No

(-) no turbidity, no growth of microorganisms, (+) turbidity as a consequence of growth of microorganisms; turbidity should not be present in at least two of the five tubes

The test for total number of microorganisms test, direct method was applied. It is performed in at least two replicates for each medium, and as a result, the mean value of the obtained results was used.

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Whereas in all samples was no colony growth, colony counters were not used and TAMC and TYMC values were not determined. The presence of E. coliis characterized by the growth of red, unsettled colonies, sometimes surrounded by reddish-zone precipitates. As can be seen from the Figure 2, the colony growth was not observed. Salmonella sp. is characterized by the growth of well-developed red colonies with or without black centers. Growth of these colonies in tested samples was not observed.

Fig 2: MacConkay agar after incubation - absence of E. coli colony growth

The absence of growth of the yellow-green colonies indicated the absence of P. aeruginosa in the investigated samples (Figures 3,4,5).

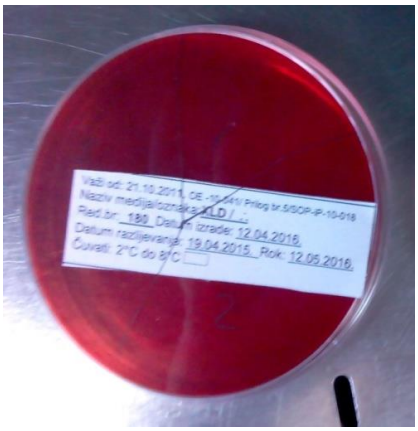

Fig 3: The absence of Salmonella spp. colony growth on XLD agar after incubation

Fig 4: MSA agar after incubation - absence of Staphylococcus aureus colony growth

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Fig 5: Cetrimd agar after incubation - absence of P. aeruginosa colony growth

Evaluation of the results was based on the inhibition of microorganism growth in the medium at the end of incubation period. The growth of microorganisms (turbidity CB) was observed in all tubes indicating that the tested samples were inefficient for the concentration of 105 CFU/mL.The active time of the tested samples was 8 minutes. Bacterial growth inhibition did not occurr even after 30 minutes. Therefore, the initial bacterial suspensions were diluted to 10^3 CFU/mL, and the tests were repeated by the same procedure.

It was found that at the concentrationof 10^3 CFU/mL sample A showed activity, after 20, 30 and 40 minutes, as expected, because it was an ex tempore prepared solution. Sample B showed different efficacy in two test replicates, which may be due to a two-day interval between the test runs. The sample analyzed 90 days after preparation, which was not opened until the moment of analysis (Sample C), did not show antimicrobial efficacy. It is therefore recomended to determine the content of ethacridine lactate in all samples, which could facilitate interpretation of the microbiological efficacy results. It is considered that the decrease of the content of ethacridine lactate can reduce the efficacy of the antimicrobial action.
Microbial contamination can reduce or even eliminate the therapeutic effect of medicines and cause medicines-induced infections.

Microorganism presented in medicines may change the chemical and physical properties and makes them hazardous from the infectious standpoint [8]. Therefore, microbiological purity criteria for sterile and non - sterile pharmaceutical products were established and the requirement for final microbiological control was introduced [4].

Oia and Kamiya investigated the bacterial contamination of commercially available solutions of etacridine lactate and antiseptic (0,1% ethacridine lactate) soaked cotton balls [9]. The results showed that 7 out of 56 solutions of ethacridine lactates were contaminated with 10^1 to 10^4 CFU/mL Burkholderia picketti.
Opposite that, no microbial contamination was observed in 15 soaked cotton balls in 0.1% ethacridine lactate solution (500 mL volume) during use in reduced amounts [10].

In this study all the test solution were not contaminated with E. coli, Salmonella spp, P.aeruginosa, S. aureus. This can be explained by adequate conditions for preparation and storage of the test solutions, and also by adequate methods of sample preparation and analysis.

CONCLUSION

Microbiological efficacy control confirmed the efficacy of ex tempore prepared sample, reduced but acceptable sample efficacy 30 days after preparation and insufficient efficacy 90 days after preparation. Based on specific tests and tests on the total number of aerobic microorganisms, it can be concluded that all samples tested, complied to specifications for microbilogical quality Therefore, irrespective of possible concentration decreas or efficacy reduction, it may be considered that the ethacridine lactate solutions three months after the preparation are complied to specifications for microbilogical quality and from that aspect there is no risk to the patient.

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