Original Article

Indicator of Antimicrobial Activity as Criteria for Selection of Concentration of Substances in the Medicinal Composition

Alina Koval *, Galyna Biloklytska, Lena Davtian, Serhii Ubohov

National Medical Academy of Postgraduate Education named by P. L. Shupyk, Ukraine.

Abstract

At present, for the production of soft medicine for local application substances such as macromolecular compounds (plasticizers, surfactants, etc.) are widely used. When creating the drug composition, a study was conducted aimed at obtaining soft medicine with certain consumer properties, in particular physicochemical (pH, osmotic activity, homogeneity, etc.), physical, and mechanical properties (rheological parameters).

Keywords: antimicrobial activity, soft medicines, metronidazole, benzyl benzoate, benzoyl peroxide

INTRODUCTION

When choosing excipients and their concentrations, it is necessary to take into account the functions [1, 2] and characteristics of each of them [3, 4], if they can affect the functional properties of the drug (stability, bioavailability, etc.) or the possibility of its production. For this purpose, we obtained model compositions containing different ratios of excipients and studied their physicochemical and physicomechanical properties.

Module 3 "Quality" of Guideline 42-3.6: 2004 requires confirmation of the absence of interaction of excipients with each other, and the formation of compounds that may adversely affect the effectiveness of the drug (drug). To confirm the lack of interaction, an organoleptic analysis of the created model compositions was performed: color, odor, homogeneity, both immediately after manufacture and during storage in natural conditions. The result of the study was the choice of the basis in which the planned introduction of active pharmaceutical ingredients (API) in quantities widely used in dermatological diseases (acne), with purulent-inflammatory processes in the sebaceous glands.

In the theoretical direction, bibliosemantic research (literature sources, Internet content, the State Register of Medicinal Products of Ukraine, etc.) was conducted, a patent search was conducted, analogs and prototypes were identified. Based on the results of the analysis and processing of the obtained data, the scientific methodology of drug composition development is formulated. The following substances were selected for this purpose:

- Metronidazole (Chemlaborreactiv, Germany), the quality of which meets the requirements of the monograph of the European Pharmacopoeia and has a certificate of conformity of the European Pharmacopoeiax [5, 6];
- Benzyl benzoate (Chemlaborreactiv, China), the quality of which meets the requirements of the monograph of the European Pharmacopoeia and has a certificate of conformity of the European Pharmacopoeia [5, 6].
- Benzoyl peroxide (Chemlaborreactiv, Germany) whose quality meets the requirements of the monograph of the European Pharmacopoeia and has a certificate of conformity of the European Pharmacopoeia [5, 6].

The work aimed to determine the spectrum and strength of antimicrobial activity of the studied compositions, to compare them with the activity of substances, and to select the most optimal concentration for further combination with each other.

Address for correspondence: Alina Koval. National Medical Academy of Postgraduate Education named by P. L. Shupyk, Ukraine.
Email: alinasposts @ gmail.com

How to cite this article: Koval, A., Biloklytska, G., Davtian, L, Ubohov S. Indicator of Antimicrobial Activity as Criteria for Selection of Concentration of Substances in the Medicinal Composition. Arch Pharma Pract 2020;11(3):138-42.

Materials and Methods

Materials for the study – soft medicines based on cetyl alcohol, Emulgade Sucro Plus, Polyvinylpyrrolidone, Carboxymethylcellulose, Tween 80, Triethanolamine, Propylene glycol, vaseline oil, which as API contains metronidazole, benzoyl peroxide, benzylbenzene.

The specific antimicrobial activity of the samples was tested in accordance with Order № 167 of the Ministry of Health of Ukraine dated 05.04.2007 by the standard macro-method of serial dilutions in a liquid nutrient medium and by the method of diffusion into the agar. The minimum inhibitory concentration (MIC) of API (substances) was determined.

The selection of test strains of microorganisms was carried out on the basis of literature data on the biocenosis (opportunistic pathogenic microflora) of the facial skin in patients suffering from (acne), with purulent-inflammatory processes in the sebaceous glands. At these pathological processes the following clinical types of microorganisms are sown (table 1).

Table 1: Clinical types of microorganisms.

Yeast-like fungi	Bacterias	
	Gram-positive	Gram-negative
Malassezia furfur ***Candida albicans***	*Propionibacterium acnes* Streptococcus haemolyticus viridans *Staphylococcus aureus* *Staphylococcus haemolyticus* *Staphylococcus epidermidis* *Clostridium perfringens*	*P. aeruginosa* *E. coli*

Mandatory members of microbial associations are the dominant species: *M. furfur*, *C. albicans*, *P. acnes*, and a representative of the parasitofauna of the skin Demodex folliculorum. Test strains were also selected according to the pharmacological properties and antimicrobial action of the above active pharmaceutical ingredients. Thus, Metronidazole acts on protozoa (Trichomonas, amoebae, Giardia), as well as on anaerobic bacteria (*Clostridium perfringens*); Benzoyl peroxide inhibits the development of gram-positive anaerobic microorganisms *Propionibacterium acnes* in acne; Benzyl benzoate has acaricidal activity (against various species of mites, including scabies mites (*Acanis scabiei*) and mites of the genus *Demodex*) and antipediculosis activity and also affects the yeast-like fungus *Malassezia furfur*. Museum cultures of bacteria and fungi available in the laboratory were used in the research [7].

As test strains were used, standard typical cultures of microorganisms were obtained from the Museum of the Research Institute (RES) of Microbiology and Virology. D.K. Zabolotny NAS of Ukraine, namely:

- *Staphylococcus aureus* 6538
- *Pseudomonas aeruginosae* ATCC 9027
- *Candida albicans* ATCC 10231
- *Clostridium sporogenes* ATCC 19404

Test strains of microorganisms (m/o) were grown on a suitable nutrient medium (table. 2):

- SKA: *Staphylococcus aureus* 6538, *Pseudomonas aeruginosae* ATCC 9027 at a temperature of 30-35 ℃ for 18-20 h;
- CA: *Candida albicans* ATCC 10231 at a temperature of 35-37 ℃ for 18-20 h;
- TIO: *Clostridium sporogenes* ATCC 19404 at a temperature of 30-35 ℃ for 48 hours.

Table 2: List of used nutrient media, solutions, and solvents.

№ p/p	Total	Series	Term suitability	Manufacturer
1	(Tryptic soy agar Casein-peptone soymeal-peptone agar for microbiology), next - **SCA***	VM846658	13.07.2019	Merck KGaA (Germany)
2	(Sabouraud 4% dextrose agar for microbiology), next - **CA***	VM834538	27.04.2023	Merck KGaA (Germany)
3	(Fluid Thioglycollate Medium) - Thioglycollate with resazurin), next-**TIO**	F9SA28816	12.2020	Merck,Germany
4	(Antibiotic Assay Medium №1)*	0000333597	03.2023	HiMedia Laboratories Private Limited, India
5	(Nutrient broth for microbiology)	VM857743	16.10.2023	Merck KGaA(EMD Millipore Corporation), Germany
6	Saline solution (0.9% sodium chloride)	121911	05.12.2022	Private enterprise "O.L.KARA-AgroZoovet-Service", Ukraine
7	Water for injections	080813	19.08.2020	TOV Yuriy-Pharm , Ukraine
8	Dioxane	SZBE176SV	14.06.2020	Sigma-Aldrich. Germany
9	Ethyl alcohol 96%	193360	04.2023	Arterium Corporation,
10	API Suspension Medium ampules (3 ml) №100	1007352110	20.05.2021	BIOMÉRIEUX, France

Inoculum preparation

After incubation in a thermostat, working suspensions of m/o (inoculum) were prepared, and their optical density was measured in McFarland units at 550 nm using a Densimat densitometer. The daily culture was added to ARI ampoules with 0.9% saline, suspended to form a homogeneous suspension equivalent to a turbidity of 0.5 IU according to the McFarland standard, corresponding to 1.5 × 108 CFU/ml. The resulting suspension was diluted 100 times with nutrient broth, obtaining 106 CFU/ml concentration of microorganisms [7]. The number of microorganisms in the suspension was confirmed in parallel by direct culture of 0.1 ml from the suspension of 103 CFU/ml per Petri dish with sterile nutrient media SKA (for bacteria)/CA (for fungi), listing the results in CFU/ml.

Preparation of stock solutions of substances

The test substances are poorly soluble in water and even in some organic solvents. Some solutions of substances in organic solvents when mixed with nutrient broth or molten agar nutrient medium formed suspensions (precipitates), films, which made it impossible to evaluate the result. Therefore, solvents, where possible, were selected by sampling. A portion of metronidazole 100 mg was transferred to a 20 ml volumetric vessel. Approximately 15 ml of sterile water was added for injections and placed on an ultrasonic bath for 15 minutes (without heating). After the complete dissolution of the substance, the contents of the flask were adjusted to the mark with the same solvent. A stock solution of metronidazole was provided with a concentration of the active substance of 5 mg/ml (0.5% solution).

A portion of benzoyl peroxide 100 mg was weighed on analytical scales in a beaker, dioxane solution up to 2.0 g was added, and stirred thoroughly until completely dissolved. A stock solution was obtained with a concentration of the active substance of 50 mg/g (or 0.05 g/g, or 5% solution).

Test method I: macro-method of serial dilutions (in vitro).

A number of tubes were prepared for dilution of the initial solutions of substances. 0.5 ml of nutrient broth was added to each tube, then 0.5 ml of the initial solution of the test substance was added to the first tube and moved thoroughly. After stirring, 0.5 ml of the resulting mixture was transferred to the next tube; thus in each subsequent sample, the concentration of the active substance was reduced by 2 times. 0.5 ml of solution was removed from the last tube. At the same time, a number of dilutions of the test solution in broth were prepared to control sterility. A separate series of successive dilutions of the test substance solution was prepared for each m/o. The final concentration of the microorganism in each of the dilutions was approximately 5 × 10^5 CFU/ml. Positive growth control of each of the test strains included 0.5 ml of nutrient broth + 0.5 ml of suspension with a concentration of 10^6 CFU/ml. Negative control was the control of sterility of nutrient broth. All tubes were incubated in a thermostat at a temperature of 35-37 ℃ for 18-20 hours.

Data analysis

The test results were evaluated visually by the degree of turbidity (i.e. the presence of m/o growth) in the test samples compared with the growth of the test strain in the positive control. No growth should be observed in the negative control and sterility control solutions. The minimum inhibitory concentration (MIC) was considered to be the last of the dilutions in which no growth of microorganisms was observed.

Test method Ⅱ: agar diffusion method (well method).

Appropriate molten and cooled to 40-45 ℃ agar medium (SKA, CA, or antibiotic agar №1) in the amount of 100 ml was inoculated, making 1.0 ml suspension of a certain test strain with a concentration of 10^6 m/o. Immediately after the introduction of the test microorganism, the nutrient medium was poured into 20 ml Petri dishes (d = 90 mm), located on the surface of the rotating table in order to obtain a uniform layer (3-4 mm). Petri dishes with the inoculated nutrient medium were left on a flat horizontal surface until the agar solidified [7]. In the solidified medium, wells ("wells") were drilled using a sterile metal punch with an inner diameter of 6 mm and an outer diameter of 8 mm, into which equal volumes of test samples of ointments or solutions of substances were introduced using an Eppendorf stepper.

After adding ointment samples (solutions of substances, solvents for substances) to the wells, Petri dishes were kept at room temperature for 1 h (preliminary diffusion) to reduce the effect of the time difference between the introduction of the first and last components (solutions), and then were incubated at 36 ± 1 ℃ for 18-24 hours.

Data analysis

At the end of the incubation period, the obtained results were evaluated visually (+/- (presence/absence) zones) and the diameters of growth inhibition zones of microorganisms were measured (if any) with an accuracy of 0.01 mm using an electronic caliper.

Discussion

According to the method I, dilution of the initial solution of the substance metronidazole did not show antimicrobial action on the test strains m/o *Staphylococcus aureus* 6538 and *Candida albicans* ATCC 10231. The highest concentration of the test substance (1.25 mg/ml) only suppressed (delayed) *Staphylococcus aureus* 6538 (i.e. showed a bacteriostatic effect), which was confirmed by reseeding the sample solution on the nutrient medium SKA; after incubation at 35-37 ℃ for 18-20 h on the medium, there was an increase in m/o (Table 3).

Table 3: Antimicrobial activity of metronidazole on test strains of microorganisms.

Test strains of microorganisms	The concentration of metronidazole in solution (mg / ml)							
	1	2	3	4	5	6	7	K+
	1,25	**0,625**	**0,3125**	**0,15625**	**0,078**	**0,039**	**0,0195**	-
***Staphylococcus aureus* 6538**	–	+	+	+	+	+	+	+
***Candida albicans* ATCC 10231**	+	+	+	+	+	+	+	+

Note: "+" - growth m/o;
"-" - no growth m/o;
K + - control.

According to method II, the obtained test results indicate a slight antimicrobial effect of the tested substances and compositions based on them on museum test strains of microorganisms [8]. It was found that the concentration of metronidazole 0.5 g/100 g of ointment (or solution) does not affect the growth of *Staphylococcus aureus* 6538, but slightly delays the growth of *Clostridium sporogenes* ATCC 19404. To increase the antimicrobial effect on the above strains, the concentration of metronidazole should be increased to 0.7 g/100 g and above (Table 4).

Table 4: Antimicrobial activity of metronidazole on test strains of microorganisms.

Test strains of microorganisms	**Based on soft medicines**	**Soft medicines compositions with metronidazole**		Metronidazole solution
	The concentration of metronidazole, g / 100 g /			
	0 (№7)	0,5 (№1)	0,7 (№2)	0,5 (або 5 мг/мл)
Diameters of zones of inhibition of growth of microorganisms (mm)				
***Staphylococcus aureus* 6538**	0	0	14,13±0,11	0
***Clostridium sporogenes* ATCC 19404**	0	10,78±0,13	11,34±0,22	0

For benzoyl peroxide in soft medicines, the optimal antimicrobial effect against *Staphylococcus aureus* 6538 is a concentration of 2.5 g/100 g. Test strain m/o *Pseudomonas aeruginosae* ATCC 9027 was insensitive to benzoyl peroxide in both MLZ and solutions of the substance. That is, to obtain the antimicrobial effect of MLZ enough benzoyl peroxide content of 2.5 g/100 g is required (Table 5).

Table 5: Antimicrobial activity of benzoyl peroxide on test strains of microorganisms.

Concentration, g / 100g / Test strains m / o	Based on soft medicines	Soft medicines with benzoyl peroxide			Dilution of the benzoyl peroxide solution					Solvent substances (dioxane)
Concentration of benzoyl peroxide	0	2,5	3,75	5,0	0,3125	0,625	1,25	2,5	5,0	
Test strains m/o	Diameters of zones of inhibition of growth of microorganisms (mm)									
***Staphylococcus aureus* 6538**	0	10,89±0,13	9,82±0,04	9,85±0,02	11,45	11,25	11,18	10,89	10,76	0
***Pseudomonas aeruginosae* ATCC 9027**	0	9,24±0,05	8,61±0,09	0,0	10,69	10,75	11,53	12,04	10,63	9,16

The antimicrobial action of benzyl benzoate was investigated on *Candida albicans* ATCC 10231, which after 24 h of incubation was not sensitive to the action of both the native substance and the ointment of which it is a part. After 48 h, the bacteriostatic effect of the ointment was observed, i.e. the zones of inhibition of culture growth (but not complete cessation!) increased from 7.8 mm to 12.78 mm (Table 6).

Table 6: Antimicrobial activity of benzyl benzoate on test strains of microorganisms.

Concentration, g / 100g, / Test strains m / o	Based on soft medicines	Soft medicines with benzyl benzoate	Benzyl benzoate substance
The concentration of benzyl benzoate	0	15,0	–
Test strains m / o	Diameters of zones of inhibition of growth of microorganisms (mm)		
***Candida albicans* ATCC 10231**	0,0	7 ÷ 8 *	7 ÷ 8 *

Note: * - areas without a clear edge, with small colonies of microorganisms.

Conclusion

1. The analyses of the compositions of MLZ and the substance revealed the presence of a slight antibacterial (bacteriostatic) effect against the test strains of microorganisms.
2. The prospect of the study is to study the antimicrobial activity of the model compositions depending on the technological method of the introduction of API into the base.

References

1. Shivhare R S, Awachat P, Mahapatra D K, Ingole A R, Borkar S S. Development of Wound Healing Ointment Formulation containing Active Extracts of Tridax procumbens, Calendula officinalis, Murraya koenigii, and Aloe barbadensis. Int. J. Pharm. Phytopharm. Res. 2019; 9(6): 99-104.
2. Subash Chandran M P, Prasobh G R, Jaghatha T, Aswathy B S, Remya S B. An Overview on Liposomal Drug Delivery System. Int. J. Pharm. Phytopharm. Res. 2019; 9(2): 61-68.
3. Alhmoud H A. Regulation of drug transmission within the matrices of different polymers and the models of drug delivery. J. Adv. Pharm. Edu. Res. 2020; 10(3): 17-23.
4. Jabar E G, Taghi H S, Hasson K J, Alkhalifa I I. Evaluation of innovated formula of Bisacodyl suppository following the dissolution profile and stability data by using developed HPLC method. J. Adv. Pharm. Edu. Res. 2020; 10(2): 5-10.
5. Lena D, AlyonaV. The study of the properties of the matrix for periodontal films based on Metronidazole, Chlorhexidine and Glucosamine. Int. J. PharmTech Res. - 2016. - Vol. 9. - №2. - R. 9–12.
6. Lyapunov M, Georgievsky V, Bezugla O, Pasichnyk M, Krychevska O, etc., developers. Quality guidelines. Medicines. Pharmaceutical development. Guideline 42-3.1: 2015: laws and legislation. Kyiv: Ministry of Health of Ukraine, Gos. Science. center doctor. means; 2017. (48 p.).
7. Lock M, Nguyen VK. An anthropology of biomedicine. John Wiley & Sons, 2018 (28-186).
8. Laatsch H. AntiBase: the natural compound identifier. Weinheim: Wiley-Vch 2017, (p.15-158).