Validation of the method for determining the specific activity of the gel with recombinant endolysin

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Abstract. A method for determining the specific bactericidal activity of the gel with the recombinant modified endolysin of the bacteriophage ECD7 has been developed. Bacterial strains to determine the specific bactericidal activity were selected and acceptance criteria were determined. The developed methodology was validated according to the indicators "Specificity", "Accuracy" and "Intermediate precision". The developed method has been successfully validated and is suitable for determining the specific bactericidal activity of a gel with a recombinant modified endolysin.
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1 Introduction

Biotechnological substances and drugs are complex products, the quality indicators of which can vary from batch to batch depending on many external and internal factors (temperature, humidity, pH, production conditions, used producer strains, etc.).In this regard, approaches and requirements for determining the quality of biotechnological products differ from those for chemicals. Thus, biotechnological products often lack a correlation between their quantitative content and biological activity (the ability of a product to cause a certain biological effect in a biological system (organism, cell culture, etc.)), and therefore, the determination of the quantitative content of a particular product does not indicate its quality [1]. As a result, the quality indicator "Assay" for a biotechnological product is replaced by the indicator "Specific activity", which indicates the biological effect characteristic of a given substance or drug [1].

Specific activity is determined according to a method developed considering the characteristics of the analyte and the biological effect that it should have. Since the method is often developed individually for each new substance, the question of the validity of this method arises, for the confirmation of which it is necessary to carry out validation.

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The aim of this work is to validate the method for determining the specific activity of a gel containing recombinant modified endolysin (an enzyme synthesized by a bacteriophage at the end of the lytic cycle and capable of destroying the cell wall of gram-negative and some gram-positive bacteria) LysECD7-SMAP [2].

2 Materials and methods

2.1 Objects of the study

The object of the study is the recombinant endolysin LysECD7 (NCBI AN: ASJ80195.1), containing at the C-end of the molecule SMAP (N-terminal myeloid antimicrobial peptide of sheep), which enhances the permeabilizing properties of the enzyme [2, 3], as well as a drug in the form of a gel containing this endolysin at a concentration of 2 mg/g, 5 mg/g and 10 mg/g.

2.2 Bacterial strains

Three bacterial strains (clinical isolates) were used to determine the specific bactericidal activity: *Acinetobacterbaumannii*TS 50-16, *Staphylococcusaureus*ATCC 25923 and *Escherichiacoli* ATCC 25922.

2.3 Determination of the specific activity of the LysECD7-SMAP substance and the drug

The determination of the specific activity was carried out according to the previously published method with some modifications (bacterial strains, buffer solution) [4].

Suspensions of bacterial cells of *A. baumannii*, *E. coli*, and *S. aureus* strains were prepared from overnight broth cultures obtained in meat-peptone broth (MPB) in a volume of 4.5-5 ml. Overnight cultures were diluted with fresh MPB and grown at +37±0.4°C in a TS-1/80 SPU thermostat (OJSC "Smolenskoe SKTB SPU", Russia) until a McFarland turbidity of 0.5 units was achieved. Turbidity was measured using a Densi-La-Meter II densitometer (ErbaLachemas.r.o., Czech Republic). The resulting cultures were centrifuged in a volume of 1 ml at 6000×g for 10 minutes, the supernatant was removed. The cells were resuspended in PBS until a McFarland turbidity of 0.5 units was reached. The bacterial cell suspension was diluted 100-fold with PBS to obtain a working suspension that was used in no more than 30 minutes to determine the sensitivity to endolysin. A working suspension of bacterial cells in a volume of 100 µl was mixed with a test sample (substance or drug) in a volume of 100 µl in a sterile flat-bottomed 96-well polystyrene plate (Eppendorf, Germany). The plates with the resulting mixture were incubated at +37±0.4°C for 30 minutes with continuous shaking at 200 rpm on a Shaker S-3 shaker (ELMI, Latvia). After incubation, tenfold dilutions in PBS were prepared from the samples. From dilutions 10-1 and 10-2, samples in a volume of 100 µl were applied to the surface of Mueller-Hinton agar (HiMedia, India) in Petri dishes and triturated with a glass spatula until completely dry on the surface of the nutrient medium. The plates were incubated at +37±0.4°C overnight, after which the colony count was performed. The assessment of the sensitivity of bacterial strains was carried out in comparison with control samples containing PBS (in the case of a substance study) or placebo (in the study of a drug) instead of the test sample. The study of experimental and control samples was carried out in three independent replicates. Specific bactericidal activity was calculated by the formula:

$$X = (1 - A/B)*100 \quad (1)$$

where *X*– specific bactericidal activity,%; *A*– the average number of bacterial cells in the experiment, CFU/ml; *B*– the average number of bacterial cells in the control, CFU/ml.

2.4 Validation

Validation of the method was carried out according to the indicators "Specificity", "Accuracy" and "Intermediate precision".

To validate the “Specificity” indicator, the specific activity for the substance and drug was determined by comparing the results with PBS and placebo, respectively. The acceptance criterion was the presence of a bactericidal effect in the case of the analysis of the substance and the drug and its absence in the case of the PBS and placebo analysis.

Determination of the "Accuracy" indicator was carried out by calculating the ratio "X_{exp}: X_{theor}" according to the formula:

$$Z_i = (X_{exp} / X_{theor}) * 100 \quad (2)$$

where Zi – desired ratio,%; X_{exp}–experimental value of specific activity,%; X_{theor}– theoretical value of specific activity,%.

The acceptance criterion was the value of the ratio “X_{exp}: X_{theor}”in the range of 90-100%.

To determine the Intermediate precision, the analysis of three laboratory batches of the drug was carried out independently by two analysts in duplicate, after which the results of analyst 1 were compared with the results of analyst 2 by calculating the relative standard deviation (RSD,%) using the formula:

$$RSD = \frac{\sqrt{\frac{\sum(X_i - \bar{X})^2}{n-1}}}{\bar{X}} \quad (3)$$

where X_i– specific bactericidal activity, %; $\bar{X}$ – average value of specific bactericidal activity, %; n – the total number of values.

The RSD value for all results should not exceed 10%, and for each pair of results "analyst 1: analyst 2" - 5%.

3 Results

3.1 Specificity

When determining the specific activity of the substance and the drug, a pronounced bactericidal activity was observed, while it was not in the analysis of PBS and placebo. The determination was carried out in three independent replicates. The results are shown in Table 1.

Thus, the method met the acceptance criteria and is specific.

3.2 Accuracy

To determine the Accuracy, the theoretical specific activity of the studied endolysin concentrations in relation to the bacterial strains on which the test was carried out was determined. The determination was carried out by analyzing a solution of endolysin at three concentrations: 2, 5 and 10 mg/ml. The theoretical specific activity was calculated as the

arithmetic mean of the results of five independent replicates. The data obtained are presented in Table 2.

Table 1. Determination of the specificity of the method

Iteration	**Specific bactericida lactivity, %**						
	Substance			Drug			PBS/ Placebo
	2 mg/g	5 mg/g	10 mg/g	2 mg/g	5 mg/g	10 mg/g	
S. aureus ATCC 25923							
I	57,60	58,28	56,43	59,20	53,79	52,53	0
II	56,29	47,37	57,36	57,31	49,21	52,12	0
III	48,72	56,41	50,79	51,68	52,86	55,33	0
Mean value	**54,20**	**54,02**	**54,86**	**56,06**	**51,95**	**53,33**	**0**
A. baumannii Ts 50-16							
I	100,00	100,00	100,00	99,77	100,00	99,89	0
II	100,00	100,00	100,00	100,00	100,00	99,89	0
III	97,77	100,00	100,00	99,38	99,76	99,78	0
Mean value	**99,26**	**100,00**	**100,00**	**99,72**	**99,92**	**99,85**	**0**
E. coli ATCC 25922							
I	94,89	100,00	100,00	95,99	99,70	99,26	0
II	96,95	100,00	100,00	99,50	98,61	98,77	0
III	94,20	100,00	99,93	93,53	98,66	99,43	0
Mean value	**95,35**	**100,00**	**99,98**	**96,34**	**98,99**	**99,15**	**0**

Table 2. Theoretical values of the specific activity of endolysin, %

Strain	**Endolysin concentration, mg/ml**		
	2	**5**	**10**
A. baumannii TS 50-16	97,39%	99,73%	100,00%
E. coli ATCC 25922	96,72%	100,00%	99,92%
S. aureus ATCC 25923	53,89%	53,20%	56,11%

Then the analysis of three laboratory series of thedrug was carried out in three independent replicates and the ratio "X_{exp} : X_{theor}". Table 3 shows the values of "X_{exp} : X_{theor}", all values meet the specified criteria.

Table 3. Valuesof"X_{exp} : X_{theor}", calculated in the course of determining the Accuracy

Strain	**"X_{exp} : X_{theor}", %**
A. baumannii TS 50-16	100,81
E. coli ATCC 25922	99,28
S. aureus ATCC 25923	98,91

3.3 Intermediate precision

When determining the Intermediate precision, the analysis of three laboratory batches of the drug was carried out in two independent replicates by two analysts on different days using the same equipment.

The calculated RSD values are presented in Table 4.

The determined indicators met the acceptance criteria, and the method was successfully validated for this indicator.

Table 4. Determination of Intermediate precision

X_i		Stand. dev.	RSD
Analyst 1	**Analyst2**		
S. aureus ATCC 25923			
59,20%	56,35%	2,02%	3,49%
51,68%	48,76%	2,06%	4,11%
53,79%	52,00%	1,27%	2,39%
52,86%	53,39%	0,37%	0,71%
52,53%	56,11%	2,53%	4,66%
52,12%	55,87%	2,65%	4,91%
Values for all results			
Stand. dev.		2,76%	
RSD		5,14%	
A. baumannii TS 50-16			
99,77%	100,00%	0,16%	0,16%
100,00%	100,00%	0,00%	0,00%
100,00%	100,00%	0,00%	0,00%
100,00%	99,84%	0,11%	0,11%
99,89%	100,00%	0,08%	0,08%
99,78%	100,00%	0,16%	0,16%
Values for all results			
Stand. dev.		0,09%	
RSD		0,09%	
E. coli ATCC 25922			
95,99%	98,30%	1,63%	1,68%
99,50%	98,08%	1,00%	1,02%
99,70%	99,65%	0,04%	0,04%
98,61%	99,30%	0,49%	0,49%
99,26%	94,28%	3,52%	3,64%
98,77%	98,97%	0,14%	0,14%
Values for all results			
Stand. dev.		1,63%	
RSD		1,66%	

4 Conclusion

Thus, in the course of this work, the method for determining the specific activity of the LysECD7-SMAP recombinant endolysin substance and the gel containing it was successfully validated.

All determined indicators met the acceptance criteria, which indicates the suitability of the method for determining the Specific activity.

References

1. J.C. Robinson, C. Jones, Bioanalysis, **3**(1), (2011) doi: 10.4155/BIO.10.161
2. Antonova NP, Vasina DV, Rubalsky EO, et al. Biomolecules, **10**(3) (2020) doi:10.3390/biom10030440
3. Antonova N.P., Vasina D.V., Lendel A.M., Usachev E.V., Makarov V.V., Gintsburg A.L., Tkachuk A.P., Gushchin V.A. Viruses, **11**, 3(2019) doi: 10.3390/v11030284