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Determining The Bioefficacy of An Ointment Containing Levantine Viper Venom

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Abstract

The bioefficacy of an anti-inflammatory ointment prepared based on levantine viper venom specifically - the release of the bioactive substance from the base was studied. The membrane diffusion method, an in vitro technique, was employed for this purpose. This method is particularly significant for studying the absorption of a medicinal substance released from an ointment through the skin, due to its convenience and relatively high accuracy. A membrane was used as a skin model. The amount of released substance was determined using HPLC.

Keywords: In vivo, in vitro, dialysis, buffer solution, quantitative analysis, HPLC, membrane diffusion method, ointment with levantine viper venom, acetonitrile.

Introduction

Bioefficacy expresses the speed at which a drug enters the circulatory system, i.e., its therapeutic activity. Drug efficacy is evaluated either through pharmacodynamic methods, based on therapeutic effect, or through pharmacokinetic methods, based on the degree and rate of absorption in the body. These methods are referred to as "in vivo." In vivo methods are employed during the final stages of drug research, within clinical trial regimes, or in the process of registering generic drugs to prove their equivalence to original medicines. In vivo methods are conducted under clinical conditions on laboratory animals and sometimes involve human volunteers [1].

However, during the development of new or generic drugs, it is important to study the influence of various pharmaceutical factors on therapeutic effectiveness. For this purpose, it is most convenient to investigate drug absorption processes in the body using different in vitro models.

Typically, in vitro methods are used in drug development to determine the impact of pharmaceutical factors on therapeutic effectiveness. The first stage of studying a drug's biological activity involves determining the release (dissolution) rate of the substance from the dosage form, which constitutes the main essence of in vitro methods [1,2].

In soft dosage forms, including ointments, bioefficacy is studied by determining the release of the active substance from the base. Factors such as the base composition, pH, used excipients, structural-mechanical properties, particle dispersion of the drug, and application methods all directly influence the release and absorption process [2,3,4].

Ointment drug release rates can be studied using both in vitro and in vivo experiments:
In vivo – biological experiments conducted on living organisms or their separated parts;
In vitro – methods based on physico-chemical and microbiological experiments.
Due to the high cost and time requirements of in vivo experiments, it is not always possible to conduct them. Therefore, ointment bioefficacy is often evaluated using simple and fast in vitro comparison experiments [9].

Common In Vitro Methods:

Agar Plate Method. This method is based on the reaction between the active ingredients in the ointment and an indicator within the agar medium to form colored compounds. The diffusion rate of the drug is evaluated by the size of the stained area in the gel [5].
Microbiological Method. Applied for ointments with antiseptic or bactericidal properties. It determines the boundary of microorganism growth in agar nutrient media.
Direct Diffusion Method. Used when the ointment base is in direct contact with the medium where the drug is intended to diffuse.
Chromatographic Method. In this method, filter paper is moistened with an indicator solution. The ointment sample is placed in the center of the filter paper. The diffusion rate is measured by the distance from the ointment application site to the outer boundary of the stained area on the filter paper.
Diffusion Through Semipermeable Membrane. This uses a dialysis apparatus (L. Kruvchinsky type) with a container (100–500 ml) and a 15 cm dialysis tube. One end of the tube is sealed with a dialysis membrane, often made from non-lacquered cellophane film, eggshell membrane, animal skin, or other natural materials. The external surface of the membrane is in contact with a liquid medium, and the ointment sample is placed on the internal surface. Drug diffusion occurs at 32 ± 0.5°C for 5–6 hours. Samples (5 ml) are taken every 30 minutes (up to 180 min) and analyzed using physico-chemical methods to determine drug quantity [5,8].
These in vitro methods are widely used in modern biopharmaceutics. Each method is selected based on the drug's physico-chemical properties, dosage form, and application feasibility.

Materials and Methods:

The bioefficacy of the ointment containing Gyurza snake venom, whose composition and technology were previously developed [7], was studied using the dialysis method.
This ointment has anti-inflammatory and analgesic properties. The nonivamide substance in its composition improves local blood circulation, ensuring faster absorption of the snake venom and enhancing its effect.
The release rate of the venom from the base was studied using an equilibrium dialysis method (Franz diffusion cell with donor and acceptor chambers) at 37±0.5°C in a 50 ml dialysis medium through a semipermeable membrane (0.45 μm). A buffer solution (pH = 7.4) was used as the receptor phase. The procedure was as follows: 0.5 g of the ointment sample was applied thinly on a membrane layer and secured with rubber to one end of a 15 cm long open-ended dialysis tube (donor chamber). The dialysis tube was placed in a beaker containing the buffer solution so that it did not touch the bottom. The buffer solution was continuously stirred at 300 rpm using a magnetic stirrer. The venom components diffused through the membrane into the solution maintained at

37°C. Samples of the dialysate (1.0 ml) were taken at 0.5, 1, 2, 4, 6, and 8 hours. After each sampling, an equal volume of fresh buffer solution was added to maintain a constant volume.
The amount of snake venom released into the dialysate (Figure 1) was determined by high-performance liquid chromatography (HPLC).

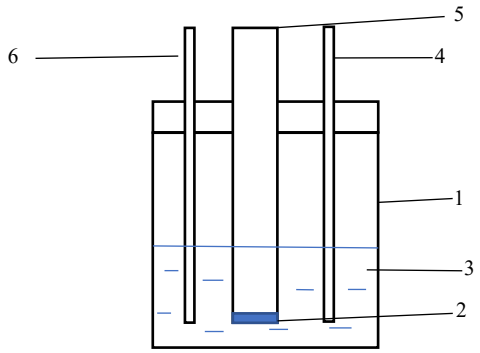

Figure 1: Apparatus for determining the bioefficacy of the ointment using the dialysis method.

1 - beaker with buffer solution, 2 - dialysis membrane (cellophane), 3 - buffer solution, 4 - pipette, 5 - dialysis tube, 6 - thermometer

Before starting the dialysis experiments, the HPLC device was calibrated using a standard solution of Levantine viper venom.
A stock solution (1 µg/ml) of dry Gyurza venom in acetonitrile was prepared and diluted to obtain standard solutions of 0.5, 1.0, 1.5, 10, 25, and 50 µg/ml. 20 µl of each solution was injected into the PerkinElmer Flexar HPLC with a UV detector, using the following conditions: Column: Agilent 5 HC-C18, 250×4.6 mm, zorbax Eclipse XDB C-18, 5 µm particle size, mobile phase: acetonitrile-water (1:1), flow rate: 1.0 ml/min, injection volume: 20 µl, column temperature: 30°C, detection wavelength: 220 nm.
Chromatograms were recorded, and peak areas were calculated. A calibration graph was plotted based on the results.
After the calibration process was completed, a quantitative analysis of the snake venom ointment, the bioefficacy of which was tested in vitro, was carried out.
1 ml of the dialysate (receptor chamber) was taken for 0.5, 1, 2, 4, 6, 8 hours and injected into the injector of a PerkinElmer Flexar HPLC liquid chromatograph with a UV detector. The concentration in the solution was determined using the calibration equation.

Results:

Standard solutions prepared from dry Levantine viper venom were injected into the PerkinElmer Flexar HPLC, and chromatograms were obtained under the conditions described above. Peak areas were calculated and used to construct a calibration curve (Table 1, Graph 1).

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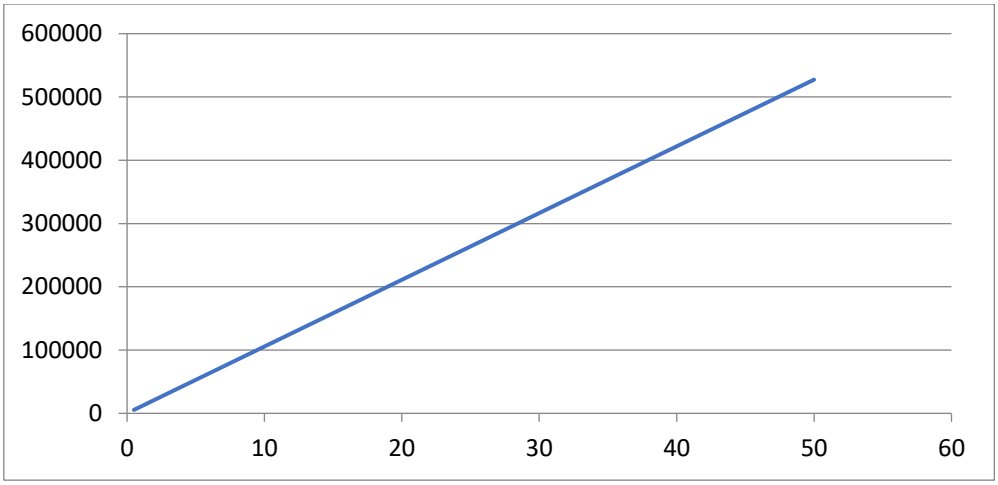
Table 1 Calibration Data

Peak Area (mV·s)	Concentration (µg/ml)
5490	0,5
10761	1
52933	5
105649	10
263793	25
527369	50



Graph **1: HPLC Calibration Curve**

After constructing the calibration curve, the concentration of drug released from the ointment was determined using the above in vitro method. The concentration of venom in the dialysate was calculated using the calibration equation (Table 2):

$x = (y - 1200) / 10530$

Table 2 Concentration of Venom in Tested Solutions

Time (h)	Concentration (µg/ml)	Cumulative Release (%)
0,5	1.208	10.5
1	2.521	22.0
2	4.805	38.4
4	7.023	55.1
6	8.224	63.0
8	8.109	63.5

Calculation of Release Rate (J) and Permeability Coefficient (P):

The release rate is calculated using the following formula:

$J = dQ / (dt \times A)$

Where:

dQ/dt – rate of change of substance in the receptor chamber (µg/s)

A – surface area of the dialysis membrane

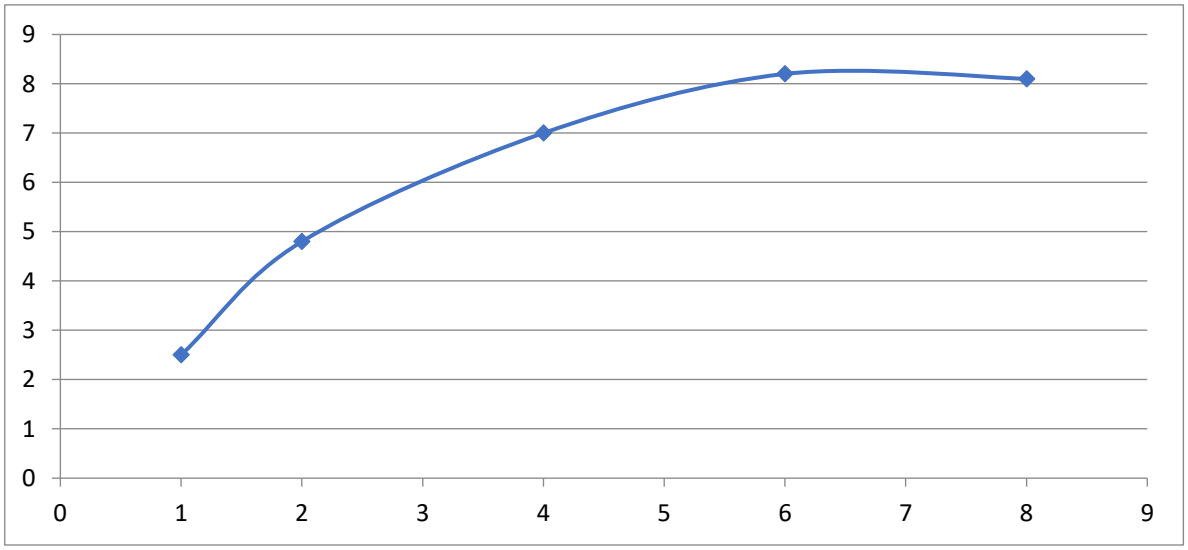
C_d – concentration of venom in the donor chamber

The results are presented in tabular form below (Table 3).

Table 3 Release Rate and Permeability Coefficient Results

Time (h)	Concentration (µg/ml)	Accumulated amount (%)	Flux J (µg/s·cm²)	Permeability Coefficient (cm/s)
0,5	1.208	10.5	1.47	$7.34*10^{-5}$
1	2.521	22.0	1.30	$6.50*10^{-5}$
2	4.805	38.4	0.62	$3.11*10^{-5}$
4	7.023	55.1	0.34	$1.69*10^{-5}$
6	8.224	63.0	-0.028	$-1.41*10^{-5}$
8	8.109	63.5	-	-

As seen from the table, the venom concentration in the receptor chamber increases over time, reaching a maximum of 8.2 ± 0.3 µg/ml after 6 hours. The diffusion process slows down afterward, and after 8 hours, the cumulative release reaches 63.5 ± %. Based on the results obtained, a graph was constructed showing the concentration of venom accumulated in the receptor chamber versus the release time (Graph 2).



Graph 2. Venom Concentration vs. Time in Receptor Chamber

Conclusion

The bioefficacy of the developed ointment containing Levantine viper venom was studied using the membrane diffusion method simulating human skin. The amount of active substance released from the base was determined using modern physico-chemical analysis (HPLC). The results indicate that the ointment shows high therapeutic activity within 8 hours, after which the drug release rate decreases.

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