AMPHOTERICIN B AS A CHOLESTEROL IDENTIFIER IN HUMAN ERYTHROCYTE'S MEMBRANE

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Abstract. Conditions were determined for the identification of cholesterol in erythrocyte membranes by the fluorescent method using amphotericin B as a probe. We performed a comparative analysis of electronic absorption and fluorescence spectra of cholesterol, amphotericin B, and the products of their interaction, and determined the optimal wavelengths for excitation (328 nm) and recording (468 nm) of fluorescence. Amphotericin B stained cholesterol in membranes was visualized by fluorescence microscopy. It was shown that the registration of emission band at 468 nm on fluorescence spectra of free and membrane-bound cholesterol can serve as a marker of the presence of this lipid in the samples.

Keywords: cholesterol, membrane, red blood cells, the electronic absorption spectra, the fluorescence spectra, fluorescence microscopy.

List of Abbreviations

AmB – Amphotericin B
RBC – red blood cells
EAS – the electronic absorption spectrum

Introduction

The lipid composition of the membrane is one of the main factors that determine its functional properties, permeability and curvature, set and activity of membrane-associated proteins. Cholesterol plays an important role in the body as a precursor to steroid hormones, bile acids, and vitamin D, a component of eukaryotic cell membranes. Although membrane cholesterol (MC) is a vital component of the cell, its excess increases the viscosity of membranes and reduces their elasticity. The content of cholesterol in the membranes of erythrocytes is modulated by long-term concentrations of serum lipoproteins, and can serve as a marker for assessing the risks of developing cardiovascular diseases along with the level of serum cholesterol (Nunes & Pretorius, 2019). However, methods for quantitative analysis of the lipid composition of membranes are laborious, expensive, and are rarely used in routine laboratory practice. At the same time, information on the localization and amount of cholesterol in membranes is one of the key aspects for understanding the development mechanisms and developing approaches to the treatment of diseases associated with impaired cholesterol metabolism (Schoop *et al.*, 2021).

Amphotericin B (AmB) is a polyene antibiotic derived from Streptomyces nodosus. The drug molecule (Fig. 1) consists of a macrolide lactone ring and a carbohydrate moiety; one side of the ring is represented by a rigid lipophilic chain of seven conjugated double bonds, and the opposite side contains the same number of hydroxylated carbon atoms (Ulozas, 2010).

The basis of the biological activity of amphotericin B molecules is the formation of structural ion channels of molecular size in lipid membranes in complex with cholesterol, ergosterol and other sterols (Pashazade & Kasumov, 2021; Sultanova & Kasumov, 2021).

The product of the interaction between amphotericin B and cholesterol is capable of fluorescence, which makes it possible to propose this antibiotic as a fluorescent probe for the localization and quantification of cholesterol content in plasma membranes (Yang *et al.*, 2016).

In connection with the above, the aim of our work was to determine the conditions for testing the presence of cholesterol in membranes by fluorescent probes, which included the analysis of electronic absorption and fluorescence spectra of cholesterol, amphotericin B and their interaction products in the free state and in erythrocyte membranes.

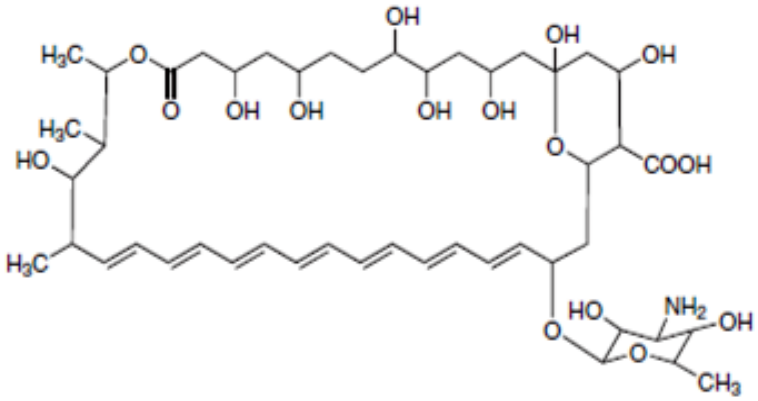

Fig. 1. Chemical structure of amphotericin B

Materials and Methods

Obtaining red blood cells

For the research, we used the heparinized blood of donors. The blood was obtained from the Voronezh Regional Blood Transfusion Station. Our study was performed according to the Good clinical practice standard. All the procedures were performed in compliance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Red blood cells (RBC's) were washed 3 times at 3000 rpm on the MPW-340 (Poland) centrifuge for 10 minutes. The resulting suspension of erythrocyte cells was adjusted to a concentration of 10^6 cells/ml (which corresponds to an optical density of D_{495}= 0.8––0.9) and then used in experiments (Artyukhov *et al.*, 2016).

Obtaining erythrocytes membranes

RBC membranes were obtained by hypoosmotic hemolysis of erythrocytes with 10 mmol/lTris-HCl buffer (pH 7.6) containing 10 mmol/l EDTA (hemolysis medium). The suspension of washed erythrocytes was rapidly and vigorously mixed with 20 volumes of a cooled hemolyzing medium and kept at +4 °C for 15 min. The hemolysate was sedimented on a MiniSpin centrifuge (Eppendorf AG, Germany) at 13,400 rpm for 15 min. The supernatant was removed, the membranes were washed three times with 20 volumes of 10 mmol/l Tris-HCl buffer (pH 7.6), each time precipitating in the same mode.

Obtaining electronic absorption spectra and fluorescence spectra

For record electronic absorption spectra and fluorescence spectra, crystallized cholesterol (1 mg) was dissolved in 10 ml of 96.4% ethanol. Dimexide (99% solution of dimethyl sulfoxide (DMSO) (OOO «Iodine Technologies and Marketing», Russia) was diluted with distilled water to a concentration of 0.001%. 1 mg of Amphotericin B (Synthesis, JSC, Russia) was dissolved in 20 ml of 0.001% DMSO solution, thus the concentration of Amphotericin B solution was 5,4×10-5 mol/l. To stain cholesterol, its alcohol solution (2 ml) was mixed with 2 ml of a solution of amphotericin B in DMSO. Erythrocyte membranes (1 ml of suspension) were stained with solutions of amphotericin B in DMSO prepared as described above, the mixture was incubated for 30 min at +4 °C.

The electronic absorption spectra of the studied samples were recorded using an automatic spectrophotometer UV-2401PC (Shimadzu, Japan) in the wavelength range 190–700 nm. Quartz cuvettes with a width of 1 cm were used. The optical density of the solutions was recorded throughout the entire studied range at 1 nm with a spectral gap width of 1 nm.

Fluorescence spectra of cholesterol samples, 0.001% DMSO, amphotericin in DMSO, cholesterol with amphotericin in DMSO, and amphotericin-stained membranes were recorded on an RF-1501 spectrofluorimeter (Shimadzu, Japan) in the wavelength range of 370–500 nm at an excitation wavelength of 328 nm.

Fluorescence microscopy of erythrocyte membranes

A suspension of erythrocyte membranes stained with amphotericin B was applied to glass slides and dried in air. Then the samples were examined on a NIKON ECLIPSE Ni-E fluorescent microscope (Nikon, Japan) at a magnification of 40x. The excitation wavelength is 340 nm, the emission wavelength is 480 nm.

Statistical analysis of the results

Statistical processing of the experimental results was carried out using the statistical software package «STADIA 8.0/educational» (InCo, Russia). The results are presented in the format «mean ± standard deviation».

Results

On the electronic absorption spectra of amphotericin B solutions, absorption maxima were revealed at 208, 223, 293, 328, 385, 408, 418 nm (Fig. 2).

The most intense absorption maximum for amphotericin B was recorded at λ = 328 nm, which allows us to assume the possibility of using this wavelength to excite fluorescence.

The maximum at 199 nm was detected on the electronic absorption spectra (EAS) of cholesterol solutions in ethyl alcohol (Fig. 3).

On the EAS of cholesterol samples incubated with amphotericin B, 7 absorption bands of different intensity were detected in the UV and visible wavelength ranges (Fig. 4). The absorption maxima at 194, 272, 283, 364, 386 and 408 nm are obviously the result of the superposition of the absorption spectra of cholesterol and amphotericin B. The appearance of peaks at 346 and 364 nm and a marked increase in the intensity of the maxima at 386 and 408 nm are probably the result of the appearance of products of interaction between cholesterol and amphotericin B and/or changes in the microenvironment of the macrolide lactone ring of antibiotic molecules.

An intense peak at 204 nm and maxima at 414, 542 and 576 nm were recorded on the electronic absorption spectra of erythrocyte membranes (Fig. 5). The absorption band in the UV range of the spectrum is due to lipid (involving

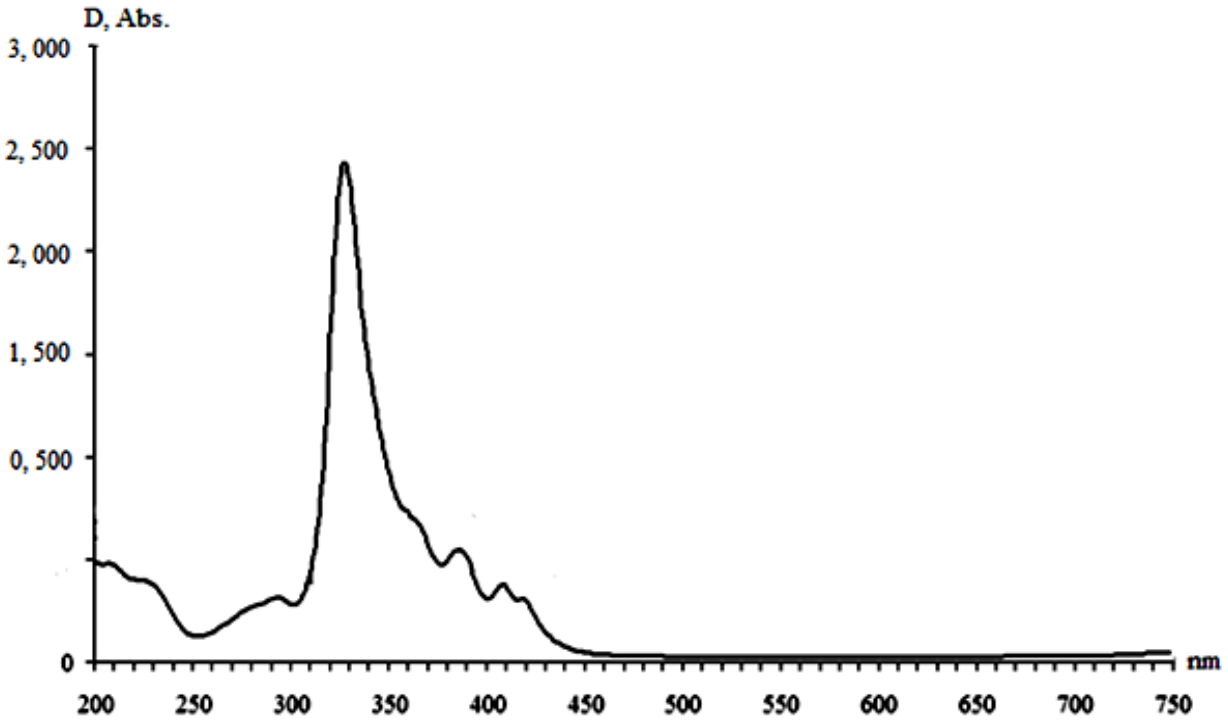

Fig. 2. The electronic absorption spectrum of amphotericin B in DMSO

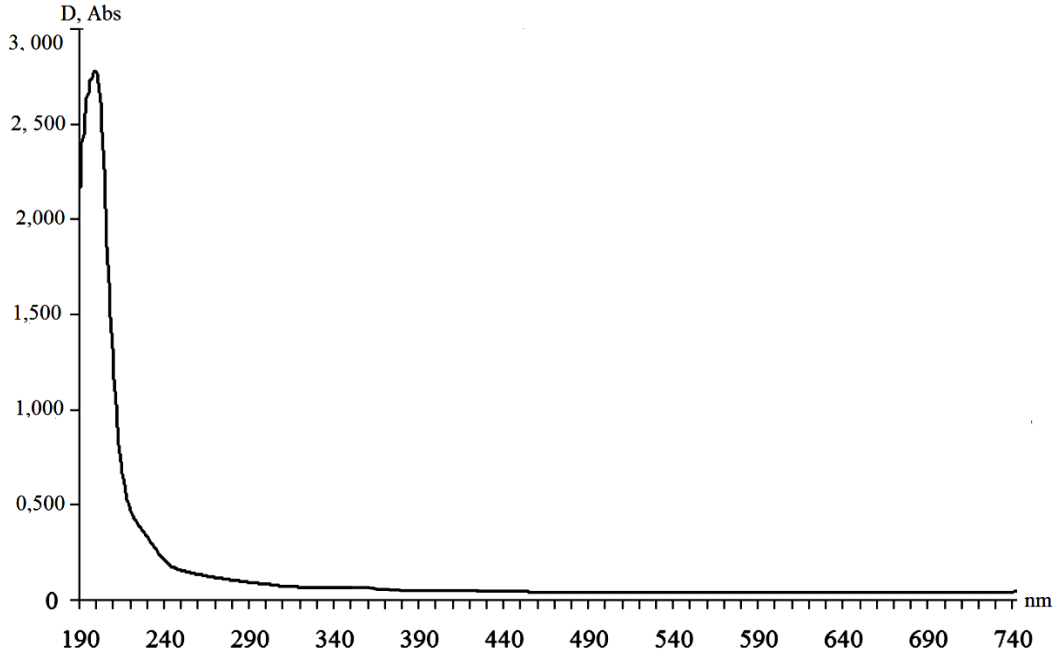

Fig. 3. The electronic absorption spectrum of cholesterol solution in ethyl alcohol

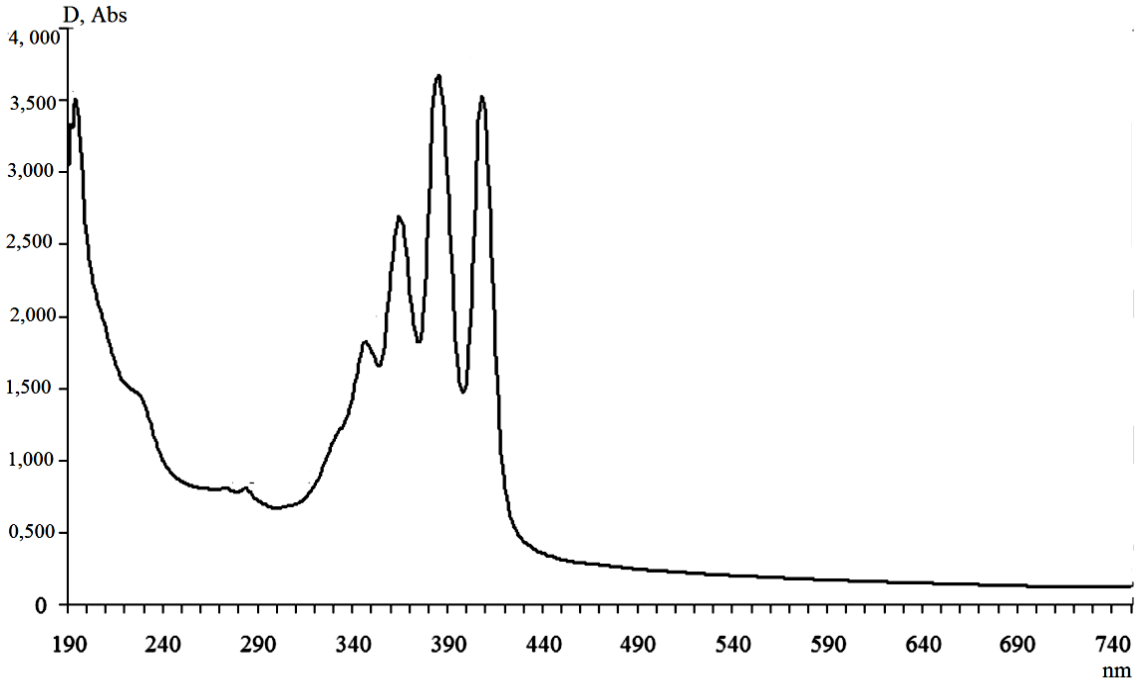

Fig. 4. The electronic absorption spectrum of the cholesterol–amphotericin B mixture

cholesterol) and protein components of the membrane, the bands in the visible region are associated with membrane hemoglobin.

The fluorescence spectra of amphotericin B were characterized by a very weak emission band at 475 nm (I_{fl} = 0.81 ± 0.34 units). Thus,

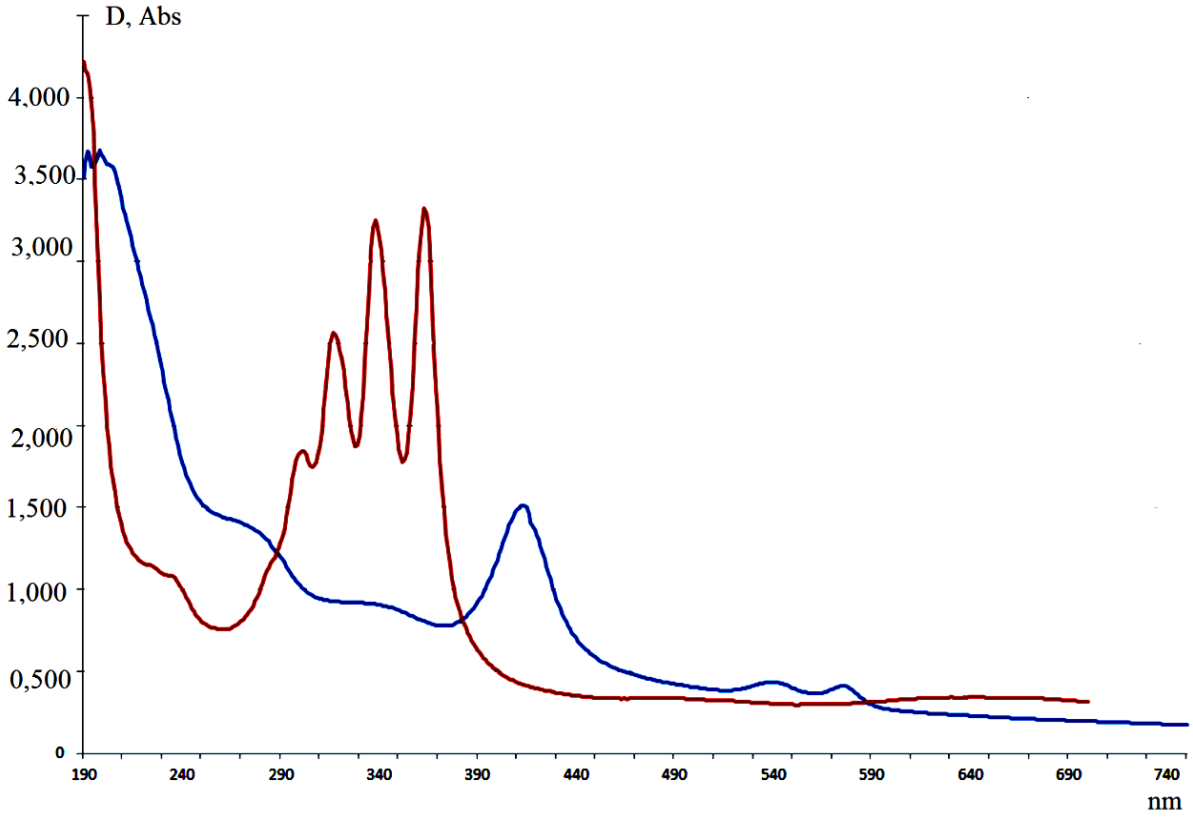

Fig. 5. The electronic absorption spectrum of intact (blue) and stained by AmB (red) erythrocyte membranes

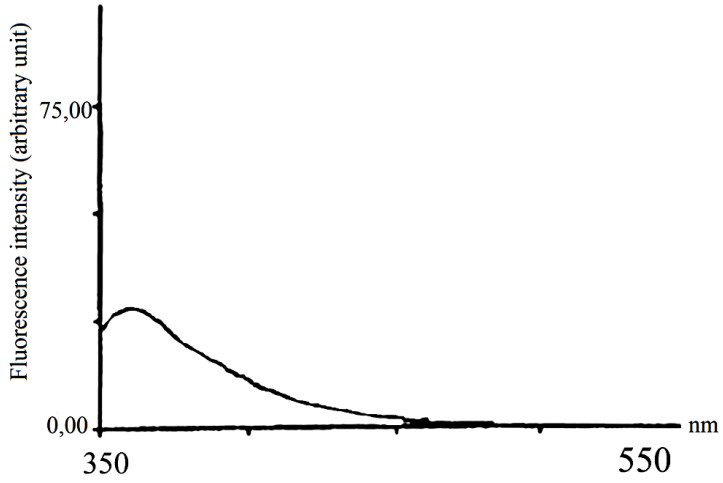

Fig. 6. The fluorescence spectrum of cholesterol in ethyl alcohol

amphotericin B does not have the ability to express fluorescence in the free state in the wavelength range 350–500 nm.

The maximum emission was detected on the fluorescence spectra of cholesterol solutions in ethyl alcohol (Fig. 6) using the excitation wavelength of 328 nm: at 365 nm (I_{fl} = 27.95 ± ± 0.41 units).

On the fluorescence spectra of cholesterol–amphotericin B mixture (Fig. 7), when using λEx = 328 nm, an emission band was detected at 468 nm (I_{fl} = 16.57 ± 2.28 units).

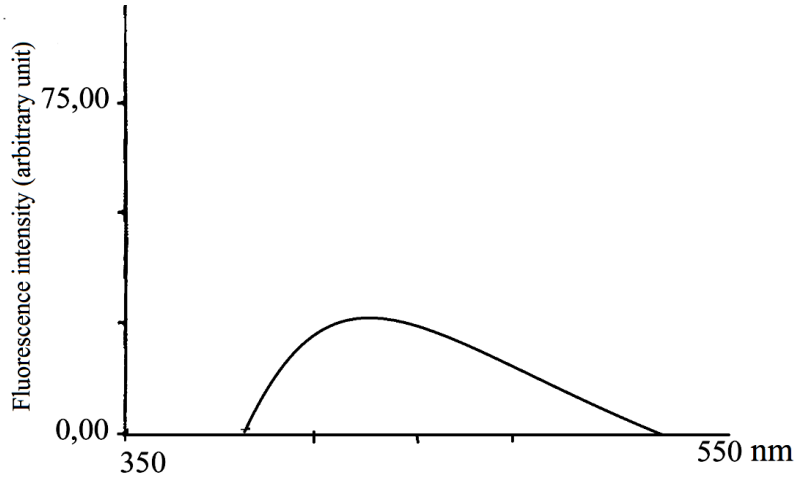

Fig. 7. Fluorescence spectrum of the cholesterol–amphotericin B mixture

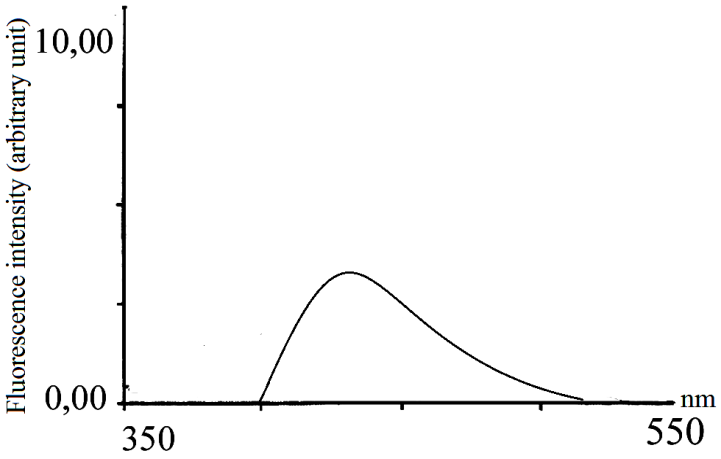

Fig. 8. Fluorescence spectrum of erythrocyte membranes stained by amphotericin B

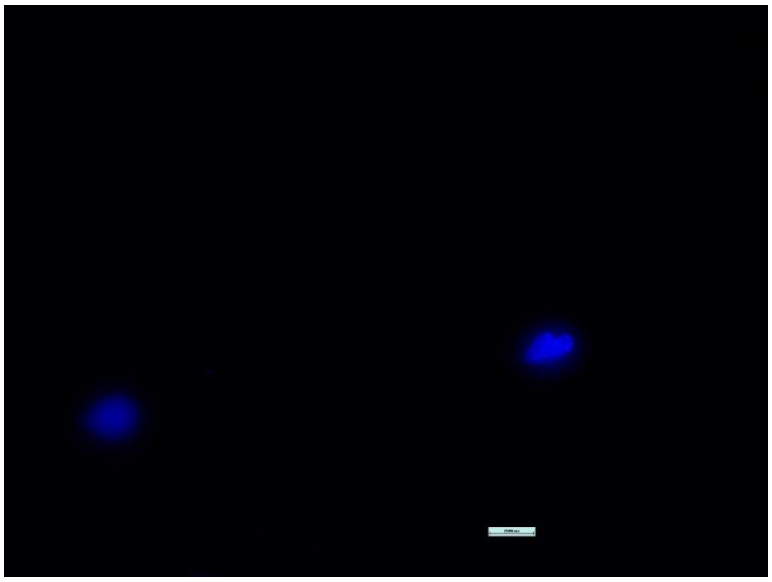

Fig. 9. Micropreparation of erythrocyte membranes stained by amphotericin B (EX 340-380 DM 400 IA 435–481, magnification 40x)

Erythrocyte membranes stained with amphotericin B were characterized by a noticeable glow at 466-470 nm: the fluorescence intensity was 4.5 ± 1.5 units (Fig. 8).

Micrographs of erythrocyte membranes stained with amphotericin B showed fluorescent areas, apparently due to the glow of cholesterol stained by amphotericin B (Fig. 9).

Discussion

After staining the membranes with amphotericin, the number and position of absorption maxima changed. Intense light absorption was still recorded in the range of 190–220 nm, maxima were detected in the near UV region at 328, 346, 364 and 386 nm (Fig. 5). Similar patterns of EAS of the cholesterol–amphotericin B complex and amphotericin-stained erythrocyte membranes suggest that the antibiotic binds to cholesterol in the membranes as well as to free cholesterol.

In this way, it is shown that amphotericin B absorbs light in the near UV and visible ranges, combines with cholesterol in the composition of membranes, therefore, it seems to be a promising object for use as an identifier of the specified lipid in membranes by fluorescence when using the wavelength of fluorescence excitation of 328 nm.

To confirm the assumption about the possibility of using amphotericin B as a fluorescent probe for cholesterol detection, we investigated the fluorescent characteristics of amphotericin B, unstained cholesterol, free cholesterol incubated with amphotericin B, unstained and stained erythrocyte membranes.

Unpainted erythrocyte membranes did not fluoresce in the studied wavelength range. A comparative analysis of the fluorescence spectra of individual compounds (cholesterol, amphotericin B) and products of their interaction showed that the emission band at 468 nm results from the «cholesterol amphotericin B» association or from a change in the microenvironment of the macrolide lactone ring of amphotericin B and can serve as a marker for the presence of this lipid in samples.

Conclusion

To sum up, we have shown that «cholesterol amphotericin B» associations are capable of fluorescence in solution and within membranes and allow the visualization of sterol-containing areas of the plasmalemma.

Amphotericin B is a sensitive probe for the identification of cholesterol in solution and in membranes and can be used to develop methods for the quantitative analysis of cholesterol content in samples.

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