DEVELOPMENT OF A METHOD FOR ASSESSING THE DEPTH OF PENETRATION OF ETHOSOMES WITH METHYLENE BLUE INTO THE SKIN DURING APPLICATION AND PHOTODYNAMIC EXPOSURE

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Abstract

A wide range of literature sources report on the potential benefits of transdermal drug delivery. Among these advantages, the following are distinguished – minimal injury, reduction of side effects, and prevention of degradation or metabolism in the gastrointestinal tract or liver. However, transdermal delivery of most molecules often excludes due to the barrier function of the skin, which prevents the penetration of exogenous substances. To overcome this barrier and increase skin absorption, ethosomal complexes use, by means penetration into the deep layers of the skin and/or systemic circulation is possible. This work devotes to the development of a non-invasive method for assessing the depth of penetration by ethosomes with methylene blue (MB) into the skin during application and photodynamic exposure. MB as photosensitizer (PS) was chosen, since there are a sufficient number of publications on its positive effect on the restoration of the cell's respiratory chain of various organs and therefore the restoration of their metabolism. Besides MB has proven to be an effective PS, destructed pathogenic microbes and viruses, including SARS-CoV-2. However, for more effective Covid-19 therapy and antibiotic-resistant microbial diseases, the penetration of MB into the vascular system of the epidermis or mucous tissue is required. Nowadays, the existing methods for assessing the penetration depth of PS are time consuming and require the use of animal skin or model samples. The LESA-01 BIOSPEC system with specially designed optical adapters that allow assessing the drug fluorescence intensity on skin surface and at a depth of up to 2 mm in the investigation was used.

Keywords: ethosomes, transdermal drug delivery, methylene blue, penetration depth, effectivethickness of the drug layer.

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РАЗРАБОТКА МЕТОДА ОЦЕНКИ ГЛУБИНЫ ПРОНИКНОВЕНИЯ ЭТОСОМ С МЕТИЛЕНОВЫМ СИНИМ В КОЖУ ПРИ АППЛИКАЦИОННОМ ПРИМЕНЕНИИ И ФОТОДИНАМИЧЕСКИМ ВОЗДЕЙСТВИИ

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Резюме

В широком спектре литературных источников сообщается о потенциальных преимуществах трансдермальной доставки лекарственных веществ. Среди данных преимуществ выделяют следующие – минимальная травматичность, снижение побочных эффектов, предотвращение деградации или метаболизма в желудочно-кишечном тракте или печени. Однако трансдермальная доставка большинства молекул часто исключается из-за барьерной функции кожи, которая препятствует проникновению экзогенных веществ. Для преодоления данного барьера и увеличения кожного поглощения могут быть использованы этосомальные комплексы, с помощью которых возможно проникновение в глубокие слои кожи и/или системное кровообращение. Данная работа посвящена разработке неинвазивного метода оценки глубины проникновения этосом с метиленовым синим в кожу при аппликационном применении и фотодинамическом воздействии. Именно метиленовый синий был выбранв качестве фотосенсибилизатора (ФС) в работе, поскольку имеется достаточное количество публикаций о его положительном влиянии на восстановление дыхательной цепи клеток различных органов и, тем самым восстановлении их метаболизма. Кроме того, метиленовый синий проявил себя как эффективный ФС, разрушающий патогенные микробы и вирусы, в том числе вирус SARS-CoV-2. Однако для более эффективной терапии Covid-19 и антибиотикорезистентных микробных заболеваний требуется проникновение метиленовый синий в сосудистую систему эпидермиса или слизистой ткани. Наданный момент существующие методы оценки глубины проникновения фотосенсибилизаторов являются трудоёмкими и требуют использования кожи животных или модельных образцов. В работе была использована система ЛЭСА-01 БИОСПЕК со специально разработанными оптическими адаптерами, позволяющими оценивать интенсивность флуоресценции препарата наповерхности кожи и на глубине до 2 мм.

Ключевые слова: этосомы, трансдермальная доставка лекарств, метиленовый синий, глубина проникновения, вирусы, микробы.

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Introduction

In recent decades, there has been a diverse and widespread use of lasers in dermatology and cosmetology. Laser methods to correct age-related changes and various skin pathologies are used [6]. Such a desire for laser therapies has led to the development of highly effective, minimally invasive and sparing methods of treatment [1], one of which is photodynamic therapy, which in the treatment of various skin diseases is used. In addition to cancerous and precancerous skin changes, PDT for cosmetic purposes in photo-rejuvenation is used [2]. Improvement of the skin condition during photoaging, prevention of actinic keratoses, the possibility of repeated procedures and a limited number of side effects make the PDT procedure very promising for skin rejuvenation [3]. One of the photosensitizers in PDT is methylene blue (MB). Several clinical studies indicate the effectiveness of MB in the treatment of basal cell carcinoma, Kaposi's sarcoma, melanoma, viral and fungal infections are used [4]. The authors [5] also note the antioxidant effect of MB and prove that its independent use can effectively protect the skin from oxidative stress and slow down skin aging.

The MB photosensitizer differs from a number of other photosensitizers, since during photoexcitation it oxidizes NAD(P)H, which is localized in the mitochondrial matrix, which defines MB as a photosensitizer of mitochondrial action [4,6]. In fact, targeting mitochondria is an important subject of research in PDT, since damage to mitochondria can induce an apoptotic cascade [4,7,8].

Brief description of the structure, functions and ways of penetration into the skin

The skin is the outer and largest organ of the human body with a complex structure. The skin performs a protective function and acts as a barrier to the penetration of exogenous substances from the external environment into the body. Stratum corneum (SC) is a "brick" organization consisting of corneocytes embedded in the lipid domain. The strengthening of cell walls is due to the presence of covalently bound lipids and cross-linked proteins, and the connection with neighboring cells occurs through desmosomes. Directly under the SC is a viable epidermis with keratinocytes, formed in the basal layer of the epidermis. Then there is a slow upward migration of these cells to the surface of the skin. Melanocytes, Langerhans cells, migrating macrophages and lymphocytes in the epidermis were also found. Under the epidermis there is a dermis containing structured collagen and elastin fibers. The epidermis and dermis perform an important function in the process of percutaneous absorption. The hypoderm under the dermis is located and is a layer of subcutaneous adipose tissue, provides the main food supply, physical protection and thermal insulation [9,10]. For the vast majority of penetrants, diffusion through SC associated with an obstacle and a restriction on the penetration rate. Since SC consists of dead cells where there is no metabolic activity, the penetration process occurs in a passive way. Such an obstacle to penetration with the composition and structure of the SC itself is related [11]. At the same

time, the only continuous region in SC is the lipid domain mainly consisting of ceramides, free fatty acids, cholesterol and cholesterol esters. A distinctive feature is the difference from other biological membranes, which consist mainly of phospholipids. Such a unique composition of SC prevents the penetration of ionic, high-polar substances and macromolecules. Highly polypophilic molecules, passing through the SC, do not easily diffuse into the hydrophilic epidermis and dermis [9].

There are three potential methods of penetration to deep layers by application: the intercellular pathway, the transcellular pathway and the accessory pathway (Fig. 1) [12]. Since sweat glands and hair follicles occupy only 0.1% of the total body surface, the accessory pathway does not contribute much to the penetration of medicinal substances [9,12]. Although, when the penetration of slowly diffusing compounds and substances with high molecular weight, such as nanoparticles, occurs, the accessory pathways may have an important role [13]. However, it is generally assumed that the intercellular pathway is the main one for the penetration of most molecules [14].

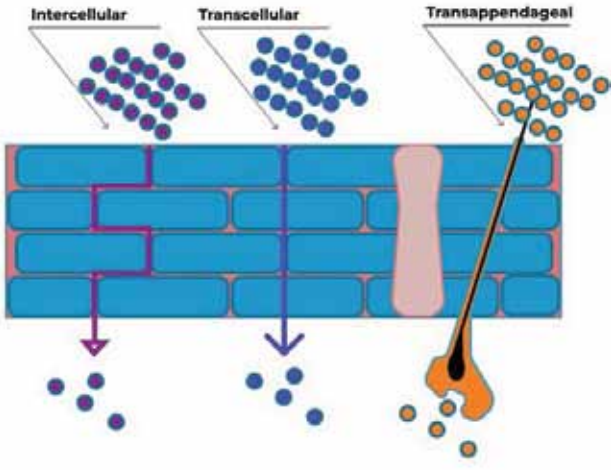


Рис. 1. Возможные пути доставки лекарства сквозь роговой слой кожи
Fig. 1. Impossible routes of drug delivery trough stratum corneum

To achieve the therapeutic amount of the drug in the deep layers of the skin and systemic circulation, appropriate penetration enhancers can be used, which affect the properties of the skin barrier and/or penetrate. Four main methods of enhancing penetration through the skin are most often considered: microneedle delivery, the use of electrical impulses, chemical reinforcement and the use of innovative vesicular carriers [9].

Innovative vesicular carries

Liposomes as a drug penetration enhancer during topical application of the drug by Mezei about two decades ago were first investigated [15,16]. According to further studies, it was shown that such rigid particles increase accumulation in the upper layers of the skin and do not lead to an increase of medicinal substance in the deep layers of the skin [17,18]. Therefore, efforts to synthesize lipid vesicular systems that can facilitate the penetration of the drug into the underlying layers of the skin and allow transdermal absorption were made [19,20]. Innovative vesicular carriers should include ethosomes. The main components of the ethosomes are phospholipids, ethanol (20-45%) and water. In special cases, propylene glycol (PG), carbopol and isopropyl alcohol to the composition are added [21].

Eggs, soy, polysynthetic and synthetic products can be used as a source of phospholipids. The high concentration of alcohol in the composition provides a soft shape and allows you to destroy the lipid bilayers of the skin. Ethanol and isopropanol as alcohol can be used. Neutral liposomes tend to stick together, and this leads to leakage of the medicinal substance (MS) from the vesicles. However, ethosomes contain ethanol in their composition, which modifies the total charge of the system, which leads to resistance to agglomeration. The increase of ethanol

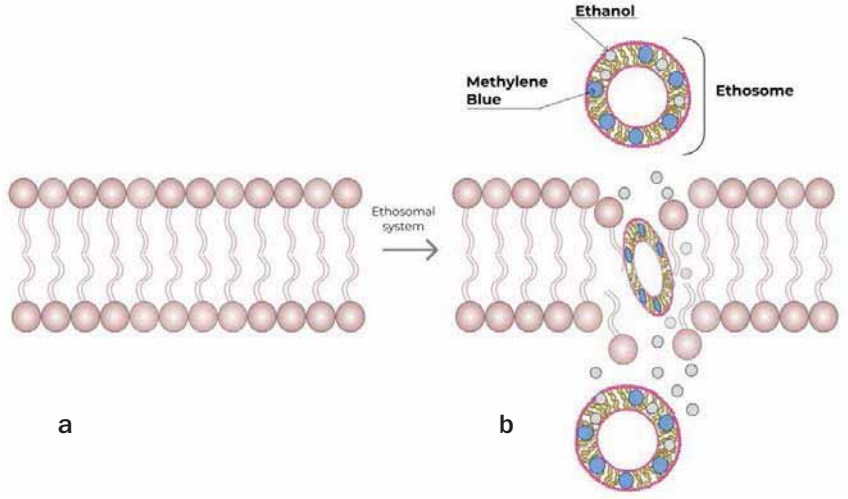


Рис. 2. Предполагаемый механизм проникновения этосомальной системы через мембрану роговой слой кожи:
a – упорядоченные липидные бислои
b – липидный бислой, разрушенный этанолом и накопившиеся мягкие, податливые этосомы
Fig. 2. The proposed mechanism of penetration of the atosomal system through the membranes of the stratum corneum:
a – ordered lipid bilayers
b – lipid bilayer, disturbed by ethanol and accumulated soft, malleable ethosomes

concentration from 15 to 45% leads to membrane fluidity, thereby increasing the efficiency of MS capture. However, a further increase in concentration may lead to a violation of the tightness of the membrane. Glycols act as surfactants (surfactants), they enhance the penetration of vesicles. Propylene glycol or transcutol among glycols are used. Additional stability of vesicles by adding cholesterol (0.1-1%) to the composition can be achieved. Oxidative degradation from light of lipids of ethosomal vesicles can be minimized by using the antioxidant α-tocopherol (a type of vitamin E and has the number E307).

Fig. 2 shows the structural form and the principle of penetration of liposomal vesicles loaded with MB.

Materials and methods

Materials

Ethosomes

Egg phospholipids, propylene glycol (PG), bidistilled water (DDW), ethyl alcohol (EtOH), carbopol were used for the preparation of ethosomal vesicles, and PS methylene blue as a therapeutic substance and a method for identifying the depth of penetration on a confocal microscope was used.

LESA-01 BIOSPEC system

The diagram of the portable system used in Fig. 3 is shown. The signal from the laser or lamp (1) via a U-shaped optical fiber (3) to the tissue under study is transmitted. The distal end of the fiber (5) receives scattered a fluorescent signal to the receiving fibers that surround the exciting central fiber. At the output end of the optical fiber connected to the spectrometer (2), the fibers form a straight line (6). At the entrance to the spectrometer there is a narrow-band light filter that reduces the intensity of the laser signal scattered backwards. The received signal is digitized and displayed on the screen of a PC with integrated UNO (4) software in real time [26]. The diagram of the portable system used in Fig. 3 is shown. The signal from the laser or lamp (1) via a U-shaped optical fiber (3) to the tissue under study is transmitted. The distal end of the fiber (5) receives scattered fluorescent signal to the receiving fibers that surround the exciting, central fiber. At the output end of the optical fiber connected to the spectrometer (2), the fibers form a straight line (6). There is a narrow-band light filter at the entrance to the spectrometer that reduces the intensity of the laser signal scattered backwards. The received signal on the screen of a PC with the built-in UNO (4) software in real time is digitized and displayed [26].

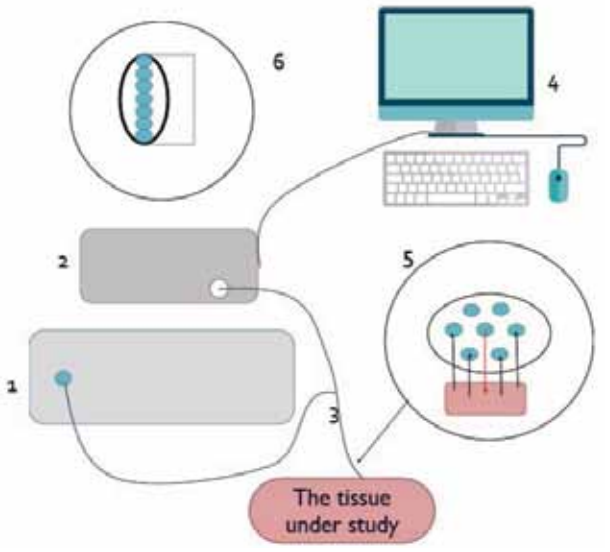


Рис. 3. Схематическое изображение экспериментальной установки, состоящей из: 1 –источника лазерного сигнала, 2 – спектрометр, 3 – оптические волокна, 4 – ПК с программным обеспечением UNO, 5 – торец диагностического катетера, 6 – выход

Fig. 3. Schematic representation of an experimental setup consisting of: 1-laser signal source; 2 – spectrometer; 3 – optical fibers; 4 – PC with UNO software; 5 – working part; 6 – output

Methods

After preparing the formulations for transdermal delivery by cold method, they at the temperature of 3-4°C in the refrigerator were stored. Ethosomal vesicles by the following methods were characterized.

1. Bubble size determination using dynamic light scattering (DLS) and zeta potential using a zeta meter.
2. The content of MS in ethosomal systems can be determined using a spectrophotometer.
3. The study of the drug release kinetics from the ethosomal system in this work when determining the MS formation in vesicles at different ambient temperatures was carried out 4°C, 27°C and 37°C at regular intervals.
4. Using the LESA-01 BIOSPEC spectroanalyzer, studies on human skin and pork skin using a special adapter to determine fluorescence at depth and on the skin surface were carried out.
5. Skin penetration study: the ethosomal preparation ability to penetrate the skin layers was determined using laser confocal scanning microscopy (LCSM).

Results and discussions

Synthesis of ethosomal samples

The classic method of ethosomes synthesis was used. Phospholipid and MB in ethanol were dissolved. Twice distilled water slowly in a thin stream with constant stirring at a speed of 700 rpm using a blender for 5 minutes was added. The ethosomal system at a temperature of 30°C during synthesis to room temperature was kept and then cooled.

Determination of vesicle size and morphology

Fig. 4a shows the results of the size distribution for

samples MB0-MB4. The average size of vesicles dissolved in ethanol, determined by Malvern Zetamaster, was 240.68 nm for the sample CM3.

The size distribution of this ethosomes ranges from tens of nanometers to microns. The size of ethosome depends on can composition of the system. For example, the graph shows an increase in the size of the ethosomes when PG to the samples MS2, MS3 and MS4 is added. Another example may be the formation of a step in the size distribution when carbopol to the composition of MS 4 is added. The smooth surface of the bubbles using SEM was confirmed (Fig. 4b).

Drug release kinetics from ethosomes and penetration depth

The stability of the colloidal solution by Malvern Zetamaster for each sample was determined. Fig. 5 shows the distribution of the zeta potential. According to the data obtained, the ethosomes containing a large amount of ethanol in their composition, which causes a modification of the total charge of the system and gives it a certain degree of steric stabilization, leads to an increase in the stability of the system to agglomeration. According to the graph of the zeta potential of the MS4 sample, there are impurities can be concluded.

All samples underwent studies on the kinetics of MB release at different temperature conditions of 27°C, 37°C and exposure time of 10, 20 and 30 minutes. The optical density of the supernatant on a spectrophotometer was determined. The results of the dynamics of the release of MS from ethosomes under various temperature conditions in the Fig. 6ab.

The exposure time increases, the optical density increases, which indicates the release of MB from vesicles. At the same time, there is an increased release at a temperature of 37°C, which indicates an increase in the release rate when penetrating into the deep layers of the skin.

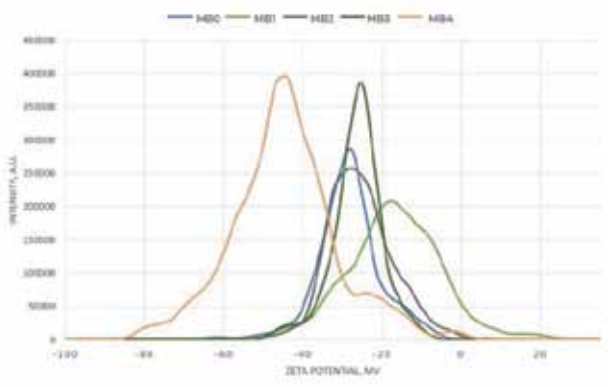
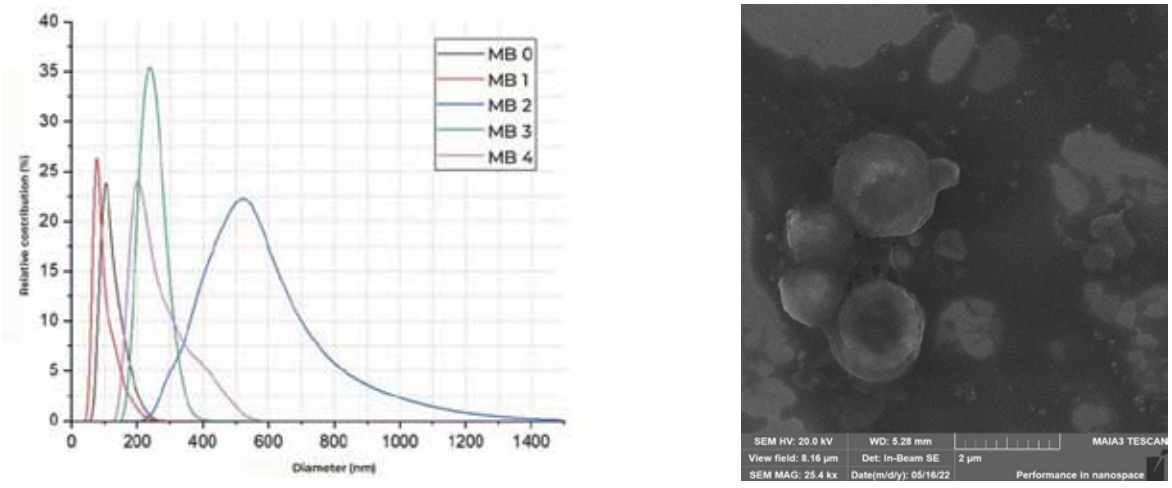


Рис. 5. Распределение дзета-потенциала для образцов MB0-MB4
Fig. 5. Zeta potential distribution for samples MB0-MB4

The kinetics of the release of MB from ethosomes on the patient's skin and pork skin using the LESA-01 BIOSPEC spectrometer were also determined. Obviously, fluorescence wavelength at depth and on the surface of the patient's skin is different, so this method allows you to determine the concentration and depth of MB penetration (Fig. 7a). The fluorescence wavelength of samples (T1, I, II) and pork skin fluorescence after irradiation in the NIR spectral region does not change during measurement (Fig. 7b). This also proves the above-mentioned removal of MB from vesicles at a temperature of 37°C. The depth of penetration of MB into pork skin on a confocal microscope in the area with and without the SC was studied (Fig. 8).

Based on the obtained results, graph 10 was constructed, which shows that a large concentration of ethosomal complexes in the SC is found, and then

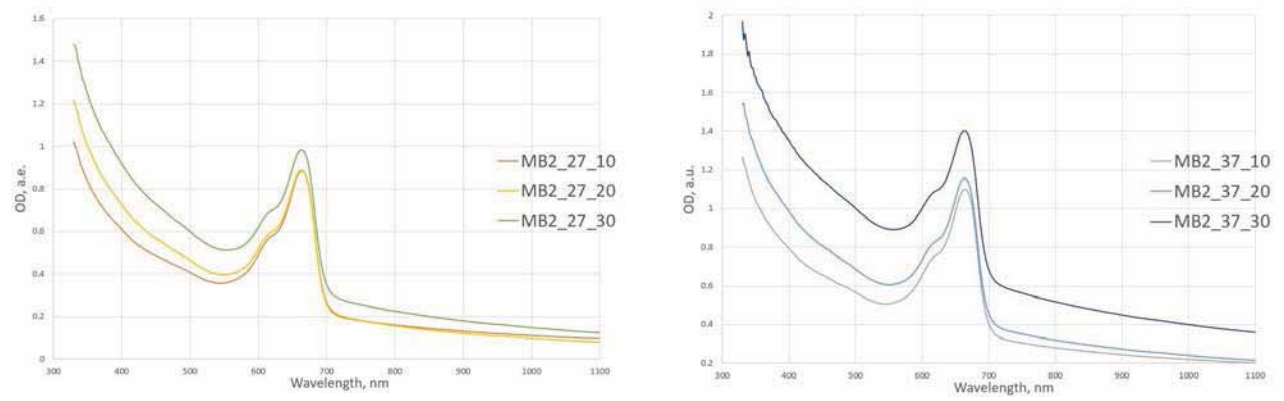


a b

Рис. 4.
a – Распределение по размерам образцов; b – Результаты, полученные после сбора осадка в этаноле с помощью СЭМ
Fig. 4.
a – Distribution of samples by size MB0-MB4; b – SEM results obtained after collecting sediment in ethanol

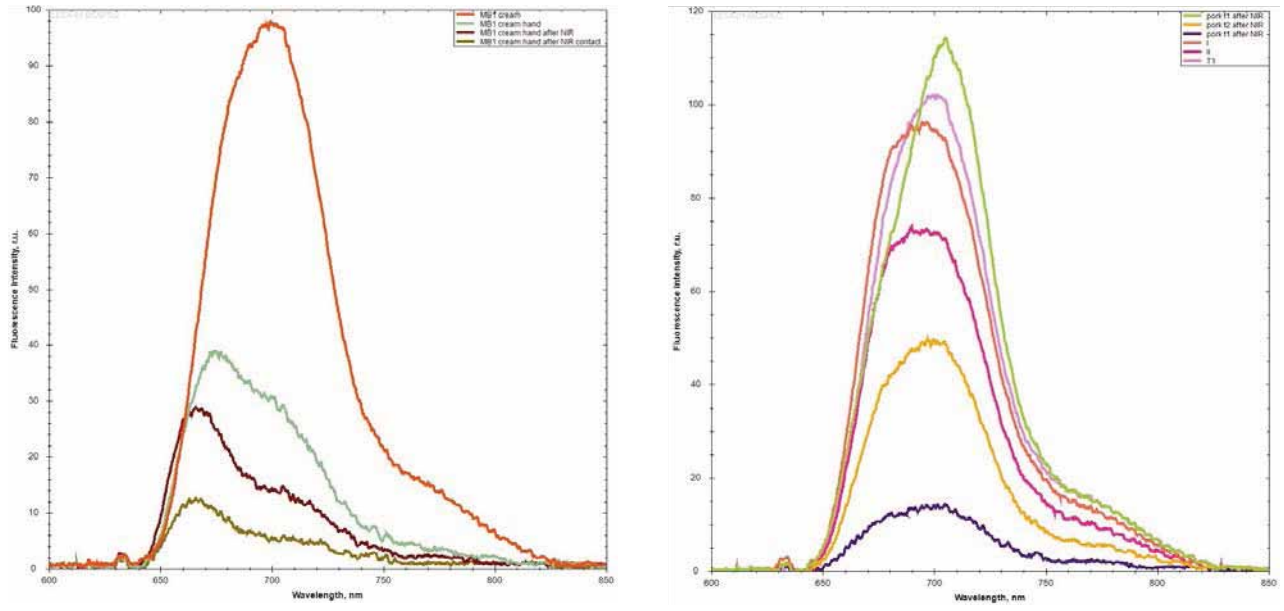
decreases and a local maximum in the basal layer is observed. There is a sufficient concentration of MB at a depth of more than 1.1 mm, which indicates penetration into the dermis. The decrease rate of the MB concentration is significantly lower in comparison with the generally accepted value manifested by the diffuse mechanism of the drug substance distribution. This means that the use of these ethosomal complexes and their stimulation with light significantly increase the depth of MB penetration.

After characterization of the complexes, it was necessary to determine the effective thickness of the cream layer in order to maintain the constancy of the results obtained. In this study, the LESA-01 BIOSPEC system was used. The above histogram shows the results after applying the M3 sample to the arm with a thickness of 0.3, 0.5 and 0.8 mm. The histogram shows that after irradiation in the NIR region of the spectrum, the intensity value decreases from 0.3 to 0.8 mm. Then, after PDT, the fluorescence intensity on the surface and



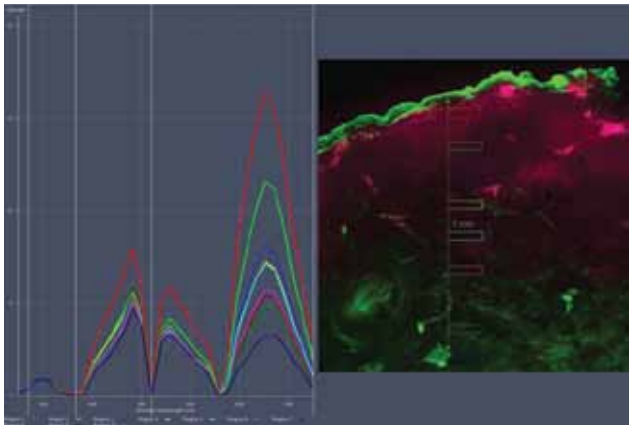
a b

Рис. 6. Зависимость оптической плотности от длины волны для образца МС2 при температуре: a – 27°C; b – 37°C
Fig. 6. Dependence of the optical density on the wavelength for the MB2 sample at an exposure time of 10, 20 and 30 minutes under temperature conditions: a – 27°C; b – 37°C



a b

Рис. 7.
a – спектр флуоресценции образца MB1 cream перед нанесением, спектр флуоресценции образца MB1 cream после нанесения на кожу пациента, спектр флуоресценции образца MB1 cream after NIR на поверхности кожи после облучения в БИК области спектра, спектр флуоресценции образца MB 1 cream hand after NIR contact в глубоких слоях кожи до 2 мм после облучения в БИК области спектра; b – T 1, I, II – спектры флуоресценции образцов, pork t1 after NIR, pork t2 after NIR, pork t3 after NIR – спектр флуоресценции образцов на поверхности кожи свиньи после облучения в БИК области спектра
Fig. 7.
a – MB1 cream sample is the sample fluorescence spectrum before application, after application on patient's skin, after irradiation in the NIR spectral region, after NIR contact is the fluorescence spectrum of the sample in the deep layers of the skin up to 2 mm after irradiation in the NIR region of the spectrum; b – T1, I, II – fluorescence spectra of samples, pork t1 after NIR, pork t2 after NIR, pork t3 after NIR – fluorescence spectrum of samples on the surface of pork skin after irradiation in the NIR spectral region



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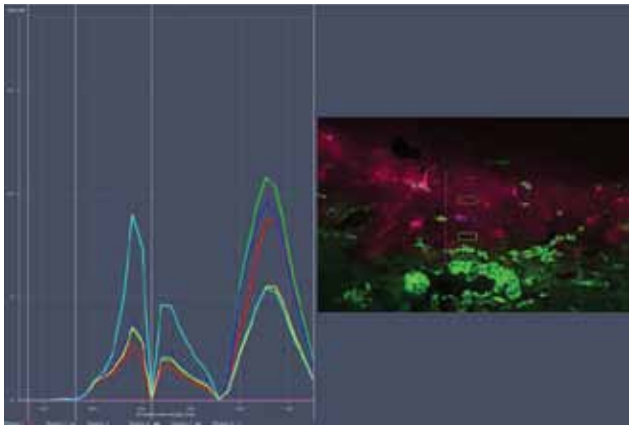

b

Рис. 8.
a – результаты, полученные с помощью LCSM, в области с роговым слоем; b – результаты, полученные с помощью LCSM, в области без рогового слоя
Fig. 8.
a – LCSM results in the area with the stratum corneum; b – LCSM results in the area without the stratum corneum

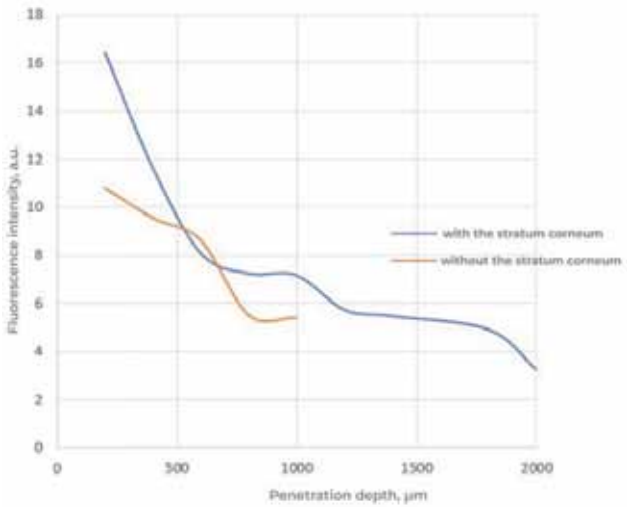

Рис. 9. Зависимость интенсивности флуоресценции от глубины проникновения в роговой слой волны при длине волны 676 нм
Fig. 9. Fluorescence intensity dependence at a wavelength of 676 nm on the penetration depth of the stratum corneum

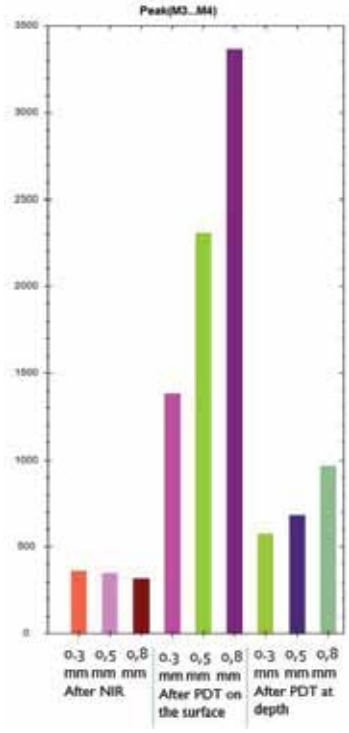

Рис. 10. Гистограмма интенсивности флуоресценции после нанесения крема, облучения в области БИК в течение 20 мин, проведение ФДТ на поверхности и глубине в течение 20 мин
Fig. 10. Fluorescence intensity histogram after applying the cream to the hand and irradiating the NIR for 20 minutes; and conducting PDT for 20 minutes on the surface and at depth

at a depth of up to 2 mm using an optical adapter was assessed. The data after PDT after an increase in laser radiation intensity are presented. According to the data obtained, applying a cream 0.3 mm thick, there is a decrease in accumulation at depth. Therefore, using a cream thickness of 0.3 mm, there is a maximum accumulation at a depth after NIR and maximum photobleaching after PDT.

Conclusion

The research shows that the LESA-01 BIOSPEC system to assess the depth of MB penetration is able, as well as to assess the concentration of accumulated PS in the epidermis and dermis. This system to provide information about the release of PS from vesicles is also able. The effective thickness of the applied preparation was determined. The article presents a comparison of the results of a confocal microscope and the used LESA-01 BIOSPEC system. The LCSM has disadvantages such as labor intensity and the use of animal skin or model samples. The investigation shows the MB introduction from vesicles when working with patient skin and pork skin is different. This is also a plus of working with the LESA-01 BIOSPEC system.

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