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Effect of ethanol on the transport of methylene blue through stratum corneum

Elina A. Genina*, Alexey N. Bashkatov, Valery V. Tuchin

Research-Educational Institute of Optics and Biophotonics, Saratov State University, 83, Astrakhanskaya Street, Saratov 410012, Russian Federation

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Abstract

Diffusion of methylene blue (MB) dissolved in both saline (0.9% NaCl) and 40% ethanol/saline in skin was investigated with reflectance spectroscopy. Experiments have been carried out with rat skin samples *in vitro* at 20 °C. The present studies have shown that 40% concentration of ethanol in solution greatly enhances transport of MB across stratum corneum. The diffusion coefficient of MB in skin *in vitro* has been estimated. The average value of diffusion coefficient is $(2.2 \pm 0.9) \times 10^{-6}\,cm^2/s$.

Keywords: Methylene blue; Skin; Diffusion coefficient; Ethanol

Introduction

Selective enhancement of absorption properties of a target living tissue is widely used for diagnostic purposes as well as for therapy and surgery. Effective change of absorption properties of living tissues can be provided by selective staining using biocompatible dyes. Application of dyes as contrast agents for imaging of blood flows, determination of blood volume, cardiac output, or hepatic function has been known [1,2]. Biocompatible fluorescent dyes are used for visualization of atherosclerotic plaques and localization of hidden tumors [3,4]. Photodynamic therapy also utilizes photosensitizing dyes and light [5–8]. Endogenous and exogenous dyes can be applicable for local increase of tissue absorption, that is used for precise microsurgery [9], selective laser thermolysis of tumors [10] and blood vessels [11,12], treatment of skin and follicle lesions [13,14], hair removal [15,16], etc.

The transdermal transport of dye molecules has become of great interest with the development of the above-mentioned methods. The problem of molecular diffusion through tissue and cell has a prolonged history in the context of drug delivery into different tissues, in particular, into skin with estimation of diffusion coefficient of water and drug in epidermal and dermal tissues [17–20]. The intrinsic routes and mechanisms for transport of drug across the human stratum corneum (SC) and enhancement of molecule's transport by both physical and chemical enhancers of permeation are problems under investigation [21–27]. However, despite numerous investigations related to drug delivery, the problem of estimating dye penetration rate in tissues has not been studied in detail. The knowledge of the diffusion coefficient and other features of diffusion, in particular for widely used photodynamic dye methylene blue (MB), is very important for providing correct dose

*Corresponding author. Tel.: +7 8452 210716; fax: +7 8452 278529.
E-mail address: eagenina@optics.sgu.ru (E.A. Genina).

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of laser radiation during photodynamic therapy, for increasing the effectiveness of laser selective photothermolysis of tumors, for treatment of acne lesions, etc.

MB is a commercially available medical dye. In spite of numerous papers dealt with application of the dye in photodynamic therapy of different diseases [28–34], the MB diffusion coefficient in skin has not been measured yet.

MB molecules are hydrophilic, thus their diffusion across the SC is hindered. Therefore, temporal disruption of skin barrier function may have a result in the enhancement of skin permeability for MB. It is well known that pretreatment of skin by ethanol significantly increases its permeation for polar molecules [35–38]. Based on the concept of ethanol-induced SC permeation, in this study we have investigated penetration of MB dissolved in 40% ethanol/saline into skin samples across epidermis.

Materials and methods

Materials

Ten skin samples were taken from the abdomen area of white rats within an hour after sacrificing. Both hair and subcutaneous fat layers were removed. The area of the samples was about $5 \times 5\,cm^2$. The thickness of each sample was measured at the initial moment with a micrometer in 10 points over the sample surface and averaged. The average value was 0.78 ± 0.02 mm.

MB (Aldrich Chemical Co., USA) was dissolved in both saline (0.9% NaCl) and 40% ethanol/saline solution with a concentration of 1 mg/ml. The transmittance spectra of the solutions with concentration 5 mg/l were measured with a spectrometer (LESA-5, BioSpec, Russia) using a 1 cm quartz cuvette in the spectral range 400–800 nm.

MB is a tricyclic phenothiazine dye with the chemical formula $C_{16}H_{18}ClN_3S$ and a molecular weight of 319.85 [1]. The chemical structure and absorption spectra of MB solutions are presented in Fig. 1a, b.

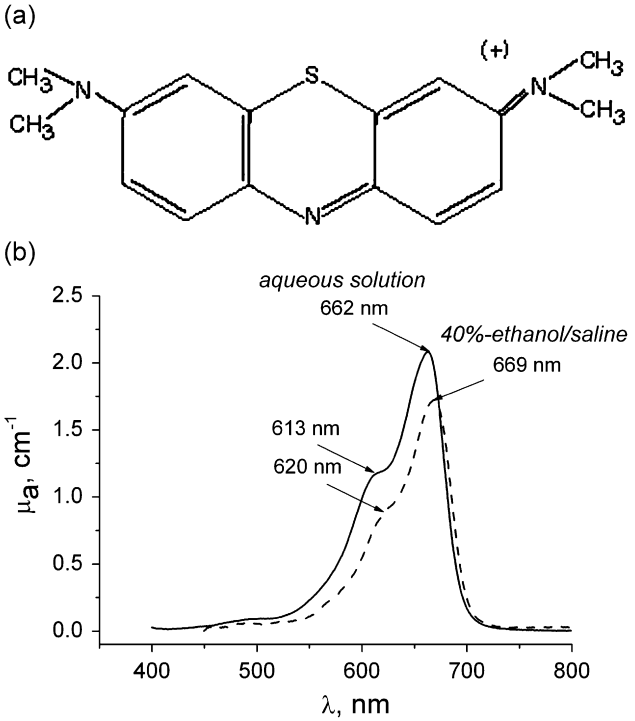

Fig. 1. Chemical structure (a) and absorption spectra (b) of both methylene blue solutions: aqueous (solid line) and in 40% ethanol/saline (dash line). Concentration of the dye in both solutions is 5 mg/l.

Experimental setup

The spectral measurements of skin reflection were performed at room temperature (approx. 20 °C) in the spectral range of 450–1000 nm using a commercially available optical multichannel spectrometer LESA-5 (BioSpec, Russia) with a fiber-optical probe. The scheme of the experimental setup is shown in Fig. 2.

The fiber-optical probe consisted of seven optical fibers. All fibers had a 200-μm core diameter and a numerical aperture of 0.22. The central fiber S (see Fig. 2) delivered incident light to the tissue surface, and six fibers D (the fibers were placed around the central fiber) collected the reflected light. The distance between the delivering and receiving fibers was 290 μm. As a reference, a white slab $BaSO_4$ with a smooth surface was used. For the spectrophotometric measurements, each tissue sample was fixed on the cuvette with a solution of MB. Dye solution penetrated into skin dermis across the SC and epidermis. The reflectance spectra of a sample were measured before administration of MB solution and every 1 min during the first 30–40 min of the interaction. Then, the measurements were carried out each 5 min up to the moment when the changes of the spectrum were negligible.

Measured reflectance spectra were recalculated into the absorbance spectra with the relation

$$A(\lambda) = -\ln(R(\lambda)). \quad (1)$$

Method for estimation of MB diffusion coefficient

The method of estimation of MB diffusion coefficient is based on the time-dependent measurements of the tissue absorbance in the spectral range from 600 to 700 nm, which corresponds to the absorption bands of the dye. These measurements are used to determine the change in the absorption of the tissue due to diffusion of the dye in relation to the initial absorption.

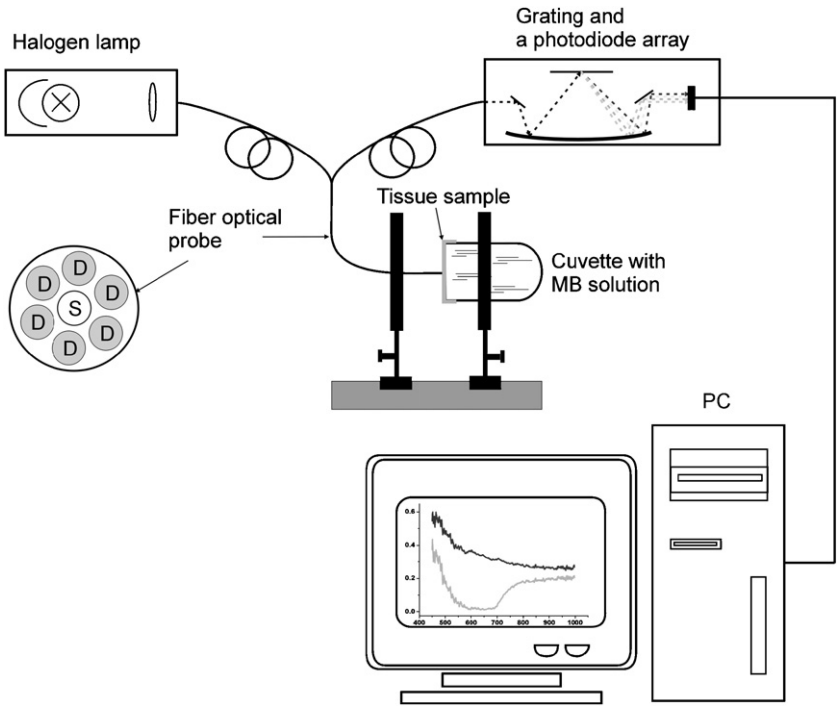

Fig. 2. Experimental setup for measurements of the spectral characteristics of skin. S is the source of irradiation, D are detectors.

The transport of MB within the tissue is described in the framework of the free diffusion model. We assume that the following approximations are valid for the transport process:

1. only concentration diffusion takes place; i.e., the flux of the dye into the tissue, at a certain point within the tissue sample, is proportional to the MB concentration at this point and
2. the diffusion coefficient is constant over the entire sample volume.

Geometrically, the tissue sample is presented as a plane–parallel slab with a finite thickness. Since lateral dimensions of the experimental samples were much bigger than their thickness, and lateral sides were fixed (not connected with the solution), the one-dimensional diffusion problem has been solved. The one-dimensional diffusion equation of the dye transport has the form (Fick's second law)

$$\frac{\partial C(x,t)}{\partial t} = D\frac{\partial^2 C(x,t)}{\partial x^2}, \tag{2}$$

where $C(x, t)$ is the MB concentration (g/ml), D is the MB diffusion coefficient (cm^2/s), t is the time (s), and x is the spatial coordinate (cm).

We also suppose that penetration of MB into the tissue sample does not change the dye concentration in the external volume. Besides, due to geometry of the measurements, penetration of the MB into the tissue sample takes place from the top surface of the tissue sample only. The corresponding boundary conditions are

$$C(0,t) = C_0 \quad \text{and} \quad \frac{\partial C(l,t)}{\partial x} = 0, \tag{3}$$

where C_0 is the MB concentration in external solution (g/ml), and l is the tissue sample thickness (cm).

The initial condition corresponds to the absence of MB inside the tissue before the measurements

$$C(x,0) = 0 \tag{4}$$

for all inner points of the tissue sample.

Solution of Eq. (2) for a slab with a thickness l at the moment t with the boundary (Eq. (3) and the initial Eq. (4)) conditions has the form

$$C(t) = C_0\left(1 - \frac{8}{\pi^2}\sum_{i=0}^{\infty}\frac{1}{(2i+1)^2}\exp\left(\frac{-(2i+1)^2 t\frac{\pi^2}{4}D}{l^2}\right)\right), \tag{5}$$

where $C(t)$ is the volume-averaged concentration of MB within the tissue sample.

For the determination of MB diffusion coefficient in tissue, the approach suggested by Mourant et al. [39] has been used. The method is based on the use of modified Lambert–Beer law and, in this case, tissue absorbance can be determined as

$$A = \mu_\alpha \sigma \rho + G, \tag{6}$$

where μ_α is the absorption coefficient, ρ is the source–detector distance, σ is the differential factor of photon path length, taking into account the lengthening of the photon trajectories due to multiple scattering, and G is the constant defined by geometry of the experiment. To simplify calculations, $\rho\sigma$ can be replaced by the parameter L, which is defined by both absorption and scattering tissue properties and source–detector distance. Since in this study the distance (290 μm) is commensurable with photon free path-length, parameter L is defined by tissue scattering properties only [40–42].

Penetration of MB into tissue increases tissue absorbance in the spectral range corresponding to the absorption bands of the dye. Thus, the tissue absorbance measured in different time intervals can be determined as

$$A(t,\lambda) = A(t=0,\lambda) + \Delta\mu_\alpha(t,\lambda)L, \tag{7}$$

where t is the time interval, λ is the wavelength, $\Delta\mu_\alpha(t, \lambda) = \varepsilon(\lambda)C(t)$ is the absorption coefficient of MB within tissue, $\varepsilon(\lambda)$ is the MB molar absorption coefficient, $C(t)$ is the MB concentration in tissue, and $A(t = 0, \lambda)$ is tissue absorbance measured at the initial moment.

Thus, the equation

$$\Delta A(t,\lambda) = A(t,\lambda) - A(t=0,\lambda) = \Delta\mu_\alpha(t,\lambda)L = \varepsilon(\lambda)C(t)L \tag{8}$$

can be used for calculation of the MB diffusion coefficient.

This set of equations represents the direct problem, i.e., describes the temporal evaluation of the absorbance of tissue sample dependent on the MB concentration within the tissue sample. Based on the measurement of the evolution of the tissue absorbance, the calculation of the MB diffusion coefficient in tissue has been carried out. The inverse problem solution has been obtained by minimization of the target function

$$F(D) = \sum_{i=1}^{N_t} (\Delta A(D,t_i) - \Delta A^*(t_i))^2, \tag{9}$$

where $\Delta A(D, t)$ and $\Delta A^*(t)$ are the calculated (Eq. (8)) and experimental values of the time-dependent absorbance, respectively, and N_t is the number of time points obtained at registration of the temporal dynamics of the absorbance. To minimize the target function, the Levenberg–Marquardt nonlinear least-squares-fitting algorithm described in detail by Press et al. [43] has been used. The iteration procedure is repeated until experimental and calculated data are matched.

Results and discussion

Figs. 3 and 4 demonstrate the wavelength dependence of skin reflectance in the process of skin interaction with MB in saline and 40% ethanol/saline solutions, respectively, in different time intervals. Fig. 3 shows that in 200 min the change of skin spectra at the interaction of MB dissolved in saline with SC is insignificant.

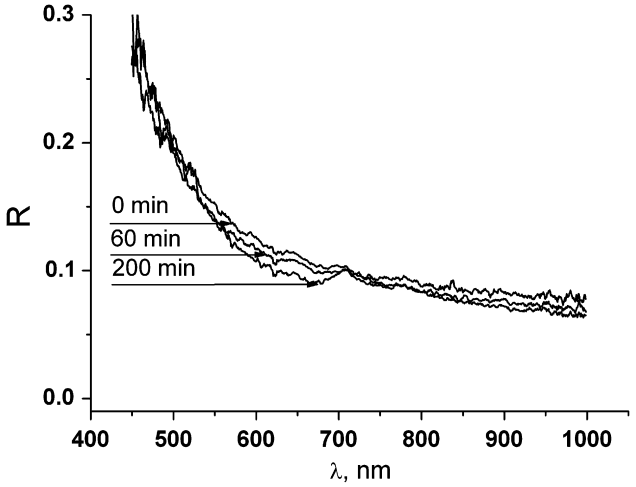

Fig. 3. The wavelength dependence of skin reflectance measured for different time intervals of methylene blue (MB) diffusion through SC. The studied solution is MB in saline.

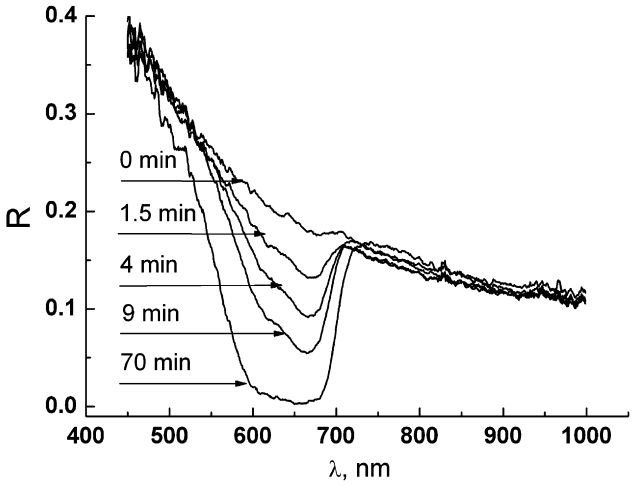

Fig. 4. The wavelength dependence of skin reflectance measured for different time intervals of methylene blue (MB) diffusion through SC. Studied solution is MB in 40% ethanol/saline.

Fig. 4 shows the change of skin spectra during penetration of MB dissolved in 40% ethanol/saline through SC and epidermis into the dermis. It is well seen that in the spectral range from 600 to 700 nm, corresponding to absorption bands of MB, skin reflectance decreases while MB penetrates into the tissue.

Fig. 5 presents differential absorbance spectra (see Eq. (8)) of one of the skin samples at different moments of its interaction with the dye. In the figure it is also seen that penetration of the dye into the tissue does not

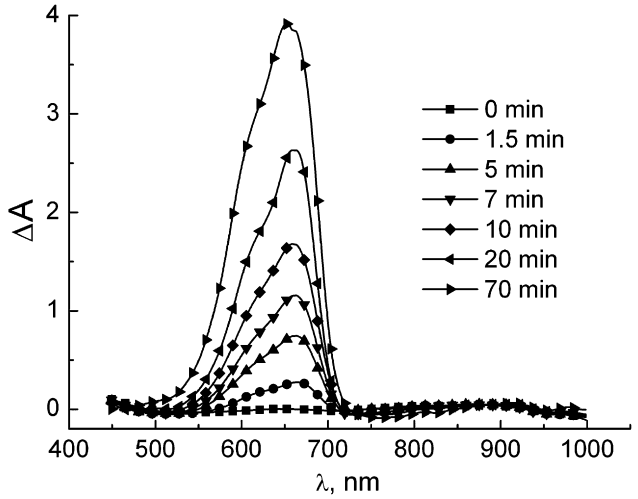

Fig. 5. The wavelength dependence of skin differential absorbance obtained for different time intervals of methylene blue (MB) diffusion. Studied solution is MB in 40% ethanol/saline.

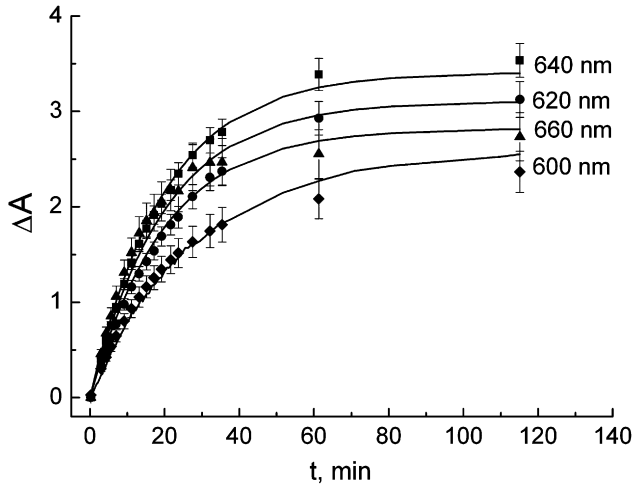

Fig. 6. Temporal dynamics of methylene blue (MB) differential absorbance measured (symbols) and calculated (solid lines) for different wavelengths. Studied solution is MB in 40% ethanol/saline.

change the scattering properties of the skin. It follows from the absence of change in the absorbance spectra in the range 750–1000 nm, where MB has no absorption bands, and the form of the spectra is determined by spectral dependence of the scattering coefficient.

Fig. 6 presents the calculated and measured dynamics of dye differential absorbance ΔA for different wavelengths. From the figure it is seen that the optimal staining time was about 60 min. Then the dye diffusion slowed down, and no absorbance change was observed.

Based on the measurements of the temporal evolution of the absorbance, the evaluation of the MB diffusion coefficient in the skin tissue has been carried out. Taking into account the thickness of the skin samples, diffusion coefficient of MB was obtained from the analysis of experimental curves by the least-squares method. The value $\varepsilon C_0 L$ was estimated from the linear region of temporal dependence of ΔA, which corresponded to complete saturation of its change. Since scattering of skin did not change during diffusion of MB into the tissue, L was considered as a constant. Calculations were made for ten wavelengths in the range 600–700 nm for each sample, and the obtained values were averaged. The mean value of the diffusion coefficient was $(2.2 \pm 0.9) \times 10^{-6}\,\mathrm{cm^2/s}$.

Depending on the physical and chemical properties of the dyes both diffusion rate and degree of staining can vary. The absorption spectrum of a phototherapy drug can also change when the drug becomes localized in the tissue and binds to cell proteins. Since MB is a weak organic electrolyte, it can be concluded that during penetration of the MB solution into the tissue the interaction between the dye ions and skin proteins is taking place. The character of dye penetration can be explained based on the analysis of the skin structure.

It is well known that diffusion of aqueous solutions of substances through SC barrier is hindered. The SC is between 10–20 μm thick. It contains the corneocytes or horny cells, which are closely packed flat non-nucleated cells, approximately 40 μm in diameter and 0.5 μm thick. Polar structures such as corneodesmosomes contribute to SC cohesion [44]. Corneocytes are embedded in a lipid bilayer matrix. The intercellular lipids are required for a competent skin barrier and form the only continuous domain in SC [44,45]. Appendages such as hair follicles and sweat glands cover approximately 0.1% of the total skin surface [46,47].

Thus, the heterogeneous nature of SC provides some possible pathways for solute transport: appendageal, transcellular (through both corneocytes and lipid bridges) and intercellular (through the lipid phase only) [47]. Lipophilic and polar permeants are transported via the lipoidal and pore pathway of SC, respectively [37].

To analyze drug penetration across the skin barrier, a two-layer model of skin was suggested, where SC barrier is in series with the epidermis/dermis layer, and where the SC is represented by a lipoidal pathway in parallel with a pore pathway [37,38,48,49]. The pathway for water diffusion across SC has been calculated to be 50-fold longer than the thickness of SC. This suggests that permeation follows a tortuous path through the intercellular lipid matrix [44,45]. The penetration of compounds into the corneocytes also takes place since immersion of skin leads to swelling of the corneocytes connected with the entry of water [44]. Thus, structure and composition of SC provides the diffusion coefficient, which is 10^3-fold less than that observed for cellular membranes [47]. It has been found that the diffusion coefficient of water through SC varies from 2.5×10^{-10} to $8.34 \times 10^{-10}\,\mathrm{cm^2/s}$ depending on the

humidity of SC [17]. Diffusion of MB in SC is supposed to be also slow. Besides tortuosity of the pathway through the lipid barrier in the process of penetration, diffusion of the dye in the tissue is accompanied by interaction with the tissue: the electrostatic interaction between the cationic dye and the negatively charged polymers on the cell surface that also decreases diffusion rate. The possible cause of diffusion deceleration is dimerisation of adjacent dye molecules bounded to the anionic site of polymers and formation of bound dimers on the cell surface [6,50]. The absence of changes in the reflectance spectra of skin during its interaction with MB solution in saline (Fig. 3) shows that SC significantly hinders diffusion of the dye into skin. During 200 min we did not see absorption bands of MB. From estimations presented in papers [51,52] the diffusion coefficient of MB in dermis is $\sim 10^{-8}\,cm^2/s$, whereas typically values of diffusion coefficients of dyes in solutions fall within the range of 10^{-5}–$10^{-6}\,cm^2/s$ [53,54].

Addition of ethanol to the dye solution induces significant increase in the diffusion rate. Ethanol is a solvent known to modify the skin barrier property. The influence of ethanol on the *in vitro* transport behavior of some polar/ionic permeants in hairless mouse skin has been investigated over different ethanol/saline concentrations in a few papers [37,38,48,49]. At high concentrations (~40%) ethanol greatly enhances pore transport due to bigger pores and/or pore density of the epidermal membrane at alcohol action. It can be explained by altered or additional pore/polar pathways, which may be formed as a result of a combination of changes in the protein conformation, reorganization within the lipid polar head regions or lipid extraction [35,36]. Lipid extraction may take place in conjunction with and/or independently of conformational alterations with protein domains [37]. The obtained value of MB diffusion coefficient is $(2.2 \pm 0.9) \times 10^{-6}\,cm^2/s$, which significantly exceeds the value of water diffusion coefficient in SC and is comparable with the diffusion coefficients of water and some solutions in the dermis ($\sim 10^{-6}\,cm^2/s$) [49].

The character of interaction of the dye solution with tissue can also be significantly changed by ethanol addition to the solution. It is confirmed by the comparison of MB spectra measured at staining of cell structures (bacteria, mucous tissue) with MB saline presented in Refs. [6,50], with MB spectra measured at staining of cell structures (epidermis) with MB 40% ethanol/saline presented in this study. The main absorption peak of the MB in 40% ethanol/saline solution occurs at 669 nm (see Fig. 1). The peak with maximum at 669 nm prevails over another peak (~620 nm) that corresponds to the monomer form of MB molecules [6]. During dye diffusion through epidermis, concentration of the dye in the area of probing increases. However, the decrease of monomer peak (hypochromic effect) as well as shift of dimer peak towards the shorter wavelengths of the spectrum (hypsochromic effect), which were observed at the interaction of MB saline with cells [6,50], did not occur. Hypsochromic effect was observed for the monomer form of the dye. The main maximum of MB spectrum shifted to shorter wavelengths from 665 nm (in 1.5 min) to 655 nm (in 70 min). Thus, dimerization of the dye did not take place, which is related, apparently, into the influence of ethanol.

Conclusion

We have presented experimental results on the study of the penetration of MB dissolved in both saline and 40% ethanol/saline into skin through SC of epidermis. Addition of ethanol to the dye solution induced significant change of character of interaction of the dye solution with tissue and increased the diffusion rate. It can be due to altered or additional pore/polar pathways, which may be formed as a result of a combination of changes in protein conformation reorganization within the lipid polar head regions or lipid extraction. The diffusion coefficient of MB in skin through SC *in vitro* has been estimated as $(2.2 \pm 0.9) \times 10^{-6}\,cm^2/s$. The results can be important for correction of the laser-radiation dose at photodynamic therapy, for effective application of laser selective photothermolysis of stained tumors, or acne lesion treatment, etc.

Acknowledgments

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Zusammenfassung

Effekt von Alkohol auf den Transport von Methylenblau durch das Stratum Corneum

Die Diffusion von Methylenblau, gelöst in physiologischer Kochsalzlösung (0,9% NaCl) und in einer 40%-igen Alkohol/Kochsalzlösung, wurde mit Hilfe

der Reflexions-Spektroskopie untersucht. Die Experimente wurden *in vitro* bei 20 °C mit Rattenhaut durchgeführt. Die vorliegenden Untersuchungen haben gezeigt, dass eine Alkoholkonzentration von 40% in Lösung den Transport von Methylenblau durch das Stratum Corneum wesentlich verbessert. Der Diffusionskoeffizient von Methylenblau in Haut *in vitro* wurde bestimmt. Der mittlere Wert des Diffusionskoeffizienten beträgt $(2.2 \pm 0.9) \times 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$.

Schlüsselwörter: Methylenblau; Diffusionskoeffizient; Haut; Alkohol

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