Effect of ethanol on the transport of methylene blue through the rat skin ex vivo

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ABSTRACT

The main goal of the study is to investigate the effect of ethanol aqueous solutions with volume concentrations 30, 40, and 50% on the transport of Methylene Blue (MB) in rat skin *ex vivo*. The concentration dependences of MB absorbance in optical phantoms mimicking skin optical properties were measured. It has been obtained, for skin-like optical phantoms with low MB concentration (less than 0.025 mg/mL) the concentration dependence of MB absorption coefficient can be taken as linear in a first approximation. The study has shown that the diffusion rate grows with the increase of ethanol concentration in the solutions. The rate is 1.6 folds higher for 50%-ethanol solution of MB than when aqueous solution of MB is used. Effective diffusion coefficients of MB in skin samples have been evaluated as $(3.34\pm0.07)\times10^{-6}$ cm^2/sec, $(3.04\pm0.07)\times10^{-6}$ cm^2/sec, $(2.59\pm0.07)\times10^{-6}$ cm^2 /sec, and $(1.85\pm0.06)\times10^{-7}$ cm^2/sec for 50%, 40%, 30% ethanol solutions and aqueous solution, respectively. The characteristic time has the following values: 24.6±4.4 min, 40.1±3.2 min, 46.8±2.7 min, and 79.4±11.9 min for 50%, 40%, 30% aqueous ethanol solutions and aqueous solution, respectively.

Keywords: Methylene Blue, skin, skin-like optical phantoms, diffusion coefficients, ethanol solutions

1. INTRODUCTION

Nowadays dyes are used in a wide class of medical supplies in optical methods of diagnostics, therapy and surgery. It is explained by their ability to absorb light of certain wavelengths. Consequently, selective enhancement of absorption properties can be reached by dyeing a specific area of tissue.

Methylene Blue (MB) is widely used in various areas of medicine because of its biocompatibility, commercially availability and safety. In response to its fluorescent properties MB is often applied in visualization techniques[1-3] and as a photosensitizer in photodynamic therapy for the treatment of cancer diseases[4]. MB is also used in photoinactivation of bacteria[5-7] and virus inactivation in donor blood[8].

The diffusion coefficient of MB in aqueous solutions was evaluated as $(6.69\pm0.42)\times10^{-6}$ cm^2/s [9], and 4.6×10^{-6} cm^2/s [10]. However, despite of MB application popularity in medicine, the diffusion of MB in tissue has not been developed sufficiently. Particularly, the features and rate of MB diffusion can differ in different tissues. This knowledge is very important for definition of necessary dyeing time and for providing correct dose and optimal exposure wavelengths. Previous works[11-13] have only focused on transport of MB in 40%-ethanol solution through stratum corneum and MB in saline in mucous and dermis. It has been shown an increase of diffusion coefficient with the presence of ethanol in the saline solution.[11] For a more thorough study of this issue, it is necessary to perform a series of measurements with different concentrations of ethanol in the MB solution.

This study analyses the impact of ethanol with different concentrations in the MB aqueous solution on the dye transport in rat skin *ex vivo* and the evaluation of the characteristic time of diffusion and effective diffusion coefficient.

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2. MATERIALS AND METHODS

2.1 Methylene Blue properties

MB belongs to phenothiazine dye group with the chemical formula $C_{16}H_{18}ClN_3S$ and a molecular mass of 378.898 and molar weight 319.85 g/mol.[14] Figure 1 shows the MB chemical structure.

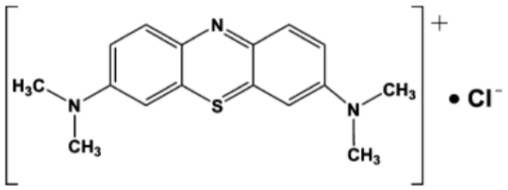

Figure 1. Chemical structure of MB+•Cl- molecule.

2.2 The optical phantoms preparation

For modeling optical properties of skin, the optical phantoms (OPs) were prepared on the base of 15% aqueous solution of a food gelatin. Scattering properties of the skin were simulated by adding a titanium dioxide (Sigma-Aldrich, USA) with nanoparticles diameter of about 100 nm into the gel. The concentration of nanoparticles in the gel was 1.5 mg/mL. MB concentration in phantoms was 0, 0.0125, 0.025, 0.05, 0.1 and 0.2 mg/mL.

2.3 Skin samples and solutions preparation

Skin samples were taken from the hip area of white laboratory rats within an hour *postmortem*. Both hair and subcutaneous fat layers were thorough removed. Sample thickness was measured before and after MB solution penetration using an electronic micrometer with an error ± 10μm.

MB was dissolved in aqueous 50%-, 40%-, 30%-ethanol solutions and in distilled water with a MB concentration 0.5 mg/mL.

2.4 The experimental setup

The experimental setup is shown in Figure 2. OP was fixed directly on the sample port of integrating sphere (ISP-80-8-REFL, Ocean Optics, USA). Tissue sample was fixed on a cylindrical cuvette with a solution of MB. Dye solution penetrated into the skin through the dermis. From the opposite side the sample contacted with a sample port of integrating sphere. The reflectance spectra were measured with a spectrometer USB4000-Vis-NIR (Ocean Optics, USA) in the spectral range 400-1000 nm. As a light source a halogen lamp HL-2000 (Ocean Optics, USA) was used. The measurements of diffusion reflectance were carried out each 5 min until the stop changing in the spectral shape.

The optimal dyeing time was reached for approximately 5-6 hours for aqueous solution, 2.5-3 hours for 50%-ethanol solution, and 3-4 hours for 40%-and 30%-ethanol solutions of MB.

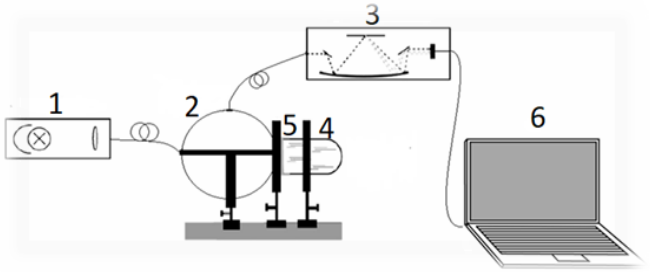

Figure 2. Experimental setup for measurements of the spectral characteristics of skin and skin-like phantoms: 1-halogen lamp, 2-integrating sphere, 3-spectrometer, 4-cuvette with solution MB, 5-tissue sample or phantom, 6-PC.

It is known, that MB has two absorption peaks for dimeric (~ 610 nm) and monomeric (~ 660 nm) forms.[15] The ratio of dimer and monomers changes in dependence on the type of the solvent and concentration of the dye in solution. In aqueous solution the dimeric form prevails. In Fig. 3 it is seen that peak with maximum at the wavelength 603 nm prevails over another peak at 663 nm. The ethanol addition induces the increase of monomers concentration in the

solutions. The greatest value of the absorption coefficient of the monomeric form of MB is observed in 50%-ethanol solution.

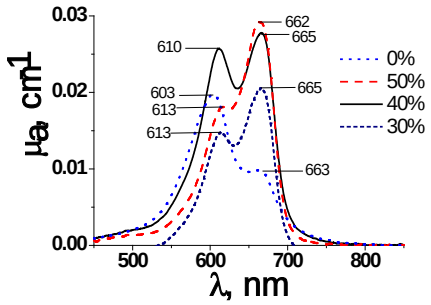

Figure 3. Absorption spectra of methylene blue in different aqueous ethanol solutions.

2.5 Effective diffusion coefficient estimation

Estimation of effective diffusion coefficient was performed on the base of measurement and analysis of skin diffuse reflectance under action of dye solution.

For a quantitative assessment of changes in the content of the dye in tissue, we used method based on the use of the modified Bouguer-Lambert-Beer law, according to which the values of effective absorbance (A) are calculated by the formula (followed by the calculation of the differential absorbance (ΔA)):

$$A = -\ln\left(R\right), \tag{1}$$

where R is skin reflectance.

Analysis of the kinetics of this process allows us to determine the diffusion rate of the dye in the medium. The study of the transport of MB through the skin can be carried out in the framework of the free diffusion model using the 2nd Fick law; in the one-dimensional case has the form:

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

where $C(x,t)$ is dye concentration in tissue sample, D is diffusion coefficient, t is time, x is spatial coordinate over tissue sample thickness.

Since the volume of the MB solution significantly exceeded the volume of the skin sample, the boundary conditions were as follows:

$$C\left(0,t\right) = C_0 \qquad \text{и} \qquad \frac{\partial C\left(l,t\right)}{\left(\partial x\right)} = 0, \tag{3}$$

where C_0 is concentration of the dye in solution, l is the tissue sample thickness. The initial condition reflects an absence of MB at all internal points of the skin sample before its incubation into the MB solution, i.e.

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

The solution of the equation (2) with boundary (3) and initial (4) conditions is:

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

The average concentration of MB inside the skin sample $C(t)$ in every time moment is:

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

or in the first-approximation:

$$C(t) = C_0\left(1 - \frac{8}{\pi^2}\exp\left(-\frac{t\pi^2 D}{4l^2}\right)\right), \tag{6}$$

Value of differential absorbance can be calculated using equation [12]:

$$\Delta A(t,\lambda) = \mu_a^{MB}(t,\lambda)l = \mu_{a0}^{MB}(t,\lambda)C_0\left(1 - \frac{8}{\pi^2}\exp\left(-\frac{t\pi^2 D}{4l^2}\right)\right)l, \tag{7}$$

where μ_a^{MB} is absorption coefficient MB in tissue, μ_{a0}^{MB} is absorption coefficient MB at concentration 1 mg/mL.

Effective diffusion coefficient *D* was determined by minimization of the target function:

$$f(D) = \sum_{i=1}^{N}\left(\Delta A(D,t_i) - \Delta A^*(t_i)\right)^2,$$

where $\Delta A(D,t)$ and $\Delta A^*(t)$ are the calculated and experimental values of the time-dependent absorbance, respectively, and *N* is the number of time points obtained at registration of the kinetics of the absorbance. To minimize the target function the Levenberg-Marquardt nonlinear least-squares-fitting algorithm described in detail by Press et al.[16] has been used. Iteration procedure repeats until experimental and calculated data are matched.

3. RESULTS AND DISCUSSION

Figure 4 shows the concentration dependence of differential absorbance of the OP. The figure shows that the measured differential absorbance increases nonlinearly with the increase of concentration of MB. A sharp increasing Δ*A* with MB concentration rise from 0 to 0.05 mg/mL is observed. With further increase of the concentration up to 0.2 mg/mL, the rate of the change of Δ*A* decreases, because OP absorbs the most part of the incident light. However, in a first approximation, at low MB concentrations in OP (up to 0.025 mg/mL), the concentration dependence of Δ*A* can be considered as linear. Obtained linear dependence of Δ*A* on the dye concentration confirms the applicability of the proposed method of effective diffusion coefficient estimation in tissues in this concentration range.

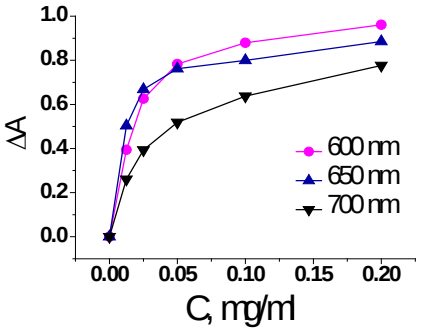

Figure 4. The concentration dependence of the measured MB differential absorbance of optical phantoms at three wavelengths at the absorption bands of MC.

Figure 5 demonstrates the spectral dependence of diffuse reflectance in different moments in the process of skin dyeing with aqueous and three ethanol solutions of MB. It is well seen that in the spectral range corresponding to the absorption

bands of MB (see fig. 3) skin reflectance decreases during MB penetration into the skin. The rate of the process gradually reduces and then it stops. Process completion time is different for different solutions: for aqueous 50%-ethanol solution it is about two hours, and for aqueous solution it is more than five hours. Besides, the process of penetration of MB in the tissue is accompanied by dimerization of dye molecules.

The differential absorbance spectra obtained for different time intervals is shown in Figure 6. The increase of the parameter during the dyeing can be clearly seen. Maximal values of ΔA in skin is not exceed 0.6 that corresponds to concentration of MB inside the skin-like phantom lower than 0.025 mg/mL at the spectral range 600-650 nm. Thus, it can be considered that concentration dependence of the dye absorption for skin lies within the linear range (fig. 4).

Figure 6 allows observation features of diffusion of MB in dermis when different solutions were used. For example, in the course of dye diffusion in dermis amount of dimer molecules increased significantly when aqueous MB solution was used. It is in a good agreement with the results presented in Ref. 17: with increasing MB concentration in bacterial suspension the ratio of the dimer to the monomer absorbance of the dye increased in a gradual manner. This effect was observed for saline solution of MB. The electrostatic interaction between the cationic dye MB and the negatively charged polymers on the bacterial cell surface induced the dimerization of adjacent dye molecules bound to the anionic site of polymers and the formation of bound dimers on the bacterial cell surface. Similar effect, apparently, took place at the interaction of MB molecules with proteins of dermis. When aqueous solution of MB was used the ratio of differential absorbance of monomers and dimmers decreased from 1.83 at 60 min to 1.4 at 180 min. It is noteworthy that the addition of ethanol in the solutions changes the ratio of dimers and monomers in tissue. The peaks of monomer and dimer forms are substantially equal in MB solution with equal parts of water and ethanol.

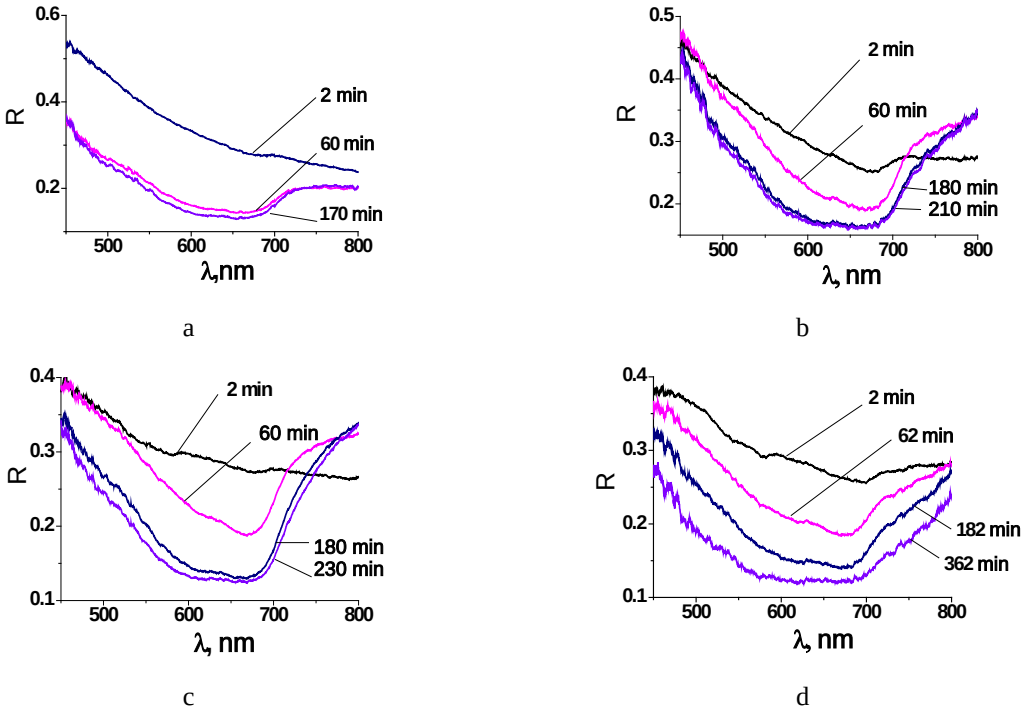

Figure 5. Spectra of diffusion reflectance of skin measured for different time intervals of MB diffusion through dermis. MB in aqueous 50% (a), 40% (b), 30% (c) -ethanol and aqueous (d) solutions.

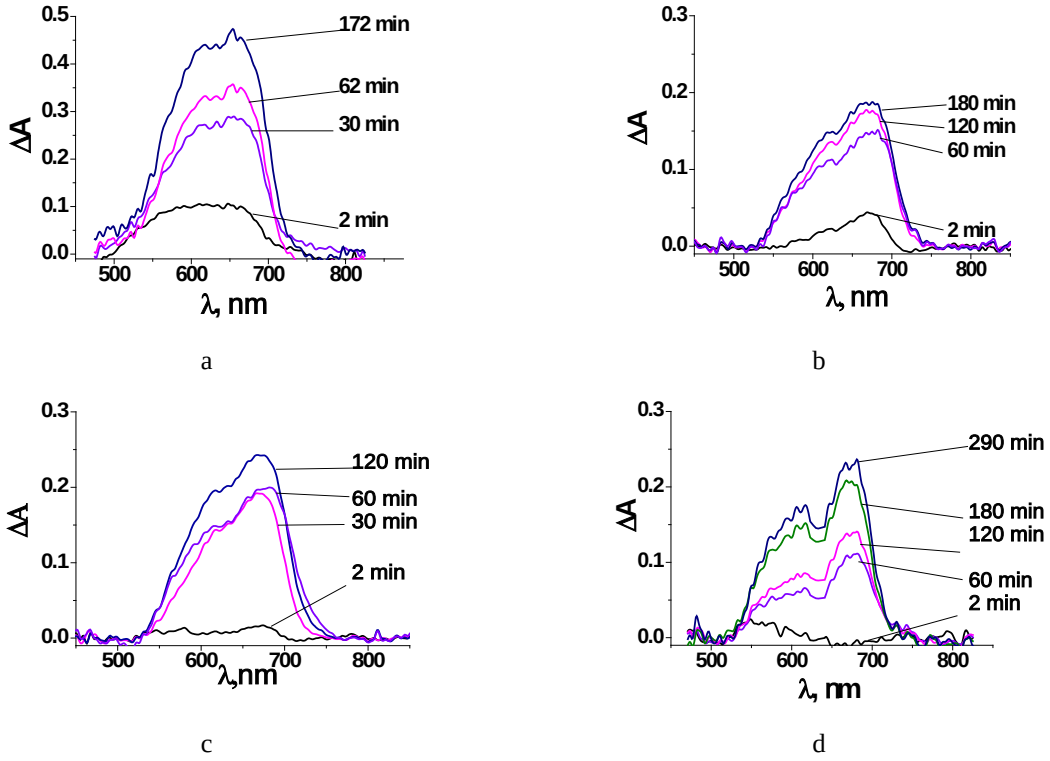

Figure 6. Differential absorbance spectra obtained for different time intervals of MB diffusion in aqueous 50% (a), 40% (b), 30% (c) -ethanol and aqueous (d) solutions.

Figure 7 demonstrates kinetics of skin differential absorbance at the wavelength 669 nm in aqueous solutions with ethanol concentrations 50%, 40%, 30%, and 0%.

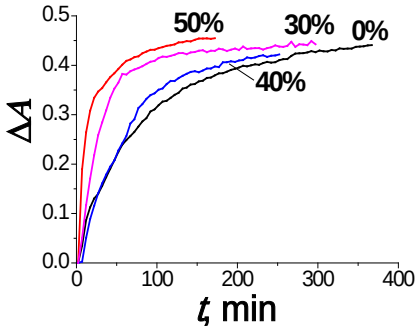

Figure 7. The calculated kinetics of MB differential absorbance at the wavelength 669 nm in aqueous solutions with ethanol concentrations 50%,40%, 30%, and 0%.

Analysis of Δ*A* kinetics has allowed evaluation of effective diffusion coefficient of MB (*D*) in skin and characteristic time (τ) of the dyeing process. Characteristic time corresponds to a relative change in the value of Δ*A* in *e* folds. Calculations for each sample have been performed in the spectral range 600-700 nm at five wavelengths. Obtained values have been averaged. The mean value and standard deviation, averaged for all the samples, and characteristic time are shown in Table 1.

Table 1. The average value of the effective diffusion coefficient (D) and the characteristic time (τ) of skin samples dyeing using the aqueous solutions with ethanol concentrations 50%, 40%, 30%, and 0%.

Ethanol concentration in aqueous solution, %	Sample thickness, mm	D, cm^2/sec	τ, min
50	0.51±0.018	$(3.34±0.07)×10^{-6}$	24.6±4.42
40	0.61±0.026	$(3.04±0.07)×10^{-6}$	40.1±3.16
30	0.49±0.016	$(2.59±0.07)×10^{-6}$	46.8±2.7
0	0.65±0.022	$(1.85±0.06)×10^{-7}$	79.4±11.92

The analysis of obtained diffusion coefficient values shows that ethanol addition into the aqueous solution of MB leads to the increase of dyeing rate of the skin. Effective diffusion coefficient achieves a maximal value for 50%-ethanol solution, and the minimal one for aqueous solution. It can be explained by ability of ethanol to enhance the tissue permeability.

4. CONCLUSION

In this study the transport of methylene blue in a rat skin in aqueous ethanol solutions with concentrations of ethanol 50%, 40%, 30%, and 0% has been studied. It has been obtained for skin-like optical phantoms with low MB concentration (less than 0.025 mg/mL) that the concentration dependence of MB absorption coefficient can be taken as linear in a first approximation. This has allowed estimating effective diffusion coefficient of MB in skin. Values of the effective diffusion coefficient of the MB solutions have been evaluated as $(1.85±0.06) ×10^{-7}$ cm^2/c in aqueous solution, $(3.34±0.07) ×10^{-6}$ cm^2/c, $(3.04±0.07) ×10^{-6}$ cm^2/c, and $(2.59±0.07) ×10^{-6}$ cm^2/c in 50%-, 40%-, and 30%-ethanol solutions, respectively. The average values of the characteristic time of skin samples dyeing have been evaluated as (24.6±4.42) min, (40.1±3.16) min, (46.8±2.7) min and (79.4±11.92) min in 50%-, 40%-, 30%-ethanol and aqueous solutions, respectively. Results of evaluation of the effective diffusion coefficient values have shown that addition of ethanol into the dye solution has led to a noticeable increase diffusion rate of the dye.

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