In vitro study of Methylene Blue diffusion through the skin tissue

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ABSTRACT

We present experimental results on study of Methylene Blue penetration into skin using a method of digital analysis of color images. Experiments were carried out with a rat skin *in vitro*. Dependence of the penetration depth of Methylene Blue on dyeing time of the tissue was researched. Diffusion coefficients of Methylene Blue within the skin *in vitro* were estimated.

Keywords: digital analysis of images, Methylene Blue, skin, diffusion coefficient

1. INTRODUCTION

Digital imaging is a method whereby images are represented by a series of numbers. Each number usually represents a measure of energy reflected from a tiny elemental portion of the structure that is being imaged. In two-dimensional imaging, this tiny picture element is called a pixel, is usually rectangular, and is displayed as a single dot in digital image. Digital images can be obtained by an analog video camera whose signal can be converted to a series of numbers using a video frame grabber computer board. The equipment used to capture an image determines the spatial resolution of the resulting digital image.

Color resolution (pixel depth) refers to the number of bits of information that are used to represent either the number of shades of gray or number of colors that each pixel can represent. Eight bits of information can represent 256 shades (0 – darkness; 255 - brightest) for each of three primary colors (red, green, and blue).

The spatial and spectral distributions of lighting are technical factors that are very important to standardize in most imaging applications. The degree to which spatial, temporal, or spectral variation in lighting will affect an imaging project depends directly on the types of information that the digital images are recording, the goals of the applications in which those digital images are going to be used, and the robustness of the imaging methods that are used.[1]

Digital imaging methods have been applied to evaluate the clinical morphology of different skin lesions (pigmentation, psoriasis, erythema, etc.), measurement of hair growth, wound healing, burn management, etc.[1-5]

Dyes have wide application as in diagnostic as in therapeutic methods of the modern medicine. It is well known the application of dyes as contrast agents for imaging of blood flows, determination of blood volume, cardiac output, or hepatic function, visualization of atherosclerotic plaques, and localization of hidden tumors.[6-9] Special photosensitive dyes are used for damage of tumor cells in photodynamic therapy.[10-17] They can be activated by light to an excited state, which in turn activates oxygen to yield oxidizing radicals. Such radicals kill pathological cells.[17] Dye application for local increase of tissue absorption is widely use for selective laser thermolysis of tumors, treatment of skin and follicle lesions (for example, acne vulgaris), and hair removal.[21-29]

Usually dyes are injected into blood or tissue, or administered topically. Thus, the research on dye penetration into skin is very important for effective applying of laser selective thermolysis of tumors; photodynamic therapy of tumors and acne disease, etc.

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We have defined the depth of Methylene Blue penetration into skin samples in dependence on the time dyeing. Diffusion coefficients of Methylene Blue within the skin *in vitro* have been estimated.

2. METHODS AND MATERIALS

Methylene Blue is a commercially available medical dye widely used for photodynamic therapy.[6, 16-19] It is a tricyclic phenothiazine dye with chemical formula $C_{16}H_{18}ClN_3S$ and molecular weight 319.85.[16] Chemical structure and the absorption spectrum of Methylene Blue are presented in Fig. 1.

Methylene Blue has a low toxicity and is used for staining cells *in vivo*.[6] In aqueous solutions, Methylene Blue is in monomer and dimmer forms. It has two peaks absorption at 668 and 609 nm. Methylene Blue can be activated by light to an excited state, which in turn activates oxygen to yield oxidizing radicals. Such radicals can cause cross-linking of amino acid residues on proteins and hence achieve some degree of cross-linking.[6, 16]

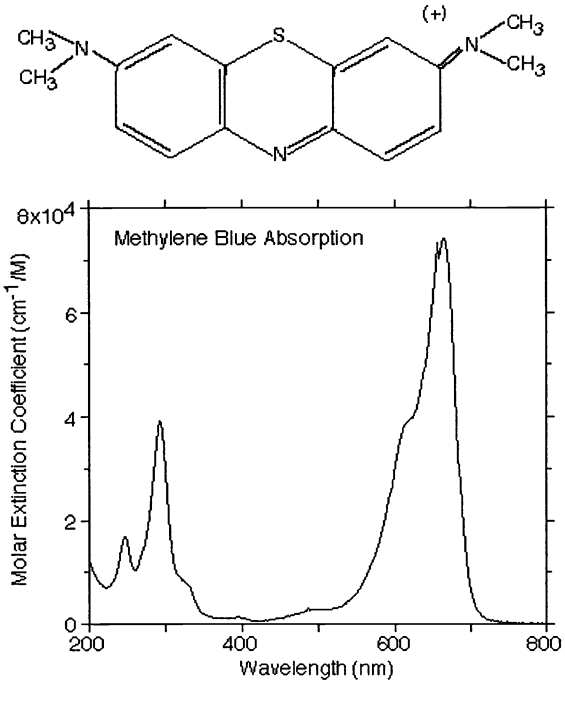

Fig. 1: Chemical structure (*a*) and absorption spectrum (*b*) of Methylene Blue. This spectrum was taken with a spectrophotometer using a 1 cm quartz cuvette filled with 10μM solution of Methylene Blue in water.[6]

Skin samples were taken from white rat in an hour *post mortem*. Both hair and adipose layer were removed. The thickness of the samples was measured by a micrometer. The value averaged on ten measurements was 0.78 ± 0.02 *mm*.

Methylene Blue (Aldrich Chemical Co., USA) was dissolved in isotonic saline solution with concentration 1 *mg/ml*. pH of the dyed solution was measured by a pH-meter as 6.7. The samples were put in a cell with the dyed solution. Experiment was carried out at the room temperature (about 20 ^{0}C). The first sample remained in the cell with the Methylene Blue solution for an hour. The second and the third ones remained in the cell for 2 and 3 hours, respectively. When the samples were taken out, cross-sections of the sample were made. The thickness of these sections was measured as 0.5 *mm*. Then these sections were put between two subject glasses and placed in a digital video-microscopic system to obtain their images.

All images were obtained in the transmission mode of illumination. The scheme of the digital imaging system is presented in Fig. 2.

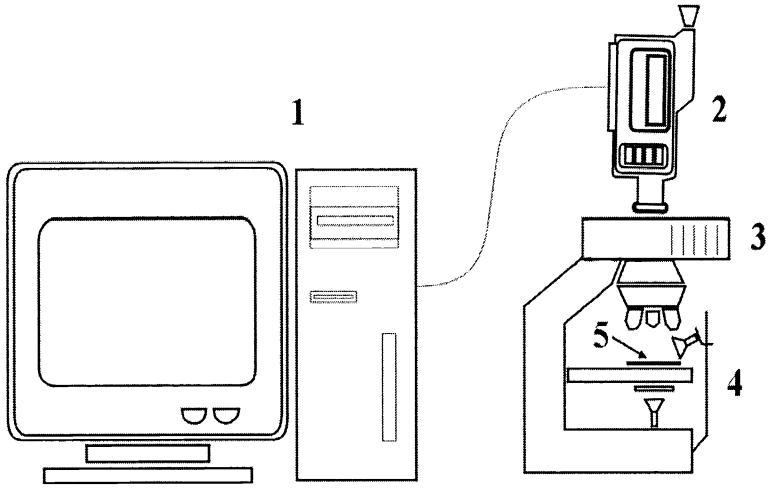

Fig. 2: The scheme of microscopic digital imaging system: 1 – PC, 2 – color video camera, 3 – light microscope, 4 - illuminating system provided as transmission as reflection modes of illumination, 5 – investigated object (skin sample).

The digital imaging system is composed of a video-microscope (SVHS Sony CCD-TR617E, PAL, Japan (2) and light microscope (3)) interfaced with a personal computer (1). The examined object is a plane plate with attached biological object (skin sample) under study (5). It is illuminated by white light, from one of two halogen lamps (4) provided either for transmittance or for reflectance modes of observation and recording of images. The size of illuminated area (4×4 mm) exceeds the field of vision of the system (1×1.5 мм). It assures the balancing illumination of the studied objects.

To process the images of the microscopic sections of skin samples we have developed the special computer program using Mathcad software (MathSoft Inc., USA). The base image (the photo of the microscopic section of the skin sample) was separated in the three color matrixes of red, green, and blue components by internal function of the Mathcad system. For the calculations we used only the red and the blue image components. The base images were scanned across skin thickness. The width of the scanning band was 401 pixels. Averaging the measured values was done within this band.

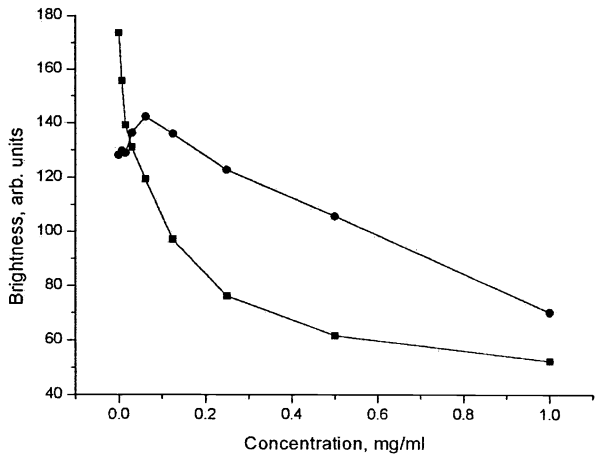

Fig 3: Calibration dependence of the brightness of both the red component of phantom's images (squares) and blue one (circles) on the concentration of Methylene Blue in these phantoms.

Calibration of this system was done using tissue-like phantoms with different concentrations of Methylene Blue. The skin-like phantom was prepared by the following method: gelatin matrix including 10%-gelatin solution in water was made turbid adding 2 *ml* of Intralipid-20% per 100 *ml* of gelatin solution. Then hemolysed blood with concentration 4 *ml* per 100 *ml* of the obtained suspension was added to provide the absorption properties of the base skin-like phantom. The thickness of the phantom was the same as one of the skin cross-sections (0.5 *mm*). Thus, this phantom was made so that the distributions of the color components of both images - this phantom and the non-dyed skin sample - obtained by the digital imaging system were coincided. The base phantoms were dyed by Methylene Blue. The concentrations of the dye in the phantoms varied from 1 *mg/ml* to 0.004 *mg/ml*. Dyed and non-dyed phantoms were combined to obtain the modeling of the skin subjected to the dye influence. All combined phantoms were photographed and processed. The values of both the red and blue components were averaged. The obtained calibration curves are presented in Fig. 3. Comparing the values of the red and blue components of the calibration phantoms and the skin samples we estimated the concentration of Methylene Blue within the samples.

The diffusion equation described Methylene Blue concentration within a skin layer can be presented in the form[30]

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2}, \tag{1}$$

where c is the Methylene Blue concentration at any moment $[c] = g/ml$, D is the diffusion coefficient $[D]=cm^2/\sec$, and x is the spatial coordinate $[x] = cm$.

Diffusion coefficients of Methylene Blue in the skin were calculated based on the free diffusion[31]:

$$\begin{aligned} &\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial t^2}, \quad C = C(z,t), \quad z \in [0,\infty], \\ &C(z,0) = C_0, \quad C(0,t) = C_1; \quad C(\infty,t) = C_0 \end{aligned} \tag{2}$$

where C is the concentration of the dye within the tissue, C_0 and C_1 are the concentrations of Methylene Blue inside and outside of the tissue, respectively, z is the depth of the dye penetration, and D is the diffusion coefficient of the dye.

The analytical solution of the task (2) was obtained by a separation of variables assuming that diffusion coefficient D is constant. This allows to describe the Methylene Blue distribution in a half-infinite homogeneous medium as a function:

$$C(z,t) = C_0\left(1 - erf\left(\frac{z}{2\sqrt{Dt}}\right)\right). \tag{3}$$

where $erf(x) = \frac{2}{\sqrt{\pi}}\int_0^x exp\left(-a^2\right)da$ is an error function.

3. RESULTS AND DISCUSSION

Series of images in Figs. 4 corresponds to different time intervals of the stay of the skin samples in the cell with Methylene Blue solution. Epidermis layer is at the right side. Dark spots in the images correspond to images of hair follicles in transmission mode. Scales of length are presented in figures.

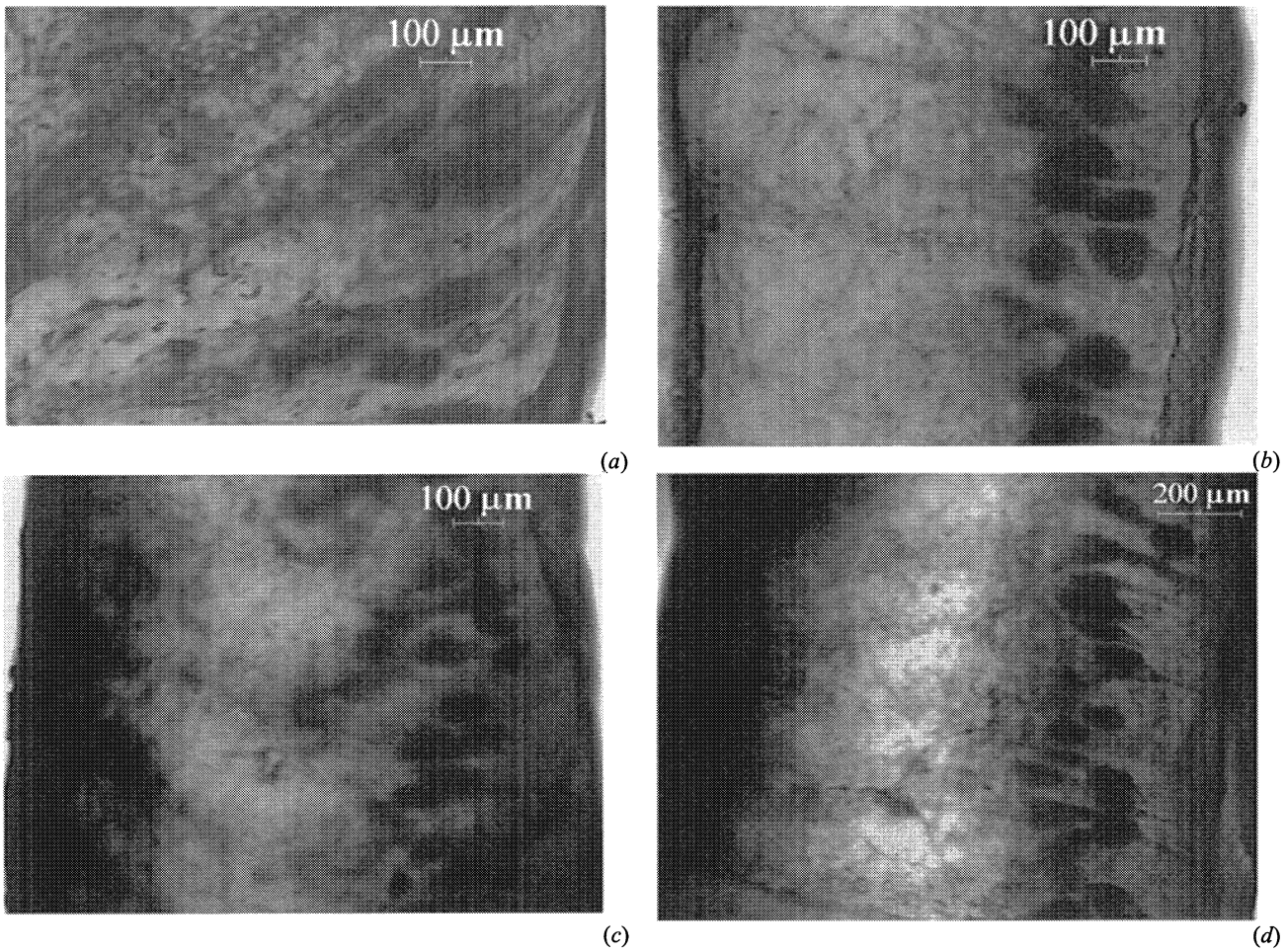

Fig. 4: Images of the non-dyed cross-section (*a*) and the dyed cross-sections of the skin samples: (*b*) 1 hour in Methylene Blue solution, (*c*) 2 hours in Methylene Blue solution, (*d*) 3 hours in Methylene Blue solution. Epidermis is at the right side.

Fig. 4 (*a*) presents the non-dyed cross-section of the skin sample photographed before it was put into the cell with the Methylene Blue solution.

From Fig. 4 (*b – d*) we can see the depth of the dye penetration into the tissue in dependence on time. The penetration of Methylene Blue took place from the side of the dermis (at the left side of the images). In the figures the dyed areas are looked as darker then non-dyed ones. We can define the depth of the dye penetration rather exactly with the system of digital analysis of this color images. Dyed areas correspond to the areas of predominance of the blue component under the other components of the image. Dependence of the concentration of Methylene Blue within tissue on the penetration depth is presented in Fig. 5.

During the first hour the dye amounted to 200 μm. During the second hour the dyed area increased up to about 600 μm. Intensity of the dyeing also increased. From the side of the epidermis the dyeing of the tissue was not observed. In three hours the sample has been dyed on the depth of about 700 μm.

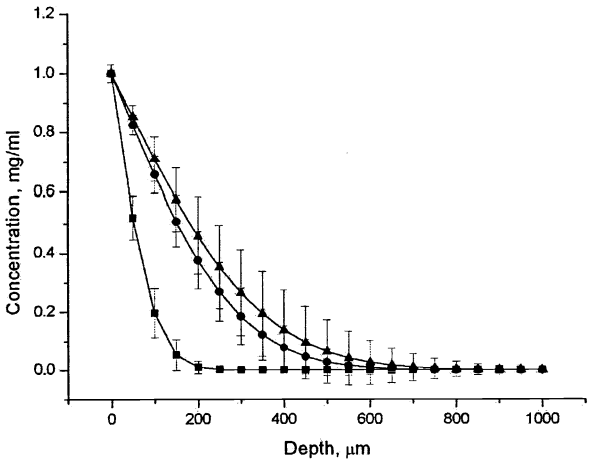

Fig. 5: Dependence of the concentration of Methylene Blue within interstitial fluid of the skin tissue on the depth of penetration of the Methylene Blue solution from the side of dermis: squares correspond to the I sample (1 hour in the solution), circles correspond to the II sample (2 hours in the solution), and up triangles correspond to the III sample (3 hours in the solution).

The values of the concentration, time and penetration depth have allowed estimating the diffusion coefficients of Methylene Blue within the skin samples from the Eq. (3). The results of the calculation are presented in the Table.

Table. Diffusion coefficients of methylene blue in the skin samples

Number of the sample	Diffusion coefficient, cm^2/sec (mean ± sd)
I (1 h. in the solution)	$0.83\times10^{-8} \pm 0.36\times10^{-8}$
II (2 h. in the solution)	$3.54\times10^{-8} \pm 1.84\times10^{-8}$
III (3 h. in the solution)	$3.35\times10^{-8} \pm 2.85\times10^{-8}$

4. CONCLUSION

We have presented experimental results on study of the penetration of Methylene Blue dissolved in isotonic saline within skin with the method of digital analysis of color images of the skin samples. This analysis has allowed to obtain the penetration depth of this dye into the tissue as from the side of the skin dermis and from the epidermis at different moments. Diffusion coefficient of Methylene Blue within the skin *in vitro* has been estimated.

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