# Methylene blue diffusion in skin tissue

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## ABSTRACT

The study of Methylene Blue penetration in both skin and subcutaneous fat is presented. Experiments have been carried out with both rat skin and human adipose tissue *in vitro* at room temperature. Microscopic analysis with digital imaging system has been applied for visualizing and investigation of the Methylene Blue diffusion in the epidermal, dermal and adipose tissue. Diffusion coefficient of Methylene Blue in skin *in vitro* has been estimated.

**Keywords:** Methylene Blue, skin, diffusion coefficient

## 1. INTRODUCTION

Control of absorption properties of living tissues is method used widely in diagnostics as well as therapy and surgery. Effective change of absorption properties of living tissues can be provided by biocompatible dyes. 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 [1-5]. For visualisation of atherosclerotic plaques and localisation of hidden tumours biocompatible fluorescent dyes are used [5-11]. Photodynamic therapy utilizes photosensitizers and light. Excitation of the sensitizer by absorption of light of appropriate wavelength in the presence of oxygen converts the sensitizer to its photoactive triple state, which in turn reacts with either a local substrate to form cytotoxic radicals, or with molecular oxygen ($O_2$). The reactive oxygen species generated then results in death of pathological cells, bacteria and viruses [10-23]. Dyes can be applicable for local increase of tissue absorption that is used for precise microsurgery [24], selective laser thermolysis of tumours [25-33], treatment of skin and follicle lesions (for example, *acne vulgaris*) [34-38], hair removal [10, 35, 39], etc.

For local influence the agents are administered topically on the skin surface or injected directly in the area of lesion. In spite of invasiveness of the last method it allows localized delivery of the agent directly to the site of action while reducing the number and extent of systemic effects [40]. For the treatment of cutaneous and subcutaneous tumours dermal administration of the dye has a number of advantages compared with other routes of administration. The objective of dermal delivery is to deliver the agent to the dermis with a minimum of systemic involvement [40, 41]. Besides it excludes the influence of protecting barrier of stratum corneum and promotes more quick and deep penetration of the agent into the skin [42-45].

It is known a lot of papers on drug delivery into different tissues, in particular into skin, and estimation of diffusion coefficient of water and drugs in epidermal and dermal tissues [40-49]. But in spite of numerous investigations related to control of tissue optical properties the problem of estimating the diffusion coefficient of dyes in tissues has not been studied in detail. Wide using dyes in the modern medicine requires the study of dye diffusion in skin: the pathways of the dye penetration, the dye concentration in skin, and diffusion coefficient. The knowledge of these parameters is very important for correct dosing laser action on the stained area during photodynamic therapy, for effective applying of laser selective thermolysis of tumours, acne disease, etc.

Among the dyes used in photodynamic therapy Methylene Blue (MB) is known widely [17-23]. It is a commercially available medical dye. MB is a tricyclic phenothiazine dye with chemical formula $C_{16}H_{18}ClN_3S$ and molecular weight 319.85 [1]. MB is related to alkaline group. Chemical structure and the absorption spectrum of MB are presented in Fig. 1.

MB has a low toxicity and is used for staining cells *in* vivo [1, 17]. In aqueous solutions it is in monomer and dimmer forms and has two peaks of absorption at 668 and 609 nm. MB can be activated by irradiation with appropriate

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wavelength in the red spectral range to an excited state, which in turn activates oxygen to yield oxidising radicals. Such radicals have the phototoxic effect on pathological cells, bacteria and viruses [17-23].

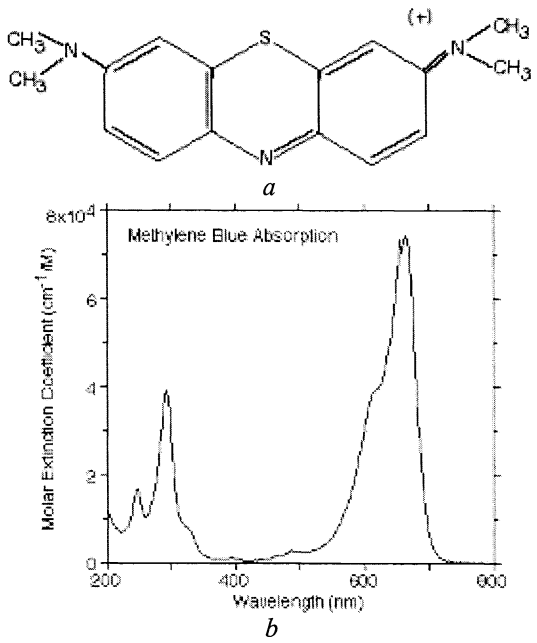


Fig. 1: Chemical structure (*a*) and absorption spectrum (*b*) of Methylene Blue. This spectrum was taken with a spectrophotometer using a 1 cm quartz cuvette filled with 10μM solution of Methylene Blue in water [5].

For the correct evaluation of the therapeutic effect, it is necessary to have detailed knowledge of the penetration behavior of the photosensitizer. We have defined the depth of Methylene Blue penetration into skin samples in dependence on the dyeing time. Diffusion coefficients of Methylene Blue in the skin samples *in vitro* have been estimated.

## 2. MATERIALS AND METHODS

*2.1. Materials*

Skin samples have been taken from white rat in an hour *post mortem*. Both hair and subcutaneous fat layer have been removed. The area of the samples was about 5×5 $cm^2$. The thickness of each sample has been measured at initial moment with a micrometer in ten points over the sample surface and averaged. The value averaged on ten measurements was 0.78 ± 0.02 *mm*. The samples of adipose tissue have been taken from human postoperative material. The volume of the samples was about 500 $mm^3$. We have used three samples of skin and three samples of adipose tissue.

Methylene Blue (Aldrich Chemical Co., USA) has been dissolved in isotonic saline with concentration 1 *mg/ml*. pH of the dyed solution has been measured by a pH-meter (HANNA, Portugal) as 6.7.

*2.2 Experimental setup*

The digital imaging system is used to visualize microscopic sections of skin and adipose tissue samples. All images of the samples have been obtained in the transmission mode of illumination. The scheme of the digital imaging system is presented in Fig. 2.

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 or fat sample) under study (5). It is illuminated by white light, from halogen lamp (4) provided

transmittance mode of observation and recording of the images. The size of illuminated area (4×4 $mm^2$) exceeds the field of vision of the system (1×1.5 $мм^2$). It assures the balancing illumination of the studied objects.

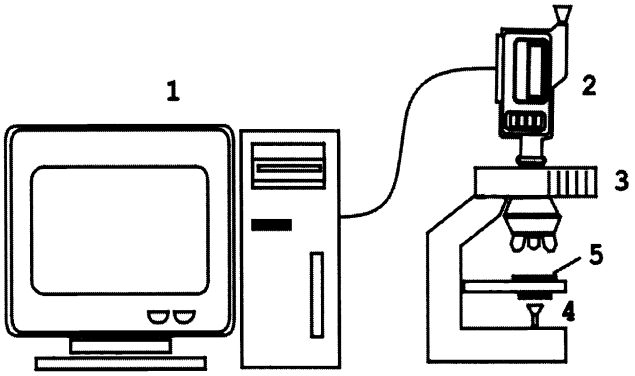


Fig. 2: The scheme of microscopic digital imaging system: 1 – PC, 2 – color video camera, 3 – light microscope, 4 - illuminating system provided as transmission as reflection modes of illumination, 5 – investigated object (skin sample).

The skin and fat samples have been put into a cell with the MB solution. Experiments have been carried out at the room temperature (about 20 $^0$C). The first sample has taken from the cell after 30 minutes. The second and the third ones have remained in the cell for 1 and 2 hours, respectively. After staining in the solution the samples have been cross-sectioned by freezing microtome MZ-2 (Tochmedpribor, Russia). The thickness of the sections was 100 ± 10 μm. Then these sections were fixed between two subject glasses and placed in the digital video-microscopic system to obtain their images.

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) has been separated in the three color matrixes of red, green, and blue components by internal function of the Mathcad system. For the calculations we have used only the red and the blue image components. The base images have been scanned across skin thickness. The width of the scanning band was 401 pixels. Averaging the measured values has been done within this band.

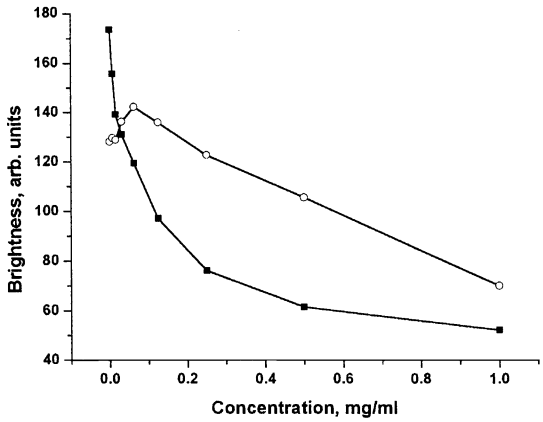


Fig 3: Calibration dependence of the brightness of both the red component of phantom's images (squares) and blue one (circles) on the concentration of Methylene Blue in these phantoms.

Calibration of this system has been done using tissue-like phantoms with different concentrations of Methylene Blue. The skin-like phantom has been prepared by the following method: gelatin matrix including 10%-gelatin solution in

water has been made turbid adding 2 *ml* of Intralipid-20% per 100 *ml* of the gelatin solution. Then hemolysed blood with concentration 4 *ml* per 100 *ml* of the obtained suspension has been 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 (about 100 $\mu m$). Thus, the skin-like phantom has been made so that the distributions of the color components of both images - the phantom and the non-stained skin sample - obtained by the digital imaging system were coincided. The base phantoms have been dyed by MB. The concentrations of the dye in the phantoms varied from 1 *mg/ml* to 0.004 *mg/ml*. Stained and non-stained phantoms have been combined to obtain the model of the skin subjected to the dye influence. All combined phantoms have been recorded by the digital imaging system and processed. The values of both the red and blue components have been averaged. The obtained calibration curves are presented in Fig. 3. Comparing the values of the red and blue components of the images of the calibration phantoms with the images of the skin samples we have estimated the concentration of MB in the samples.

*2.3. Diffusion coefficient estimation*

The analysis of the mechanism of permeability of chemical agents through human skin bases on Fick's diffusion theory since agents passively permeate through the skin [50]. The diffusion equation describing MB concentration within a skin layer can be presented in the form [50]

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

where $c$ is the MB 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 MB in the skin have been calculated based on the free diffusion [51]:

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

where $C$ is the concentration of the dye within the tissue, $C_0$ and $C_1$ are the concentrations of MB 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 the diffusion coefficient $D$ is constant. This allows describing the MB 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 MB 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.

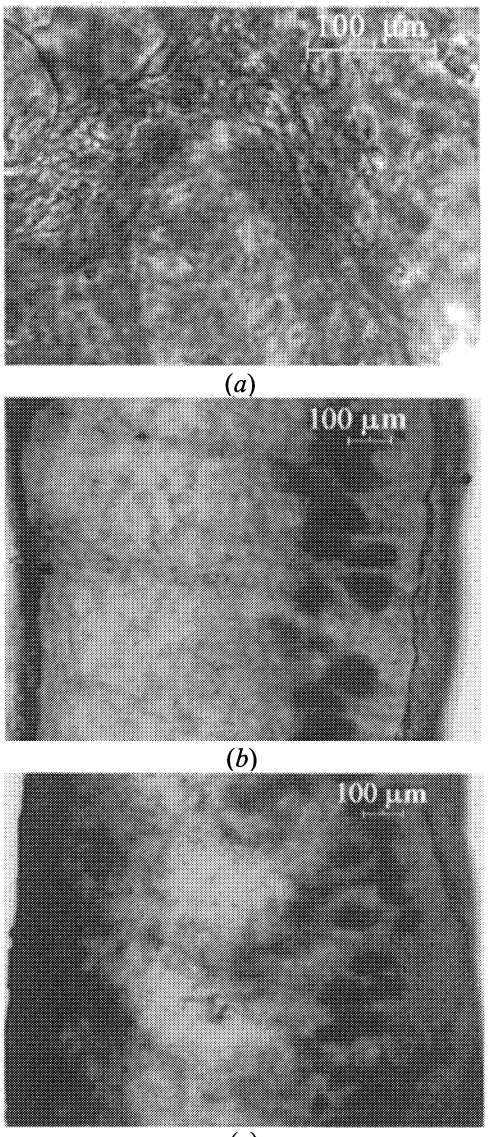


Fig. 4: Images of the stained cross-sections of the skin samples: (*a*) 30 minutes in Methylene Blue solution, (*b*) 1 hour in Methylene Blue solution, (*c*) 2 hours in Methylene Blue solution. Epidermis is at the right side.

During the fist 30 minutes staining the skin samples has been insignificant at visual observations. In the microscopic image (Fig. 4 (*a*)) it is well seen that dermal tissue has remained non-stained practically. Apparently the stained objects are only blood and lymph vessels, channels of hair follicles, and infundibula of different glands contiguous to the solution. During the experiment the MB solution has penetrated gradually into interstitial space. Fig. 4 (*b, c*) shows the depth of the dye penetration into the skin samples in dependence on time. It is well seen that MB solution has penetrated into the samples from the side of the dermis (at the left side of the images) and stained dermal fibers. At that the epidermis has remained non-stained practically during all time of the experiment. It is connected with hindered diffusion of substances through stratum corneum barrier. As well known the stratum corneum or horny layer is between 10 and 20 $\mu m$ thick. It contains the corneocytes or horny cells, which are closely packed flat nonnucleated cells, approximately 40 $\mu m$ in diameter and 0.5 $\mu m$ thick. Cellular organelles and cytoplasm have disappeared during the process of cornification [42]. They are composed primarily of insoluble bundles keratins surrounded by a cell envelope stabilized by cross-linked proteins and covalently bound lipid. Interconnecting the corneocytes of the stratum corneum are polar structures such as corneodesmosomes which contribute to stratum corneum cohesion [42]. The intercellular

region is composed of lipid. The intercellular lipid is required for a competent skin barrier and forms the only continuous domain in the stratum corneum [42, 46]. The pathway for water diffusion across the stratum corneum has been calculated to be 50-fold longer than the thickness of the stratum corneum. This suggests that permeation follows a tortuous path through the intercellular lipid domain [42, 46]. The penetration of compounds into the corneocytes also takes place as immersion of skin in bath leads to swelling of the corneocytes connected with the entry of water [42]. Thus, structure and composition of stratum corneum provides diffusion coefficient which are $10^6$-fold less than that observed for cellular membranes [42, 49].

The dermis consists mainly of conjunctive collagen fibrils packed in lamellar bundles. Within each bundle the groups of fibril are separated from each other by the large empty lacunae distributed randomly in space. Interstitial fluid contains proteins, proteoglycans, glycoproteins, and hyaluronic acid [52]. Due to their glycosaminoglycan chains, these molecules concentrate negative charges. They are highly hydrophilic and have a propensity to attract ions, creating an osmotic imbalance that results in the glycosaminoglycan absorbing water from surrounding areas. This absorption helps maintain the hydration of the matrix; the degree of hydration depends on the number of glycosaminoglycan chains and on the restriction placed on proteoglycans swelling by the surrounding collagen fibers [53]. It has been obtained that the diffusion coefficient of water through stratum corneum varies from $2.5\times10^{-10}$ to $8.34\times10^{-10}$ $cm^2/s$ in dependence on humidity of the stratum corneum [49]. On the contrary the diffusion coefficient of water and some solutions in the dermis varies in the range $10^{-5} - 10^{-6}$ in dependence on containing of solution [41].

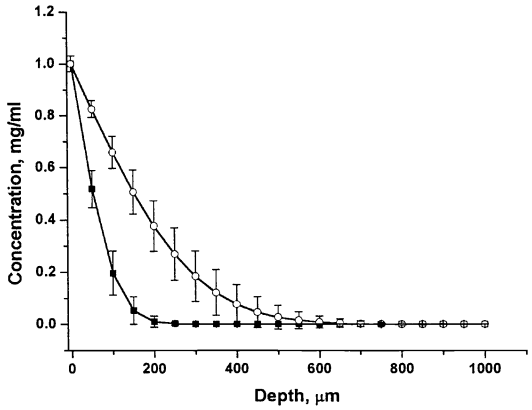


Fig. 5: Dependence of the concentration of Methylene Blue within interstitial fluid of the skin tissue on the depth of penetration of the Methylene Blue solution from the side of dermis: squares correspond to the I sample (1 hour in the solution), circles correspond to the II sample (2 hours in the solution),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

Diffusion of dyes in tissue is accompanied by interaction with tissue. In dependence on physical and chemical properties of the dyes the speed of diffusion and degree of staining can vary. MB as weak organic electrolyte accumulates in dermal tissue and provides bright staining tissue [50]. However the interaction of dye ions with molecules of proteins in tissue hinders the penetration of the dye into the tissue. We have defined the depth of MB penetration with the system of digital analysis of the colour images. Stained areas correspond to the areas of predominance of the blue component under the other components of the image. The concentration of MB can be obtained on the base of calibration dependence (Fig. 3). Dependence of the concentration of MB in the tissue on the penetration depth is presented in Fig. 5. Thus, during the first hour the dye amounted to 200 $\mu m$. During the second hour the dyed area increased up to about 600 $\mu m$. Intensity of the dyeing also increased. From the side of the epidermis staining the tissue has not been observed.

The diffusion of aqueous MB solution through the skin sample can be assumed as free [41,42,45,49,50]. The values of the concentration, time and penetration depth have allowed estimating the diffusion coefficients of MB within the skin samples from the Eq. (3). The MB diffusion coefficient value is $(0.83 \pm 0.36)\times10^{-8}$ $cm^2/s$ for the skin samples after 1

hour in the solution and $(3.54 \pm 1.84)\times10^{-8}$ $cm^2/s$ for the skin samples after 2 hour in the solution. We have taken into account the second and the third samples only as in the first sample separate fragments of the tissue have been stained.

Differences between the diffusion coefficients of MB in water and in tissue are connected with the structure and composition of the dermis interstitial fluid. As it has been shown the dermis interstitial fluid contains the proteins, proteoglycans and glycoproteins, which provide selective barrier to the diffusion of charged molecules. Thus, the penetration of MB molecules into tissue is hindered.

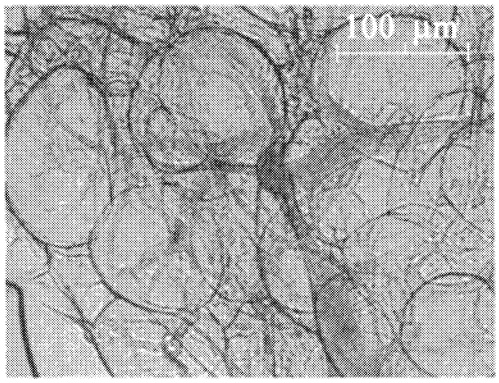


Fig. 6: Image of the stained cross-sections of the adipose tissue sample after 2 hours in Methylene Blue solution.

The staining of the vessels and fibers of connective tissue of adipose tissue is well seen in Fig. 6. During two hours of the experiment the penetration and interaction of MB solution into fat cells have not been observed due to hydrophoby of lipids.

## 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 colour images of the skin samples. This analysis has allowed obtaining the penetration depth of the dye into the tissue as from the side of the skin dermis and from the epidermis at different moments. The pathway of MB solution into the skin samples is through dermis. The epidermis has remained non-stained practically during all time of the experiment. It is connected with hindered diffusion of aqueous solutions through hydrophobic stratum corneum barrier. Diffusion coefficient of MB within the skin *in vitro* has been estimated. Low values of the dye diffusion coefficient are connected with the interaction of MB ions with molecules of proteins in tissue.

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