Study of the Diffusion of Tetracycline in the Dentin of the Human Tooth *Ex Vivo*

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Abstract. Currently, manufacturers of tetracycline and tetracycline antibiotics necessarily warn about possible side effects when using tetracycline in children under 8 years of age and pregnant women. When tetracycline is used in utero or after birth, before the teeth erupt through the gum during mineralization or calcification, the antibiotic binds to calcium ions, which causes persistent gray or brown stains or often streaks on the teeth. In the present study, the diffuse reflectance spectroscopy with an integrating sphere was used to determine the molecular diffusion coefficient of tetracycline in the dentin of a human tooth *ex vivo*, which was found as $D = (6.49 \pm 1.12) \cdot 10^{-6}$ cm^2/s. The dependences following from Fick's second law and the modified Bouguer-Lambert-Beer law, as well as restrictions for the free diffusion model, were used in the calculations. It was found that when dentinal samples are saturated with tetracycline hydrochloride, the total transmittance decreases over the entire studied wavelength range from 200 to 800 nm. These studies are important for clinical practice. © 2022 Journal of Biomedical Photonics & Engineering.

Keywords: tetracycline hydrochloride; human dentin; molecular diffusion; diffuse reflectance spectroscopy; integrating sphere; free diffusion model.

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1 Introduction

Tetracycline is a broad-spectrum antibiotic. It is produced in the form of a base and hydrochloric acid salt. It has a bacteriostatic effect due to the suppression of protein synthesis of pathogens caused by a violation of the formation of a complex between the transport ribonucleic acid and the ribosome [1]. Until the 1980s and 1990s, tetracycline was actively prescribed to patients suffering from such diseases as pneumonia and respiratory tract infections caused by Mycoplasma pneumoniae, respiratory tract infections caused by Haemophilus influenzae and Klebsiella spp., bacterial infections of the urinary and reproductive system, skin infections and soft tissues, ulcerative necrotic gingivitis, stomatitis, conjunctivitis, acne, actinomycosis, intestinal amoebiasis, anthrax, brucellosis and many others [2]. The maximum concentration of the antibiotic in the human body is reached within about 3 hours and over the next 8 hours the concentration gradually decreases. It is distributed unevenly in the body, the maximum concentration is found in the liver, kidneys, lungs, and in the spleen, lymph nodes [2]. It accumulates in large quantities in bone tissue, tumor tissues, in dentin and enamel of primary teeth [3].

Patients taking tetracycline-based antibiotics have included pregnant women and young children. At the same time, medical science had not yet fully deciphered the side effects of this drug which include permanent staining or discoloration of teeth that are not fully formed (i.e., in children under eight years of age) [4]. When using tetracycline in childhood, as well as with intrauterine or ectopic exposure before teething through the gums during mineralization or calcification of the teeth, the antibiotic binds to calcium ions in the teeth. This results in persistent permanent staining in the teeth. In dentistry, the term "tetracycline teeth" even arose. The first case of discoloration of teeth in children was reported in 1956,

followed by many others [5, 6]. The warning about this effect also extends to a number of tetracycline derivatives including doxycycline and minocycline, and others [7]. Staining and discoloration of teeth with tetracycline depends on the dosage used, the duration of treatment or exposure, the stage of mineralization (or calcification) of the teeth, and the degree of the mineralization process activity. The spots can be gray or brown in color and often appear in stripes around the teeth. During teething and exposure to light, calcium-bound tetracycline oxidizes causing enamel discoloration from fluorescent yellow to brown.

The location of the discoloration directly correlates with the stage of tooth development during tetracycline exposure. The age range for the development of pathology begins from the 2nd trimester of intrauterine development and up to 8–9 years of growing up. During this period, calcification of the teeth occurs. Calcification of primary teeth may occur before the age of 10–14 months, anterior permanent teeth between 6 months and 6 years of age, and posterior permanent teeth before the age of 8 years [8, 9]. Therefore, exposure to tetracycline during any of these periods of calcification may result in irreversible staining. Cases of discoloration in adults have been reported, minocycline causes tooth discoloration in 3–6% of adults who take a daily dose of more than 100 mg for more than one month [10, 11]. The discoloration is less noticeable in adults due to the lack of free calcium in tooth enamel.

Does tetracycline cause cavities? There is information from dental experts who believe that the drug can sometimes bind to calcium phosphate in the teeth of young children and lead to its absorption by the tissues of the teeth and form tiny pits in the enamel which contributes to the development of caries [12]. However, most dentists tend to believe that the worst thing that tetracycline can do is permanently stain children teeth, and many other factors can provoke caries [13]. Can tetracycline teeth be whitened effectively? Not always. Tetracycline stains are different from coffee, tea, or wine stains. Unlike the latter, which exist only on the surface of the teeth, tetracycline staining is found inside the teeth. This means that "tetracycline teeth" cannot be whitened with toothpaste, strips, and home whitening kits. However, this does not mean that it is impossible to get rid of tetracycline stains on teeth with whitening. Internal professional teeth whitening can improve the appearance of teeth that are not too discolored [14]. In cases of severe tetracycline damage, the dentist may suggest the use of veneers and crowns to address the aesthetic problem in order to change your confidence in your smile.

Currently, manufacturers of tetracycline and tetracycline antibiotics necessarily warn about possible side effects when using tetracycline in children under 8 years of age. Structure-activity-relationship (SAR) of tetracycline family, which shows the bioactivity, strength and selectivity to the biological target, makes it valuable for labeling with radioisotopes. Up till now there are a few numbers of analogues of tetracycline which are labeled with radioisotopes and clinically used for treatment of bacterial and non-bacterial diseases [15].

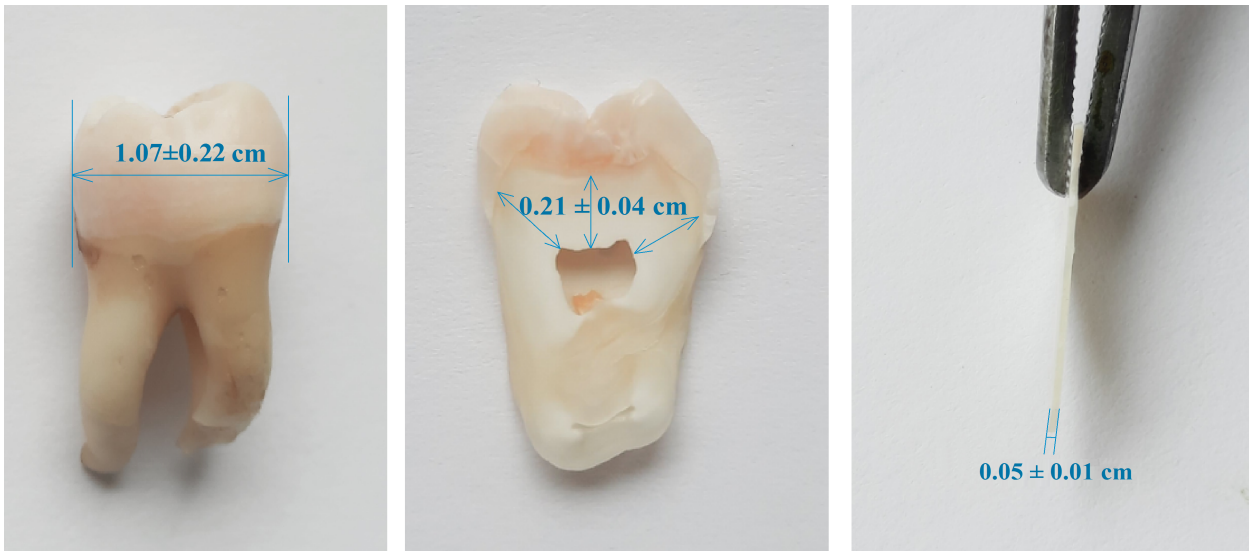
One of the effective methods of treating bacterial infections of the oral cavity, including teeth, is photodynamic therapy in which tissues are stained with a photosensitizer that mediates the generation of singlet oxygen and other reactive oxygen species (ROS) when irradiated with light of a certain wavelength. Tetracyclines are able both to kill bacteria due to the formation of ROS during staining of bacteria and exposure to light, and to prevent the growth of bacteria due to inhibition of ribosomes [16]. For example, doxycycline is effectively excited by light at a wavelength of 365 nm, and demeclocycline by UV and blue light (415 nm). The time and efficiency of staining are critical issues in the application of phototherapy in the clinic, so the measurement of the diffusion rate of a photosensitizer, in particular tetracycline, in the tooth tissue is of great importance. Quantitative parameters characterizing the penetration of a photosensitizer into tissues are necessary to determine the optimal concentration that provides safe and effective treatment. The method described in this study is also applicable to other tissues and photosensitizers [17].

So far, there are no quantitative data on the relative concentration of tetracyclines in tissues compared with blood serum, although such data are important for predicting treatment. New studies on the pharmacokinetics of tetracyclines are needed to understand the entire chain of oral tetracycline delivery from the stomach to the dentin at per oral administration. It is known that in the blood plasma tetracyclines find the maximum concentration after their administration in 2–3 hours [18]. Evidently, it is necessary to determine the entire path of molecules delivery from stomach to dentin. In this study, we offer investigation of only one important pathway in the entire chain and determine how long the tetracycline molecules will go from pulp (blood supply) to dentin and stain it.

The aim of this study was to determine the kinetic parameters of the penetration of a solution of tetracycline hydrochloride into the dentin of a human tooth. Namely, to quantify the diffusion coefficient of an antibiotic in dentin by diffuse reflectance spectroscopy using a free diffusion model.

2 Methods and Materials

The material for the *ex vivo* study was the saw cuts of human teeth, which were extracted from patients in a dental clinic for orthodontic indications. Sections were taken from three different teeth (molars) belonging to different patients aged 18, 37, 45 years (Fig. 1(a, b)). The extracted teeth were stored in saline at 5 °C in a dark place. Wet teeth were cut with a diamond disc into



(a) (b) (c)

Fig. 1 Photo of the studied samples of human teeth (molars): (a) a whole tooth; (b) the investigated saw cut with the distance from the pulp to the enamel; (c) 0.05 cm thick saw cut to study ASTH diffusion.

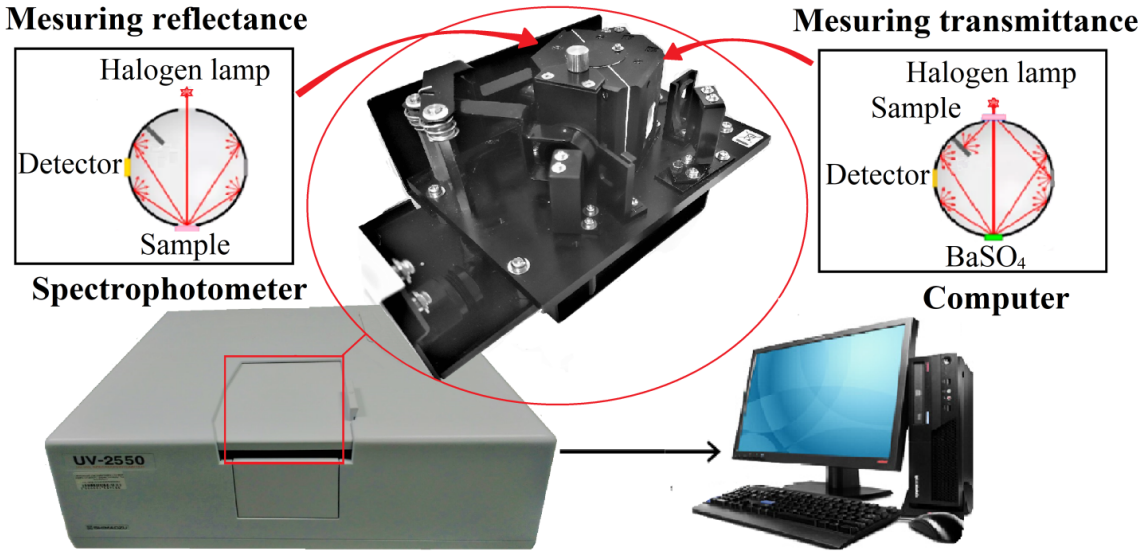


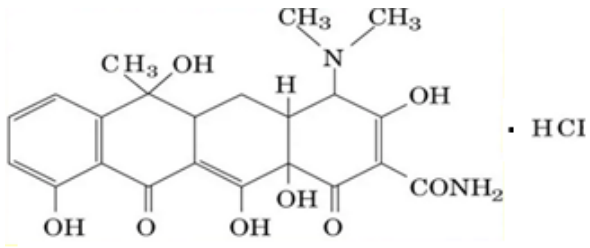
Fig. 2 Experimental scheme for measuring the spectra of total reflection and total transmittance.

longitudinal sections about 0.5 mm thick. Sections were placed for 20 sec in 35% phosphoric acid. To clean the surface from sawing products, the samples were placed in a Techsonic UD100 SH-45 L ultrasonic bath with water for 10 min and then wiped with a brush dipped in alcohol. The samples were dried in air for several days. The thickness of cuts (samples) of biological tissue was measured with a micrometer, the measurements were carried out at several points of the sample and averaged. The accuracy of each measurement is ±10 μm. In total, three cuts (samples) of different teeth were studied. The thickness of the dentin saw cut averaged 0.05 ± 0.01 cm (for $n = 3$, n is the number of samples) (Fig. 1(c)).

To measure the total transmittance and diffuse reflectance of tissue samples in the spectral range of 200–800 nm, a Shimadzu UV-2550 double-beam spectrophotometer (Japan) with an integrating sphere was used (Fig. 2). The radiation source was a halogen lamp with radiation filtering in the investigated spectral range. The limiting resolution of the spectrometer was 0.1 nm. The spectra were normalized before measurements using the $BaSO_4$ reference reflector, which has suitable UV properties. All measurements were carried out at room temperature (~25 °C) and normal atmospheric pressure. Each sample of the tissue under study was fixed in a special frame with a 0.5×0.5 cm window in a quartz cuvette so that the tissue sample was pressed against the wall of the cuvette and subjected to optical measurement. To measure the total transmission spectra (TTS), a quartz cuvette with a tissue sample was placed directly in front of the integrating sphere, collecting all radiation that passed

through the tissue sample. The diameter of the light beam incident on the sample was 3 mm.

The structural formula of tetracycline is shown in Fig. 3(a). Tetracycline hydrochloride in powder form retains its properties for more than two years. Tetracycline solutions are unstable, an alkaline solution at pH = 8.85 under normal conditions loses its effectiveness by 50% within 12 hours, in acidic solutions at pH = 3–5 it does not change slowly up to 6 days [19]. Working solutions of tetracycline hydrochloride were acidified by adding 1 drop of hydrochloric acid to pH=5. ASTH with concentration of tetracycline hydrochloride $C = 1.1 \cdot 10^{-4}$ mol/l was used.



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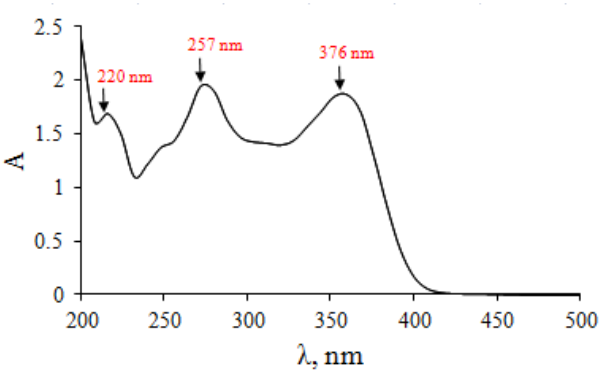


(b)

Fig. 3 Structural formula of tetracycline hydrochloride (a); absorption spectrum of an ASTH (b).

The absorption spectrum of tetracycline hydrochloride was recorded on the same spectrophotometer only in the absorption mode (without an integrating sphere).

The absorption spectrum of tetracycline hydrochloride has the form shown in Fig. 3(b). It has characteristic maxima in the ultraviolet region of the spectrum at the corresponding wavelengths: 217 nm, 257 nm, 376 nm.

The determination of the diffusion coefficient of tetracycline hydrochloride in biological tissue is based on measuring the kinetics of the optical diffuse reflectance spectra (DRS). For these measurements, each sample of dentin was fixed using a double-sided tape in a special clip in the form of a frame with a window of 0.5×0.5 cm, pressed against the wall of the quartz cuvette at the beam entrance to the spectrophotometer, so that there was free space between the surface of the sample and the cuvette wall filled with an aqueous solution of tetracycline hydrochloride (ASTH). Before measurements, the cuvette was filled with saline to wet the test sample (i.e., simulate the physiological state of the dentin). After 5 min, saline was removed using a syringe, after which the cuvette was filled with ASTH and measurements were performed during 200 min until saturated of the temporal dependence due to completion of the ASTH diffusion process (Fig. 4). The process of ASTH transport in a sample can be described in terms of the model of free diffusion [20].

3 Calculations

Geometrically, a sample of tissue can be represented as a plane-parallel plate of a finite thickness. It is also necessary to take into account some of the limitations inherent for the free diffusion model:

1) at the beginning of the experiment, we assume that the ASTH is absent at all points of the sample;

2) the volume of the ASTH is significantly larger than the volume of the dentin sample;

3) only concentration diffusion occurs;

4) we assume that the diffusion coefficient is constant at all points inside the studied dentin sample.

Using the second Fick law and performing transformations based on the use of the modified Bouguer-Lambert-Beer law, described in detail in Ref. [21], we obtain an expression for the difference between the effective optical density at the current time $A(t, \lambda)$ and at the initial time $A(t = 0, \lambda)$

$$\begin{aligned} &\Delta A(t,\lambda) = A(t,\lambda) - A(t=0,\lambda) = \\ &= \Delta\mu_{eff}(t,\lambda)L \sim C_0\left\{1-\exp\left(-\frac{\pi^2 Dt}{4l^2}\right)\right\}L, \\ &I = I_0 \exp\left[-\mu_{eff}L\right], \\ &\mu_{eff}(t,\lambda) = \sqrt{3\mu_a(\mu_a+\mu'_s)} \rightarrow \Delta\mu_{eff}(t,\lambda), \end{aligned} \quad (1)$$

where the effective optical density is determined from the measurements of DRS.

$$A = -\log R_d, \quad (2)$$

t is the time in sec during which the diffusion occurs, λ is the wavelength in nm, $\Delta\mu_{eff}$ (t, λ) is the difference between the effective coefficient of attenuation of light in biological tissue at the current time and at the initial time, 1/cm; L is the average path length of photons, which in the backscattering mode is $L \cong 2l_d$, $l_d^{-1} = \mu_{eff}$, $\mu'_s = \mu_s(1 - g)$, cm^{-1}, g is the scattering anisotropy factor (varies from 0 to 1, for dentin, $g \cong 0.93$) [18]; and for transmission $L \cong l$, l is the thickness of the sample, cm; D is the diffusion coefficient of the tetracycline hydrochloride molecules, cm^2/s; C_0 is the initial concentration of the tetracycline hydrochloride, mol/l.

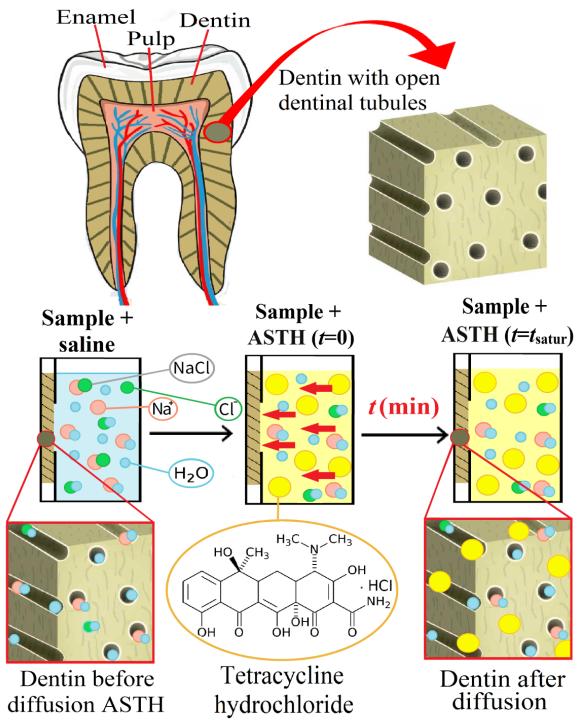


Fig. 4 Dentin samples during impregnation with an aqueous solution of tetracycline hydrochloride (ASTH).

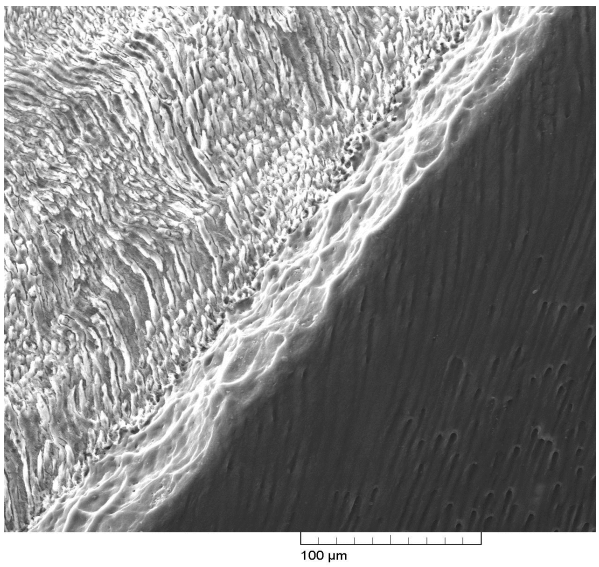
The recorded DRS ($R(\lambda)$, %) were converted using the standard Kubelka-Munk algorithm to extinction spectra $A(\lambda)$ using Shimadzu UV-2550 spectrophotometer software. An analysis of the kinetics of the difference in effective optical attenuation (ΔA) using Eq. (1) and taking into account the coefficients of approximation of experimental data made it possible to calculate the diffusion coefficient of the tetracycline hydrochloride (D).

4 Results and Discussion

4.1 Structure of Human Dentin

The crown of a human tooth is covered with enamel, the hardest tissue in the tooth (Fig. 5(a)). Dentin is the main mass inside the tooth, has a light yellow color and some elasticity; it is stronger than bone and cement, but 4–5 times softer than enamel. It consists of an intercellular substance penetrated by dentinal tubules (tubules), which determine its trophism (Fig. 5(b)) and which make a significant contribution to the diffusion of substances inside the tooth.

Using scanning electron microscopy (SEM), the number of dentinal tubules in the studied samples (n=3) was determined, which amounted to (20550 ± 800) units per mm^2. They have an inner diameter of 0.5 to 4 µm, depending on the area of the tooth. The average number of dentinal tubules per unit volume (density) in the middle part of the root dentin is significantly lower than in the middle part of the crown dentin. The density of tubules in the dentin located closer to the outer part of the tooth also differs significantly from the density located near the occlusal fissure. It has been noted that with a low density of dentinal tubules, they have a more branched structure. The size of the branches of the dentinal tubules differs depending on the location: the branches of the dentinal tubules located along the periphery have a diameter of 0.5–1 µm [22, 23].



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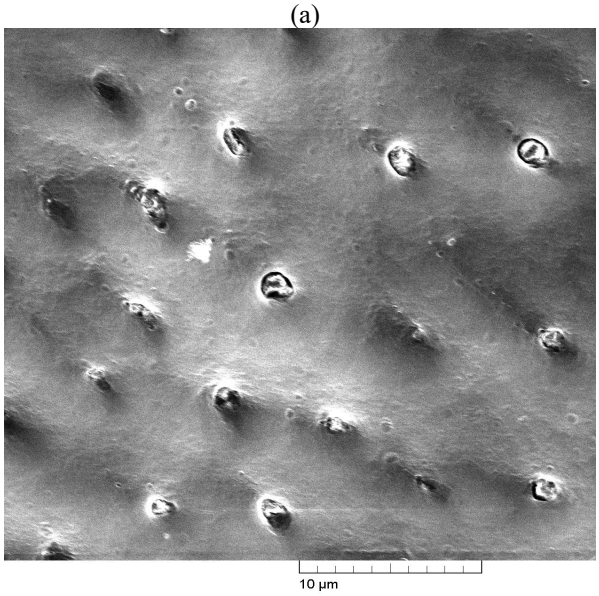
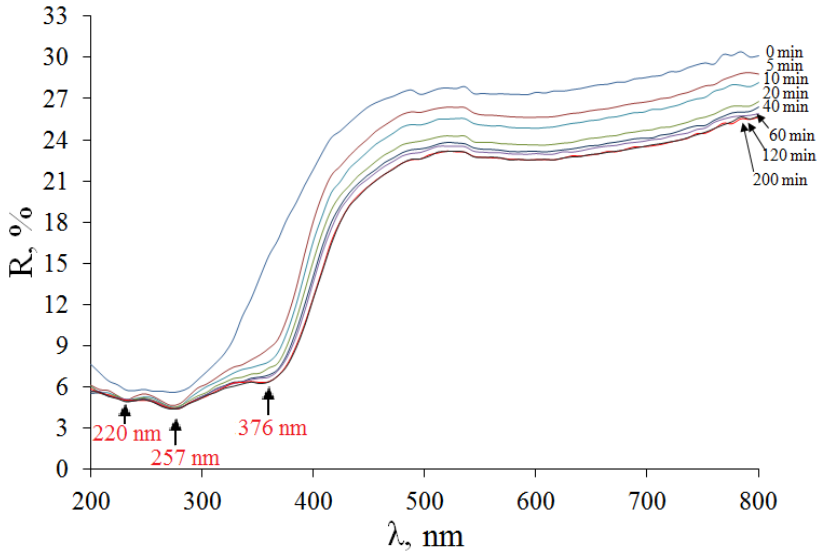


(b)

Fig. 5 Electronic micrographs (SEM) of a longitudinal cut (sample) of a human tooth: dentin-enamel junction (magnification 1k) (a); part of the saw cut of the crown (middle) (magnification 10k) (b).

4.2 Results of Spectrophotometric Analysis

DRS in the initial time has a characteristic appearance for dentin. By penetrating into dentin, ASTH changes the shape of the reflection spectra of dentin samples, which manifests itself in the form of characteristic absorption bands of tetracycline on the diffuse reflectance spectrum of dentin (Fig. 6(a)).



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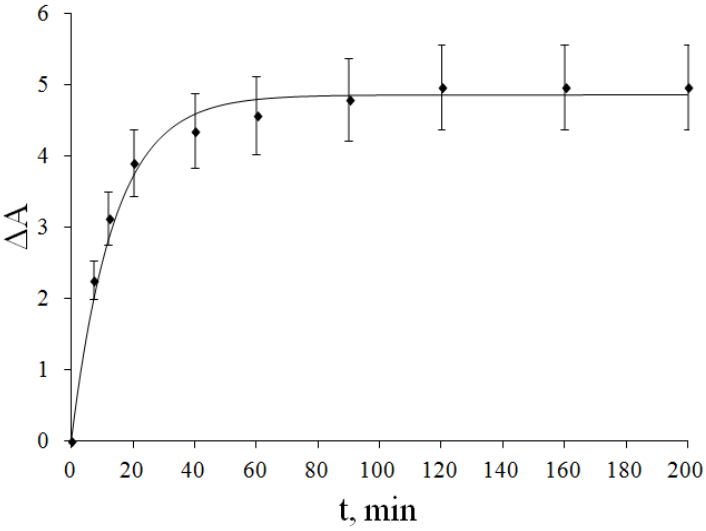


(b)

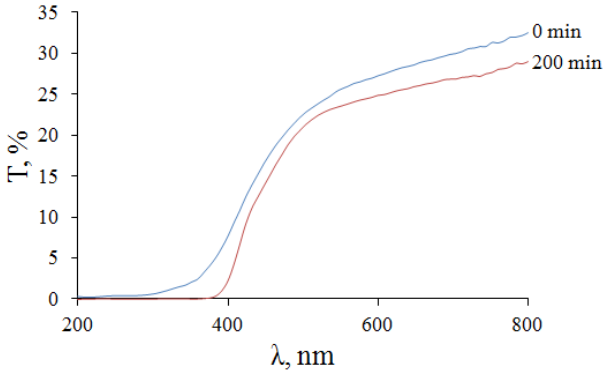
Fig. 6 Measured DRS of the dentin sample during its impregnation with the ASTH (a); kinetics of differential effective optical attenuation at λ = 376 nm calculated from experimental spectra using equations (1) and (2).

As the dentin saturated with ASTH, the diffuse reflectance decreases over the entire studied range, but most significantly from 320 to 800 nm, with a dip at 376 nm, which corresponds to one of the peaks of tetracycline hydrochloride. For the dentinal sample, the DRS of which is shown in Fig. 6(a), saturation occurred after 120 min of ASTH application, while the peaks did not shift and remained at their characteristic wavelengths. Fig. 6(b) shows a typical kinetic curve for the change in ΔA at 376 nm of the dentin sample during its impregnation with ASTH.

Diffusion coefficient of tetracycline hydrochloride was calculated using Eq. (1) and the least squares method. Calculations for each dentin sample were performed for five wavelengths in the spectral region of 360–400 nm, corresponding to the maximum absorption peak of tetracycline hydrochloride, and the obtained values were averaged. On average, the diffusion coefficient for the tetracycline hydrochloride is $D = (0.99 \pm 0.23)\cdot10^{-6}$ cm^2/s. The diffusion coefficient of an antiseptic solution of acridine dye (Rivanol) was measured as $(2.27 \pm 0.32)\cdot10^{-6}$ cm^2/s [24]. Also, the data obtained are consistent with the diffusion coefficient of methylene blue in the dentin of a human tooth, which turned out to be $(5.29 \pm 1.33)\cdot10^{-6}$ cm^2/s [25].

The permeability coefficient for 10% H_2O_2 in dentin was obtained as $7.2\cdot10^{-6}$ cm/s [26]. Using the relationship $P = D / l$ [21] and thickness of the studied dentinal samples $l = 0.5$, we get $D = 3.6\cdot10^{-7}$ cm^2/s. The diffusion coefficient of 40% glucose solution in dentin was found as $(5.4 \pm 0.8)\cdot10^{-6}$ cm^2/s [27]. The value of the diffusion coefficient is consistent with the data presented in the

work [28], where the diffusion coefficient in dentin was $0.27 \cdot 10^{-6}$ cm^2/s for water and $1.20 \cdot 10^{-6}$ cm^2/s for 40%-aqueous solution of glycerol. The scatter in the values of diffusion coefficients can be caused by a difference from sample to sample in the number density of dentinal tubules, surrounded by peritubular dentin, which is made from denser material, than the intertubular dentin, located between the tubules. In mature teeth, on transverse sections of dentinal tubules, peritubular dentin can be traced from the predentin to the dentin–enamel junction and occupies up to 60% of the area near the predentin–dentin junction, and about 2.5% at the dentin–enamel junction [29]. The number of dentinal tubules counted using SEM images in the studied cuts spreads from 6790 to 35076 per unit area 1/mm^2 with an average diameter from 1.98 ± 0.34 to 3.86 ± 1.02 μm [24].



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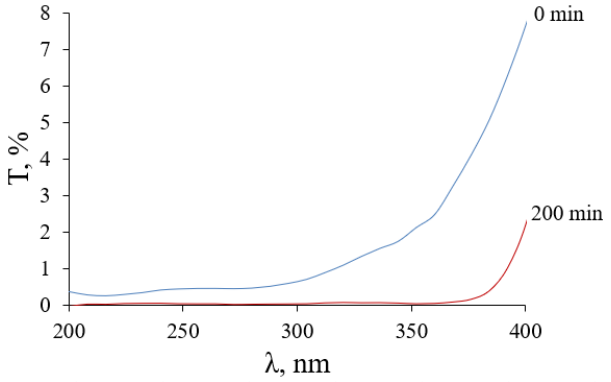


(b)

Fig. 7 Full transmission spectra of human dentin samples during diffusion of an aqueous solution of tetracycline in the wavelength range from 200 to 800 nm (a), from 200 to 400 nm (b).

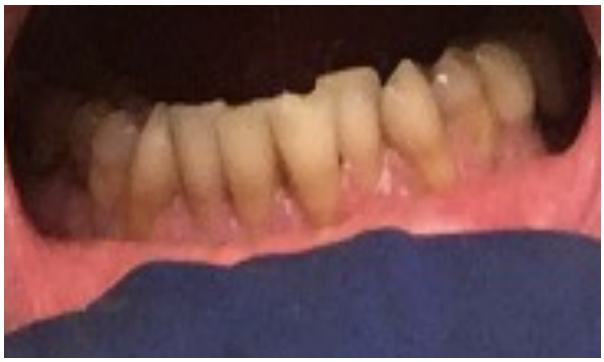
The obtained variability of diffusion coefficients in the range 10^{-6}–10^{-7} cm^2/s is well fit to variability of dentinal structures, this range is also typical for diffusion of studied molecules in other biological materials [30].

The total diffusion time τ of the tetracycline hydrochloride through entire dentin of thickness $l = 0.21 \pm 0.04$ cm (see Fig. 1b) can be calculated from experimental data for the diffusion coefficient $D = (0.99 \pm 0.23) \cdot 10^{-6}$ cm^2/s using equation valid for the one-side diffusion [31] as:

$$\tau = \left(4 / \pi^2\right)\left(l^2 / D\right) = (4.97 \pm 0.42) \text{ h}. \qquad (3)$$

The full transmission spectra of human dentin samples at the initial time and during vapor liquid diffusion are shown in Fig. 7 (a, b).

It can be seen that they correlate with the diffuse reflectance spectra over the entire wavelength range, since determined mainly by scattering on hydroxyapatite and collagen fibers. After the complete diffusion of the antibiotic in the dentin of the human tooth, the shape of the total transmission spectra of the dentin changes, the total transmittance does not significantly, but decreases over the entire wavelength range, which indicates an increase in the absorption of the sample (optically turbid medium – dentin).



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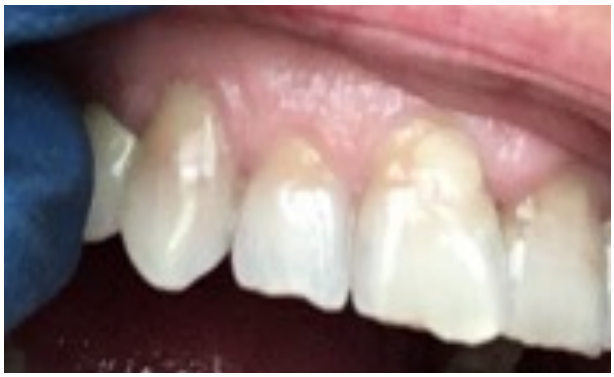

(b)

Fig. 8 Clinical cases of discoloration of teeth "tetracycline teeth" as result of the use of tetracycline antibiotics at an early age: man, 59 years old – oral tetracycline up to the age of 12 years, several courses (a); women, 28 years old – use of oral doxycycline at the age of 15 years, several courses.

4.3 Clinical Example of Tetracycline Teeth

"Tetracycline teeth" are found in people who have taken tetracycline antibiotics. The discoloration may affect the entire tooth, it may be gray, or there may be spots in the

form of horizontal stripes (e.g., streaks) that range from light yellow/gray to dark brown (Fig. 8 (a, b)).

These teeth photos show that tetracycline reaches dentin through pulp and strongly stains the tooth with regular use.

5 Conclusions

Our study is devoted to determination of the rate of diffusion of tetracycline from blood plasma through pulp to dentin. Diffuse reflectance spectroscopy using an integrating sphere was used to determine the diffusion coefficient of an aqueous solution of an antibiotic, tetracycline, into the dentin of a human tooth. The studies were carried out in the wavelength range from 200 to 800 nm. When calculating this kinetic parameter, the model of free diffusion was used. The study revealed that after complete saturation of the sample with an antibiotic, the total transmission coefficient of dentin decreases over the entire studied wavelength range. According to the well-known tetracycline pharmacokinetics after oral administration, tetracycline enters the tooth pulp after 2–3 hours. We determined that for about 4.97 ± 0.42 hours the entire dentin will be stained. The data obtained must be taken into account for the preparation of clinical protocols.

Disclosures

The authors declare that they have no conflict of interest.

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