# Pharmacokinetics of Chitosan-Ethacridine Adduct in Rats

**V.O. Antonyuk*†‡, N.O. Manko‡, I.O. Nektegaev†, R.S. Stoika†‡§**

† Danylo Halytsky Lviv National Medical University, Pekarska Str., 69, 79010, Ukraine; *e-mail: antonyukvo@gmail.com
‡ Institute of Cell Biology, NAS Ukraine, Lviv, Drahomanov Str. 14/16, 79005, Ukraine;
§ Ivan Franko National University of Lviv, Hrushevsky str.,4, 79005, Ukraine.

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*The objective of this study is to investigate the pharmacokinetics of the chitosan-ethacridine adduct in white rats. It was demonstrated that the kinetics of ethacridine release from adduct are significantly different from that for intraperitoneally introduced ethacridine. When free ethacridine was introduced, 80% of the administered amount was detected in blood of rats in 30 min. This quantity decreased gradually until 20 h, when 1.25% of the administered amount was detected. Contrary, for chitosan-ethacridine adduct, the ethacridine content in the blood slowly increased for 8 h and then decreased. Even after 72 h of the injection, 3% of the injected amount of ethacridine was detected. The experiment demonstrates a significant delay in the release of ethacridine from the chitosan-ethacridine adduct in the blood of treated rats. Thus, a possibility of using chitosan for prolongation of action of the ethacridine as an antiseptic agent was shown.*

**Keywords:** chitosan, ethacridine, chitosan-ethacridine adduct, release kinetics

# Фармакокінетика аддукту хітозан-етакридин у щурів

**В.О. Антонюк*†‡, Н. О. Манько‡, І.О. Нектегаєв†, Р.С. Стойка†‡§**

† Львівський національний медичний університет імені Данила Галицького, вул. Пекарська, 69, 79010, Україна; *e-mail: antonyukvo@gmail.com
‡ Інститут біології клітини НАН України, Львів, вул. Драгоманова 14/16, 79005, Україна;
§ Львівський національний університет імені Івана Франка, вул. Грушевського, 4, 79005, Україна;

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*Мета цього дослідження - дослідити фармакокінетику аддукту хітозан-етакридин у білих щурів. Кінетика вивільнення етакридину з аддукту в сироватці крові щурів суттєво відрізнялася від такої кінетики внутрішньочеревинно введеного вільного етакридину лактату. Коли вводили вільний етакридин, 80% введеної кількості було виявлено у крові щурів через 30 хв. Ця кількість поступово зменшувалася до 20 години, коли вона становила 1.25% від введеної кількості. Коли вводили хітозан-етакридиновий аддукт, вміст етакридину в крові щурів повільно збільшувався до 8-ої години. Після цього він повільно зменшувався, хоч через 72 год після ін'єкції все ще реєструвався на рівні 3% від введеної кількості. Експеримент демонструє значну затримку вивільнення етакридину з комплексу хітозан-етакридин у крові оброблених щурів. Таким чином, була показана можливість використання хітозану для продовження дії етакридину як антисептичного засобу.*

**Ключові слова:** хітозан, етакридин, етакридин-хітозановий аддукт, кінетика вивільнення

Chitosan is a linear polymer consisting of β-(1-4) D-glucosamine monomers. Usually, chitosan is isolated from chitin of hard shells of molluscss (shrimp, crabs, and crayfish) [1]. Chitosan is not soluble at alkaline conditions; however, it is soluble at weak acid medium (pH 3-6), in particular, it can be easily dissolved in 1% acetic acid. The dissolution rate and viscosity of chitosan solution primarily depend upon its molecular mass.

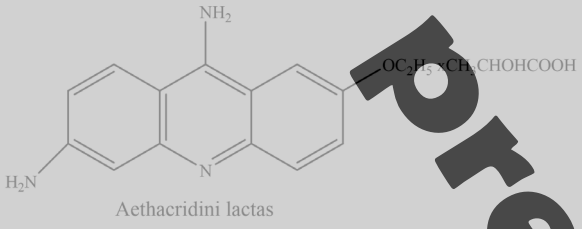
Chitosan is widely used in the medicine and food industry. Its biological properties strongly depend on the molecular structure, molecular mass, impurities, and some other parameters. For example, chitosan-based products are used for quick stop of bleeding and reducing blood loss (ex. commercial Celox). However, only chitosan samples of high molecular weight and high porosity are suitable for such application [2].

In both dissolved and undissolved states, chitosan can absorb different compounds including medicinal substances. Besides, chitosan can be chemically modified by introducing active groups to which various substances can be conjugated for their gradual release in the body. When chitosan is chemically modified, a change in the structure of the attached molecules can take place, thus affecting their biological activity [3]. Therefore, the formation of chitosan complexes without chemical modification of the conjugated biomolecules is desirable. Chitosan is used as a

carrier for medicinal substances of various chemical nature [4]. For example, chitosan was used as matrix forming agent when combined with amiodarone. In experiments were provided a prolonged release of amiodarone when compared to an industrial pharmaceutical product formulated as conventional release tablets [5]. However, the pharmacokinetics of most such complexes has not been studied thoroughly.

Here we used a synthetic drug ethacridine lactate (see Fig. 1) applied in surgery as an antiseptic in the treatment of venous leg ulcers [6], to prevent infections in local antineoplastic therapy [7] in dermatology for treatment patients with epidermolysis bullosa acquisita [8] and veterinary [9]. It is effective against coccas, especially streptococci [10]. Ethacridine is also used as an agent for second-trimester abortion [11].

The drug is of low toxicity for human body and does not induce tissue irritation. Its $LD_{50}$ is 70 mg/kg when administered subcutaneously to rats [12].




Aethacridini lactas

**Fig. 1**. Structure of ethacridine lactate, mol. mass – 361.4.

The objective of this study is to investigate the pharmacokinetics of the chitosan-ethacridine adduct in white rats. Due to the presence of numerous amino groups in the composition of chitosan, it can form unstable complexes with anions, the dissociation of which is slow. This makes it possible to prolong the action of drugs that are in such a complex. Prolongation makes it possible to avoid sharp fluctuations in the concentration of the active substance and reduce possible side effects. In addition, for substances with antimicrobial activity, which is ethacridine lactate, the creation of prolonged drugs reduces the chances of appear resistant forms of microorganisms.

However, in the study of the pharmacokinetics of chitosan complexes there are many difficulties, for example, the difficulty of measuring low concentrations of drugs in the serum. Therefore, there are not many such studies. The advantage of ethacridine is the strong fluorescence of this drug, which allows its fluorometric measurement in serum even at low concentrations. As shown earlier, it was found that the limit of detection by induced fluorescence is 1.13 μm, while for the spectrophotometric method the limit is 29.6 μm [13].

## Experimental part

*Chitosan sample and product purification.* The chitosan produced by "Tyanshi" company (China) was isolated from shells of crabs and it is recommended as a food additive. The purchased product was additionally purified from the fillers by its dissolving in 1 % acetic acid (1:20 of ratio) and subsequent precipitation with 0.1 N NaOH. The resulting precipitate was washed twice with distilled water, ethanol, acetone, and diethyl ether, afterward it was dried on air at room temperature. The product was grinded in the coffee-grinder and sifted through a 0.5 mm sieve.

*Determination of molecular mass and a degree of deacetylation.* In the obtained chitosan preparation, the average molecular mass was defined by using the viscosimetric method, as described [14]. The viscosity was measured at 25 °C with a viscosimeter Ubbehold of VPZh-4 type (VPZh-4, Soyuznauchpribor, USSR) with 0.82 mm diameter of a capillary. The viscosity of chitosan solutions was measured in a mixture of 0.1 M acetic acid: 0.2 M NaCl (1:2).

The intrinsic viscosity (η) was used for defining of molecular mass of chitosan. It was determined by measuring the relative viscosities, of a series of solutions of differing concentration. From these data the specific viscosity was calculated. An intrinsic viscosity was estimated by a fit of the Huggins equation (Eq. (1)) to reach the obtained results.

$$\frac{\eta_{sp}}{c} = [\eta] + k[\eta]^2 c, \qquad (1)$$

where $\eta_{sp}/c$ is a reduced viscosity, in $mLg^{-1}$, $\eta_{sp}$ is the relation between viscosity of the polymer in solution and the solvent, dimensionless, c is the concentration of the solution, in g $mL^{-1}$, and *k* is a constant valid for each polymer, dimensionless.

A degree of deacetylation of the obtained chitosan preparation was determined by its titration according to the following procedure: 50.0 mg of chitosan was dissolved in 0.02 N HCl. A dissolution was slow with a constant stirring for 2 - 4 h. The resulting solution was titrated with 0.1 N NaOH using phenolphthalein as an indicator of pH. A degree of deacetylation (DD, Eq. 2) was calculated by using the formula:

$$DD = \frac{203.2 \times 100}{42.0 + 1000 \times \frac{M}{C_{NaOH} \times V_{NaOH}}}, \qquad (2)$$

where m - mass in grams, $C_{NaOH}$ - molar concentration of alkali, $V_{NaOH}$ - volume of alkali in ml that was used for titration of chitosan salt.

*Preparation of chitosan-ethacridine adduct.* For preparation of chitosan-ethacridine adduct 100 mg of chitosan were stirred with 4 mL of 1 % $NaHCO_3$ solution. In order to improve wetting of chitosan powder, this mixture was stirred for 20 min in a hot water bath at 60-70 °C. The mixture was centrifuged, the supernatant was removed, 4 mL of 2 % aqueous solution of ethacridine lactate were added (in abundance). After 2 h of incubation in a test-tube, ethacridin lactate that not connected with chitosan

particles, was washed once with 10 mL of distilled water, then several times with 10 mL of 6 % aqueous NaCl solution until the supernatant was discolored, and finally, with 10 mL of 90 % ethanol. The resulting yellow powder of chitosan-ethacridine adduct was dried in a drying cabinet at 55 °C. The content of ethacridine in the obtained complex was determined spectrophotometrically according to calibration curve by measuring the extinction of the solution in 1% acetic acid at 364 nm.

*Covalent attachment of ethacridine lactate to chitosan*. 50 mg of shrimp chitosan powder (d < 0.1 mm) was mixed with 2 mL of 1% $NaHCO_3$ solution. Then 0.5 mL of 10 % $NaJO_4$ water solution was added. After 30 min incubation, chitosan out of $NaJO_4$ was water washed. To the oxidized chitosan was added 2 mL of 2 % aqueous solution of ethacridine lactate and incubated for 60 minutes. The conjugate was then washed with 1 M NaCl and water. 5 mg of sodium borohydride was added and after 30 min of incubation they were washed with 90 % ethanol, acetone and diethyl ether and air-dried.

*Experimental animals.* We used twenty-four white rats Wistar line weighing 210 ± 17 g at the experiments. They were randomly divided into four groups of six rats each (one control and three experimental). Animals were housed in plastic cages individually in a temperature- and light-controlled room.

*Study of pharmacokinetics of the chitosan-ethacridine complex.* Animals were injected intraperitoneally with 0.6 mL of 1% solution of chitosan complex containing 10 % ethacridine lactate dissolved in 0.15 M acetate buffer solution, pH 6.5. In parallel, rats were injected intraperitoneally with 0.6 mL of 0.1% solution of ethacridine lactate. In both experiments, 0.4 - 0.8 mL of blood in 1.0 mL of buffered saline that contained 2 % sodium citrate, protecting of hemolysis was collected through the same time intervals - 30 min, 4 h, 8 h, 20 h, 48 h, 72 h, 96 h. The erythrocytes were removed by centrifugation, the supernatant was diluted to 2.0 ml, and the amount of ethacridine was determined fluorimetrically on a Quantech Fluorimetr UV-VIS model QNT fluorimeter (US, Thermo Fisher Scientific) using NB 440 (excitation filter) and SC 535 (emission filter).

The content of ethacridine in the blood serum was determined fluorimetrically according to calibration curve by measuring the extinction of the well-known and experimental ethacridine lactate solutions using emission filter (SC 535).

*Study of antifungal activity*. The antifungal activity was determined using a method of counting colony-forming units (CSO). Pure chitosan, ethacridine lactate and chitosan-ethacridine complex were used in 0.1, 0.05 and 0.025 mg / mL doses and were incubated for 4 h with a suspension of yeast *Candida albicans* cells (approx. 1 million). Then, yeast cells were diluted 10,000 times, sown 200 µL on Petri dishes, and after 24 h incubation, numbers of colonies were counted. As a control (100 %), a quantity of colonies in the untreated group was taken (without entering the investigational drugs).

*Statistical analysis.* Each experiment was performed in triplicate and average values were recorded. The data were evaluated statistically using Student's t-test, and a value of $p \leq 0.05$ was statistically reliable.

## Results and Discussion

*Molecular mass and degree of deacetylation of "Tyanshi" chitosan.* Average molar weight, MW, at certain chitosan viscosity was calculated based on the intrinsic viscosity value utilizing the Mark-Houwink-Sakurada equation (Eq. (3)) [14, 15].

$$[\eta] = KM^{\alpha}, \quad (3)$$

where $K=1.81 \cdot 10^{-5} dl \cdot g^{-1}$ and $\alpha = 0.93$ are constants that depend on a solvent-polymer system [15].

From Eq. 4 we found value 1.97 $m^3/kg$ for intrinsic viscosity $[\eta]$ from the graph of dependence $\eta_{specific} = F(C)$.

$$\lg M = \frac{\lg[\eta] - \lg K}{\alpha}. \quad (4)$$

Results of determination of the viscosity of "Tyanshi" chitosan for calculated of the molecular mass are presented in Table 1.

"Tyanshi" Chitosan purified additionally from the contaminating substances, according to a viscometrical measurement, had an average molecular mass of ≈ 250 kDa.

A degree of deacetylation of studied chitosan preparation was 87±2.0 %.

**Table 1.** Results of determination of the viscosity of "Tyanshi" chitosan for calculated of the molecular mass.

| Concentration/ viscosity of chitosan | 0.25 kg/m³ | 0.625 kg/m³ | 1.25 kg/m³ | 2.5 kg/m³ |
|---|---|---|---|---|
| $\eta_{relative}$ | 1.51 | 2.38 | 4.25 | 8.18 |
| $\eta_{specifity}$ | 0.51 | 1.38 | 3.25 | 7.18 |
| $\eta_{reduced}$ | 2.04 | 2.21 | 2.6 | 2.87 |

*Characteristic of chitosan-ethacridine complex (adduct).* A developed chitosan-ethacridine complex obtained as descripted in the "Experimental part" section contained 10 ± 1.0 % of ethacridine lactate per dry powder mass (the chitosan: ethacridine molar ratio was 1 : 77). This complex is soluble in 1 % acetic acid and in 0.1-1.0 M acetate buffer with a pH of 4.0 - 6.5. At pH above 6.5, this chitosan preparation gets to be insoluble. It is not possible to administer a very acidic solution of chitosan to animal, because that can cause necrosis at the injection site. Therefore, chitosan was dissolved in 0.1 M acetic acid and overnight dialyzed against acetate buffer with pH 6.5, after which chitosan dissolves completely.

UV spectra were measured on a NanoDrop 1000 spectrophotometer 3.8.1 (USA). The UV absorption spectrum of chitosan, ethacridine and chitosan-ethacridine adduct are presented in Fig. 2.

IR spectra were measured on a SPECORD M-80 spectrophotometer in KBr tablets. The IR spectrum of chitosan-ethacridine adduct and conjugate as well as free chitosan and ethacridine lactate was measured. These spectra are presented in Fig. 2.

*The kinetics studies.* The kinetics of ethacridine lactate liberation with chitosan complex to rats' blood differs significantly from that for free ethacridine lactate. At introducing free ethacridine, in 30 min 80 % of the amount of administered drug was detected in rats' blood, and that amount decreased gradually until 20th hour, when it reached 1.25 % of the administered amount. When the chitosan-ethacridine complex was administered, the ethacridine content in blood of treated rats slowly increased till 8th h. After that, it slowly decreased, although 72 h after the injection, it was still recorded in 3 % of the injected amount (see Fig. 3 and 4).

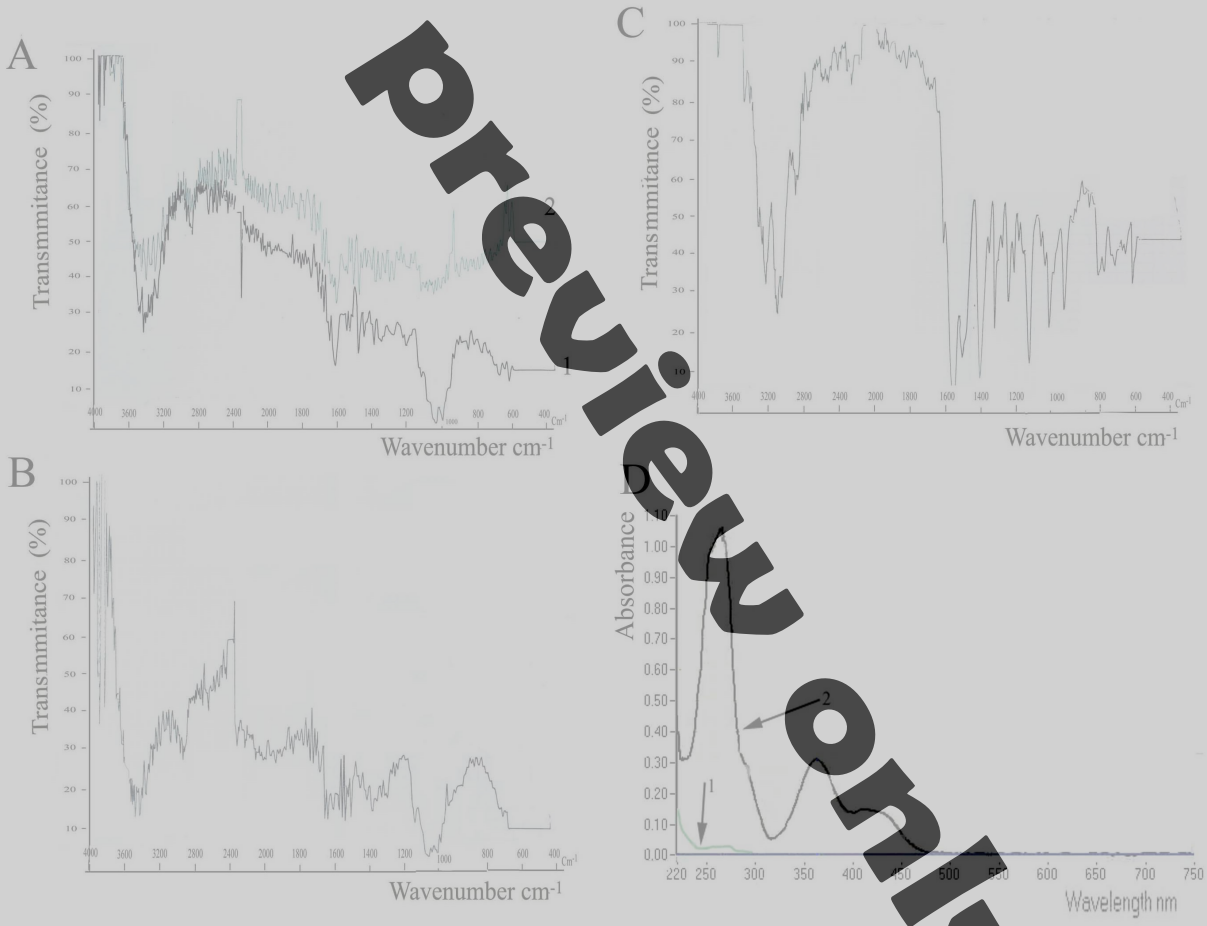



**Fig. 2.** IR spectrum of chitosan-ethacridine adduct (Fig. 2A, line 1) and conjugate (Fig. 2A, line 2), chitosan (Fig. 2B) and ethacridine lactate (Fig. 2C). UV absorption spectrum of 0.025 % of chitosan solution in 1 % acetic acid (Fig. 2D, line 1) and chitosan-ethacridine adduct (Fig. 2D, line 2). Spectrum of ethacridine lactate is almost identical to the UV spectrum of the chitosan-ethacridine adduct (Fig. 2D, line 2).

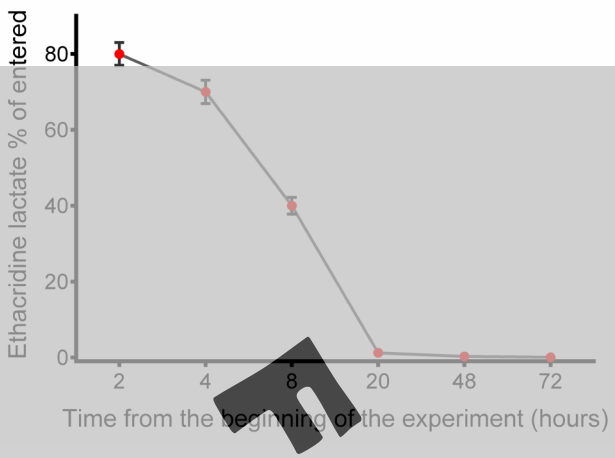



**Fig. 3.** Kinetics of a release of ethacridine lactate in blood of rats.

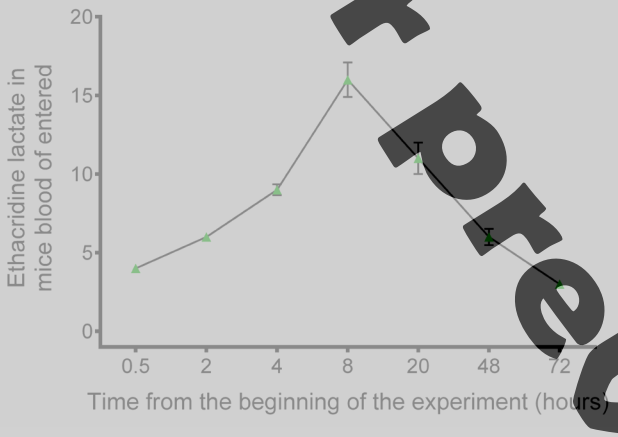



**Fig. 4.** Kinetics of a release of ethacridine lactate from the chitosan-ethacridine complex in blood of rats.

*Antifungal activity.* Next, we carried out experiments in order to compare the antifungal activity of the chitosan-ethacridine complex with that of free forms of chitosan and ethacridine lactate (Fig 5).

Thus, the chitosan-ethacridine complex at a concentration of 0.1 and 0.05 mg/ml shows higher antifungal activity towards *C. albicans* than pure chitosan or ethacridine lactate after 4 h of their action.

In this study, we aimed to create an ethacridine-chitosan conjugate by covalently attaching ethacridine to this natural polymer. To achieve this, chitosan was oxidized in an alkaline medium with sodium periodate and formed aldehyde groups of chitosan to conjugate ethacridine through its amino group. Thus, 25 % - 30 % ethacridine was detected in the resulting product. However, as was established in the experiments, the resulting complex contains both covalently attached ethacridine and ethacridine adsorbed probably, through hydrogen bonds. It is almost impossible to completely wash the adsorbed ethacridine from the covalently attached. It is difficult to determine the parts of ethacridine that is combined in various ways. In addition, the conjugate is a new compound that may have other antimicrobial properties. And what contribution to these properties of the various components of the resulting product remains unknown. To maintain the purity of the experiment, we abandoned the covalent conjugation of ethacridine and considered only the complex with non-covalently bound ethacridine (adduct).

The formation of chitosan-ethacridine complex, due to the sorption of ethacridine, raises the problem of stability and reproduction of such a complex since it washes out the sorbed ethacridine when washing out water. The amount of drug added varies depending on the ratio of the complex to water, ambient temperature and other factors. Because ethacridine is insoluble in 0 % NaCl solution, washing it provides a reproducible composition of the complex. At the final stage of its formation, the complex washed with sodium chloride remains washed with only 90 % ethanol.

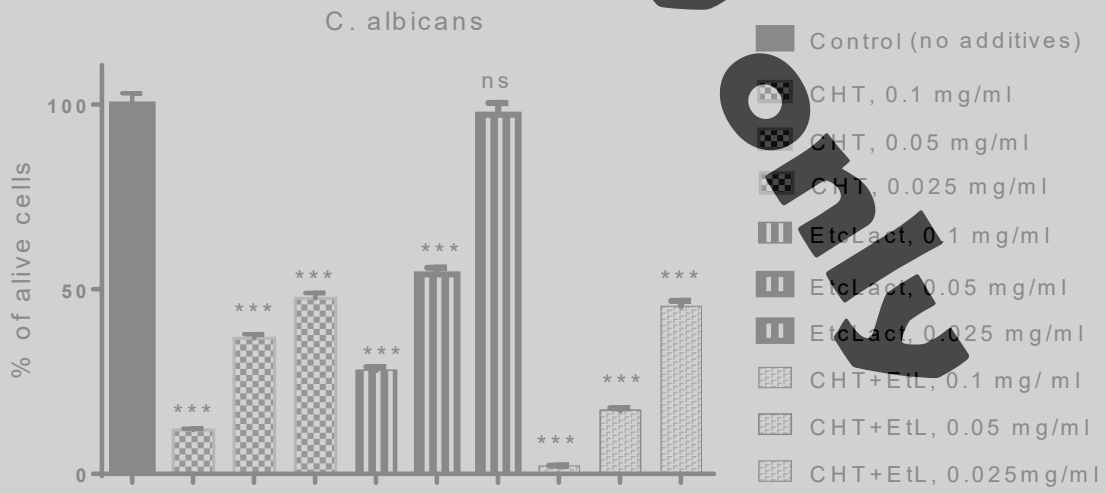



**Fig. 5.** Indicators of antifungal activity of chitosan (CHT), ethacridine lactate (EtcLact), and chitosan-ethacridine complex (CHT + EtL).

The UV spectra of the chitosan-ethacridine adduct, and conjugate did not show a difference between the native ethacridine lactate, possibly due to the slight absorption of chitosan only in the 220-230 nm range. At the same time, the IR spectra were more informative. Comparing the spectra of the chitosan-ethacridine conjugate with chitosan-ethacridine adduct, we can conclude that they coincide as a whole and result of imposition from the spectra of two compounds, chitosan and ethacridine lactate. The main difference is observed in the range of 1100-1000 $cm^{-1}$, where the bands of absorption of bending vibrations of hydroxyl groups of OH are manifested. In the adduct spectrum, a broad absorption band is observed, and in the conjugate spectrum this band is of much lower intensity, which can be explained by the fact that oxidized chitosan sodium periodate is used in the conjugate (Fig. 2).

The main difference between the absorption spectra of the adduct and conjugate from native ethacridine lactate lies in the shift of the vibrational band of the carboxyl group into the high-frequency region. Thus, in ethacridine lactate, the C = O bond stretch vibrational vibrations occur at 1552 $cm^{-1}$, and in the adduct and conjugate this peak manifests at 1632 $cm^{-1}$, which can be explained by the formation of a complex with the amino group of chitosan.

The method described in the section “Experimental part”, allows to obtain a complex of ethacridine with chitosan without significant deviations in its composition. The kinetics of ethacridine release from such an adduct can be characterized by the amount of ethacridine in the blood of experimental rats depending on time. The results of the experiments showed a significant delay in the release of ethacridine in the blood of rats from the chitosan-ethacridine adduct. Thus, it is possible to use chitosan to continue the action of ethacridine as an antiseptic agent.

A possible cause of the slow release of ethacridine lactate from the chitosan complex is the ion-exchange properties of chitosan and a decrease in the solubility of the complex at physiological values (pH 7.4). Chitosan has many amino groups that can attract anions, in particular, lactate ions in ethacridine lactate. This may prolong the release of ethacridine from the complex. The chitosan-ethacridine adduct was administered in solution at pH 6.5. It can then bind to acidic whey proteins, forming slightly soluble complexes that decompose more slowly than similar complexes with ethacridine without chitosan. Participation in this process of specific enzymes is not excluded.

To summarize, chitosan is a promising basis for the binding of biologically active substances, including antifungal agents, as it provides for their gradual release and increased local activity.

## Conclusions

A method for a formation of chitosan-ethacridine complex (adduct) due to non-covalent binding between starting materials and method for stabilizing its composition was developed. Content of bound ethacridine is reached relative to dry mass of complex in 10 %.

The kinetics of a release of ethacridine in serum blood of rats (free and in the chitosan-ethacridine adduct) differs significantly. It was established that in the case of intraperitoneal administration to rats of ethacridine lactate, its amount in the blood after 30 min is maximal (≈80 % of the injected), remains approximately at this level for 3 h, and then gradually decreases, and 20 h after the input is only 1.25 % of the input dose.

In the case of intraperitoneal administration of the chitosan-ethacridine adduct, the amount of ethacridine slowly increases to 8 hours after administration and amount to maximum of ≈16 % of the amount of ethacridine administered, and then slowly decreases, but 72 hours after injection was still recorded at 3 % quantity from the entered dose.

The influence of ethacridine binding with chitosan on antifungal activity was investigated. The chitosan-ethacridine adduct at a concentration of 0.1 and 0.05 mg/ml after their 4 h action shows higher antifungal activity towards *C. albicans* than pure chitosan and ethacridine lactate.

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