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# Influence of polyfunctional interactions between organic zwitter-ion eremomycin and carboxylic cation exchangers on forming concentration front

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**Abstract**

The nature of the polyfunctional interactions between the organic zwitter-ions of eremomycin and the carboxylic sorbents was studied. A cation exchange and hydrophobic mechanisms of interactions in the studied sorption system were determined as preponderant over other ones. The typical features of the eremomycin diffusion into the pores of the structurally segregated carboxylic sorbents were studied. The physical–chemical properties of the sorption system which influence the formation of the target product concentration zone with sharp boundary were investigated. The effect of column sizes on the eremomycin unified elution peak formation was shown. The height to diameter ratio leading to the eremomycin concentration front division into two zones was determined.


*Keywords:* Preparative chromatography; Antibiotics; Eremomycin; Carboxylic sorbents; Polyfunctional binding; Concentration profiles

## 1. Introduction

At present displacement chromatography mode is one of the most common methods of frontal preparative isolation of high purified biologically active substances [1–7]. In this method the efficiency of the target substance isolation to a great degree depends on forming the rather sharp boundary between sorption zones [8–10].

The use of polyelectrolytes in biology, medicine and in the pharmaceutical industry has made essential elucidation of the general patterns of their interaction with organic ions, in particular with ions of physiologically active substances. These patterns differ from those operative in classical ion exchange as a consequence of a number of factors, such as limited permeability of ion exchangers for complex ions, partial accessibility of functional groups even when the permeability of the cross-linked polyelectrolyte is high, polyfunctional interaction, and low rate diffusion of ions through the sorbents grains. Special attention must be paid to studying the sorption of organic zwitter-ions such as peptides and proteins, since they can behave as cations or anions, dependeing on the solution pH value. Moreover, hydrophobic interaction can often be an important factor in sorption of organic ions and can be accompanied by the Donnan effect [11–14]. From this point of view, it is important to choose the type of sorbent's functional groups with their degree of ionization being checked very carefully and to recognize how the fixed groups and counterions are disposed within polymeric matrix to yield the stable connections [14,15].

The main object of this work was eremomycin. This is an antibacterial antibiotic with a wide range of chemical–therapeutic effects, which greatly depend upon the safety the native antibiotic structure [16]. Chromatographic methods of isolating biological substances allow to create soft conditions for isolating the biologically active substances of the desired quality. Since the development of efficient preparative separation routing of the antibiotics isolation is very essential, the study of complex molecular interactions is of

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primary importance both for better understanding the sorption retention mechanisms and for developing purification schemes based on using chromatographic unit-operations.

In frontal displacement chromatography the sorbent is much more saturated than in a suspension, because in most of the column the sorbed substance is in equilibrium with initial solution. However, the desorption of organic ions is very difficult because of their strong affinity for the sorbent [3]. The eluate will contain a high proportion of the sorbed substance in the eventual desorption step only if its affinity for sorbent can be sizably reduced [14].

The aim of our work was to analyze the equilibrium, kinetics and dynamics of interaction of eremomycin with carboxylic sorbents, to find out conditions of forming the concentration zone with sharp boundary and to develop efficient chromatographic method for the preparative isolation and purification of target product.

## 2. Experimental

### 2.1. Chemicals

Purified crystalline samples of eremomycin sulfate (93.8% of purity) used in this study were prepared from a culture broth of INA-238 actinomycetes in Scientific Research Institute for New Antibiotics, Russian Academy of Medical Sciences, Moscow, Russia. Eremomycin is a zwitter-ion as it includes three amino groups ($pK^1_{\alpha 1}$ 6.9, $pK^2_{\alpha 1}$ 7.9, $pK^3_{\alpha 1}$ 9.0), phenyl groups ($pK^1_{\alpha 2}$ 9.7, $pK^2_{\alpha 2}$ 10.4, $pK^3_{\alpha 2}$ 11.35) and a terminal carboxylic group ($pK_\alpha$ 3.1) (Fig. 1). The molecular weight of eremomycin ($C_{73}H_{89}N_{10}O_{20}Cl$) is 1540 [17].

The following sorbents were tested: carboxylic cation exchangers of BDM type produced in Institute of Macromolecular Compounds, Russian Academy of Sciences, St. Petersburg, Russia by means of radical copolymerization of methacrylic acid (MA) and the hydrophobic cross-linking agent ethylene glycol dimethacrylate (EGDM) [18,19].

The aqueous buffers of needed ionic strengths were prepared from crystalline ammonium acetate (pure for analysis) and distilled water.

### 2.2. Aparatus

The pH measurements were taken with the laboratory ionmeter I-160 M manufactured by “ANTECH”, Moguilev, Belarus.

The sorption equilibrium and kinetics parameters were studied statically [9,10]. For optical density measurements we used a spectrophotometer SPh-26 manufactured by “LOMO”, St. Petersburg, Russia.

Dynamic sorption was conducted on the laboratory columns of different sizes. A pump PP-1M (Russia) and a

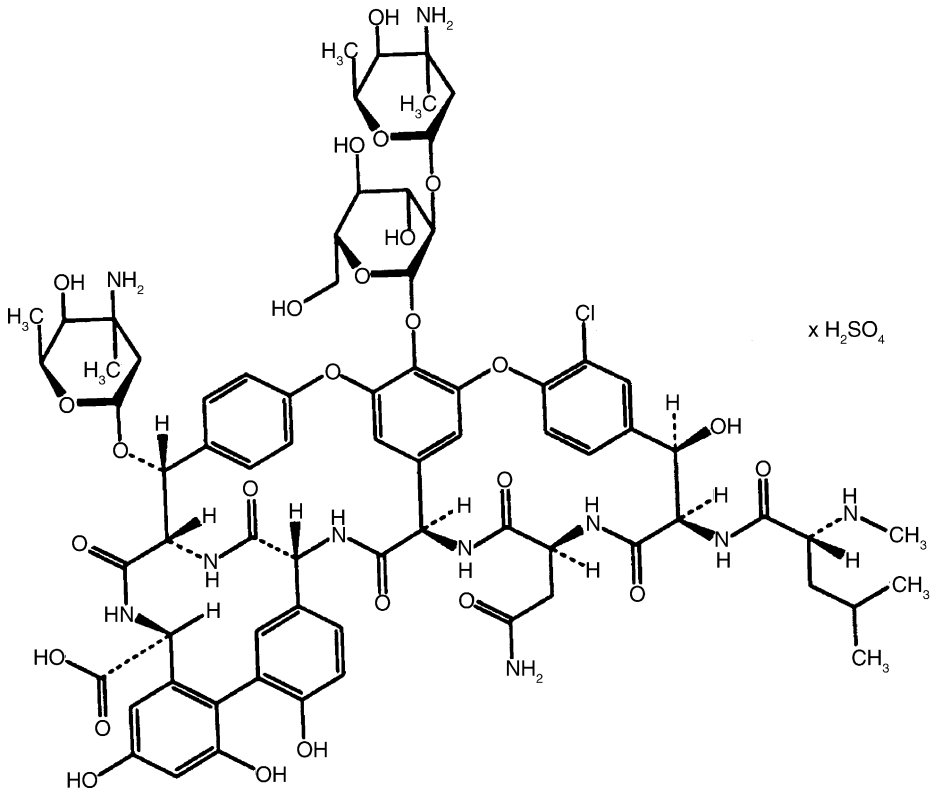


Fig. 1. Structural formula of eremomycin sulfate: molecular mass ($C_{73}H_{89}N_{10}O_{20}Cl$) = 1540; terminal carboxylic group $pK_\alpha$ = 3.1; amino groups $pK_\alpha$ = 6.9; 7.9; 9.0; and phenyl groups $pK_\alpha$ = 9.7; 10.4; 11.35.

fraction collector FCC-60 (Prague, Czech Republic) were used.

### 2.3. Methods

#### 2.3.1. Equilibrium sorption of eremomycin

The experiments on sorption of eremomycin were carried out as follows: 10 ml of eremomycin solution in 0.2 M ammonium acetate with known antibiotic content was added to 10 mg of swollen sorbent. The solution had been stirred with sorbent for 24 h until equilibrium was reached. Eremomycin concentration in the equilibrium solution was determined by registering UV absorbency at 280 nm with the use of the calibration curve. The sorption capacity of sorbent was defined as:

$$m = \frac{(C - C_{\text{equil}}) \cdot V}{m_{\text{s}}} \times 1000 \quad (1)$$

where $C$, $C_{\text{equil}}$ are initial and equilibrium concentrations of eremomycin correspondingly (mg ml$^{-1}$); $V$ is solution's volume (ml); $m_{\text{s}}$ is sorbent's mass ($g$).

#### 2.3.2. Determination of kinetic parameters

The kinetic experiments were carried out as follows: 20-ml of eremomycin solution in 0.2 M ammonium acetate with the eremomycin content of 1 mg ml$^{-1}$ was added to 50 mg of swollen sorbent. In the course of the kinetic experiment performed with continuous stirring, 0.2-ml samples were taken at regular intervals of time $t$ (s). The eremomycin concentration in the solution was determined spectrophotometrically. The fraction of the total sorption capacity of the sorbent consumed by the sorbate, $F$, was calculated as $F = m_t/m_\infty$, where $m_t$ is the sorption capacity in time $t$; and $m_\infty$ is the equilibrium sorption capacity. Main kinetic parameters were defined from kinetic curves $F = f(t^{1/2})$.

The kinetic parameters were found to be best described by "shell and core" mathematical model [9,14,15]:

$$F = \frac{1}{L} \frac{6}{\left[3 - \frac{L}{R}\left(3 - \frac{L}{R}\right)\right]} \sqrt{\frac{\bar{D}t}{\pi}}, \quad (2)$$

where $\bar{D}$ is the effective diffusion coefficient (sm$^2$ s$^{-1}$); $R$ is the radius of the swollen cation exchanger granule (μm); $L$ is the absorptive layer thickness (μm).

The average time of diffusion, $\bar{t}$ was calculated as:

$$\bar{t} = L^2 \frac{(1 + 3\rho + 6\rho^2 + 5\rho^3)}{15\bar{D}(1 + \rho + \rho^2)}, \quad (3)$$

where $\rho$ is the relative non-absorbing radius of the "core" and it equals:

$$\rho = \frac{R - L}{R}. \quad (4)$$

#### 2.3.3. Dynamic sorption of eremomycin

The frontal dynamic sorption was conducted on the laboratory columns of different sizes. Sorbent was stabilized with 0.2 M ammonium acetate, pH 7.2. The 30-ml eremomycin solution in 0.2 M ammonium acetate (pH 7.2) with antibiotic initial concentration of 1 mg ml$^{-1}$ was loaded to its working capacity. The ammonium acetate solution of different ionic strength and pH were used to elute the antibiotic from sorbent. The concentration on the column outlet was controlled spectrophotometrically.

## 3. Results and discussion

### 3.1. The equilibrium sorption of eremomycin on carboxylic cation exchangers of BDM-group

As eremomycin is a polypeptide (Fig. 1), it can show amphoteric behavior. Its actual form as basic, neutral or acid depends on the pH value and, consequently, influences sorption characteristics. The effect of the solution pH on the sorption capacity of carboxylic cation exchangers of BDM-group with respect to eremomycin is shown in Fig. 2. Since at low pH values (3.5–4.0) the BDM resins are uncharged, binding the eremomycin cations by resins may be elucidated by non-exchange (particularly hydrophobic) interactions in the sorption system. The improvement of the eremomycin sorption can be observed while pH increases from acidic to neutral values. More complete sorption is observed in the neutral pH range when both eremomycin and carboxylic sorbents (p$K_\alpha$ values about 5–7) are maximally charged. The antibiotic polyfunctionally binds to the sorbents with cation exchange dominating over hydrophobic and other types of interactions. Moreover, in these conditions eremomycin ions exist as cations, zwitter-ions and anions, and can interact each to the other forming associations [9,20]. Diffusion of the eremomycin associations into sorbents matrix is not impeded owing the BDM sorbents' high permeability.

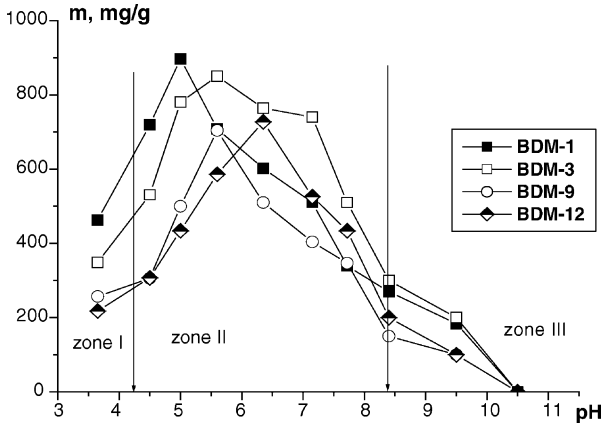


Fig. 2. Sorption capacity of the carboxylic cation-exchangers of BDM-group to eremomycin as a function of pH. The figure in the sorbent name is quantity of cross-linking agent (EGDM) in mol%. The I zone demonstrates binding zwitter-ion eremomycin with the weak charged sorbents; the II zone demonstrates polyfunctional connecting (the ion exchange dominates in binding); the III zone is related to the sorption of protonized eremomycin.

Under the further increasing of pH to alkaline values, the antibiotic amino groups become uncharged. That limits the ion exchange in the sorption system. However, both sorbents' fixed groups and the eremomycin terminal carboxylic group are negatively charged under such pH value. As a result, in high alkaline solution sorbents' fixed groups repel the antibiotic anions. This repulsion impedes any interactions and leads to the sorption discontinuance (Fig. 2).

The equilibrium experimental data have allowed us to choose conditions of the eremomycin selective sorption on the carboxylic exchangers. As it has been established, the main conditions for complete desorption of eremomycin from the sorbent phase are achieved by changing the degree of ionization of the antibiotic functional groups under varying pH value of solution.

### 3.2. Kinetic parameters of eremomycin sorption on carboxylic sorbents

Kinetics plays an essential role in formation of a sharp chromatographic zone boundary. On analyzing behavior of sorption system in irregular sorption depending on different physical–chemical conditions, it is possible to understand how heterogeneous mass transfer is realized in the studied system.

The rate limiting sorption step is easily identified in irregular process from the shape of the initial portion of the $F = f(t^{1/2})$ curves (Fig. 3). Their linear dependence demonstrates the intraparticle kinetics of eremomycin on the BDM-12.

Curve 1 (Fig. 3) demonstrates the mass transfer under conditions of the eremomycin polyfunctional interaction with sorbent. The smooth initial portion of the kinetic curve shows that the zwitter-ions gradually redistribute into the heterogeneous medium of the structurally segregated sorbent [20,21]. In an irregular sorption the diffusion process is very often accompanied by both absorbing and ousting the external solution as a result of a concentration gradient. The absorbed eremomycin dipoles are held up in the sorbent interglobular space (transport canals) mainly by hydrophobic, coulomb and other interactions [14,15]. As there are only about 10% of the fixed carboxylic groups in the transport pores of BDM sorbents, the ion exchange can proceed insignificantly [16]. But for ion exchange kinetics, the limiting step is determined by a gradient of chemical potential. Thus, the eremomycin

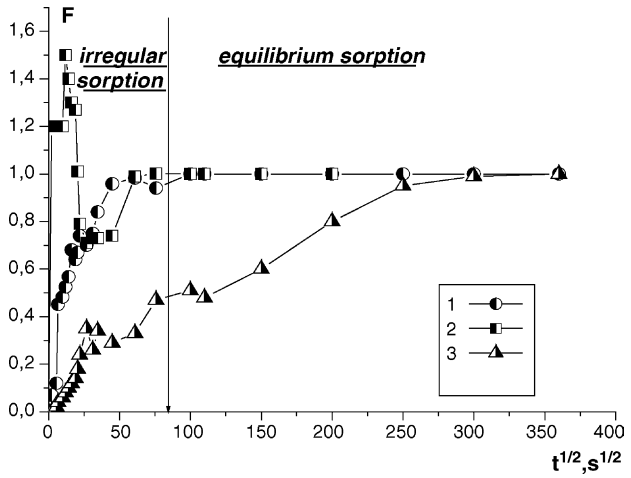


Fig. 3. Kinetics of eremomycin sorption on carboxylic cation exchanger BDM-12. Sorption conditions: (1) from 0.2 M ammonium acetate, pH 6.9; (2) from 0.2 M ammonium acetate with 0.4 M NaCl, pH 6.9; (3) from 0.2 M ammonium acetate with 50% isopropyl alcohol, pH 6.9.

ions penetrate into the microglobular pores, polyfunctionally interacting with sorption centers up to when an equilibrium is achieved.

As kinetics of the cation exchange sorption is significantly slower than that for hydrophobic interaction [22], it is important to find out differences between the mass transfer parameters of both kinetics types. Sorption kinetics with the domination of hydrophobic interaction was studied under conditions when the ion exchange between eremomycin and the BDM-12 was suppressed (Fig. 3, curve 2). Some fluctuations of the $F$ values on the initial portion of the kinetic curve refer to phenomena of Donnan's exclusion. Very often organic ions can be absorbed superequivalently, but in an irregular sorption they cannot completely bind with sorption centers and are pushed out from sorbent phase under concentration gradient [15]. As it is seen from Table 1, the equilibrium sorption capacity, $m_\infty$, under mainly hydrophobic sorption, is much less than when that one is under complete polyfunctional binding eremomycin with the BDM-12.

The cation exchange kinetics was studied under conditions of suppressing the hydrophobic interactions. For that, isopropyl alcohol was added into solution of the antibiotic. The slope of the initial portion of curve shows that the cation exchange rate is very slow (Fig. 3, curve 3). However, the $m_\infty$ parameter achieves about the same value as it is for

Table 1
Dependence of eremomycin sorption kinetics by BDM-12 on the physical–chemical conditions

| Kinetic parameters | The physical–chemical conditions of eremomycin sorption on BDM-12 | | |
|---|---|---|---|
| | A (polyfunctional binding) | B (hydrophobic interactions) | C (cation exchange) |
| $tg\ \alpha$ | 0.034 | 0.058 | 0.006 |
| $\overline{D}$ ($cm^2\ s^{-1}$) | $3.6 \times 10^{-9}$ | $1.05 \times 10^{-8}$ | $1.16 \times 10^{-10}$ |
| $\overline{t}$ (min) | 6.44 | 1.77 | 161 |
| $m_\infty$ ($mg\ g^{-1}$) | 480 | 150 | 460 |

Sorption conditions: (A) 0.2 M ammonium acetate, pH 6.9; (B) 0.2 M ammonium acetate with 0.4 M NaCl, pH 6.9; and (C) 0.2 M ammonium acetate with 50% isopropilic spirit, pH 6.9.

sorption under polyfunctional interaction of eremomycin with the BDM-12 (Table 1). Since the preliminary study of the eremomycin sorption kinetics has showed heterogeneous distribution of the antibiotic into the limited volume of sorbent, the "shell and core" model has been used to describe the main parameters of eremomycin sorption kinetics [9].

So the study of the effects of the different external factors on equilibrium and kinetic parameters of the eremomycin sorption on the carboxylic cation exchangers has allowed us to optimize the conditions for the antibiotic dynamic displacement sorption.

### 3.3. Dynamic sorption

Conditions of reversible dynamic sorption of eremomycin on the BDM-12 carboxylic exchanger have been chosen in accordance with data obtained during the equilibrium sorption experiments. For the selective binding of eremomycin with the BDM-12, the dynamic sorption was realized from 0.2 M ammonium acetate with the neutral pH value. To provide favorable conditions for the elution of the antibiotic, its degree of ionization was sharply decreased by raising the pH of the eluting solution to the high alkaline value. Fig. 4 exhibits the eremomycin front is being concentrated and sharpened with the increase of the eluent pH to more alkaline values. Gaussian shapes of the dynamic curves demonstrate that the process occurred in a regular regime.

Very often dynamic process can proceed under nonequilibrium conditions, which are introduced by low rates of heterogeneous mass transfer exchange in some sorption system. However, the mass transfer of eremomycin on BDM-12 is characterized by the high-effective kinetic parameters (Table 1), which allow the regular dynamic sorption to proceed in the wide range of the antibiotic solution flow rate. Breakthrough curves in Fig. 5 demonstrate that nonequilibrium dynamic sorption takes place only when the rate is more than 3.5 ml $min^{-1}$.

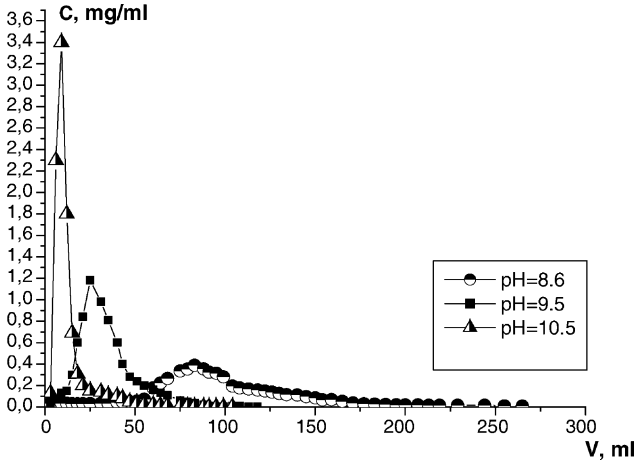


Fig. 4. The effects of eluent pH on shaping the eremomycin concentration front on BDM-12. Sorption was realized from 0.2 M ammonium acetate, pH 6.9; eluent was 0.2 M ammonium acetate, pH 8.6; 9.5; 10.5.

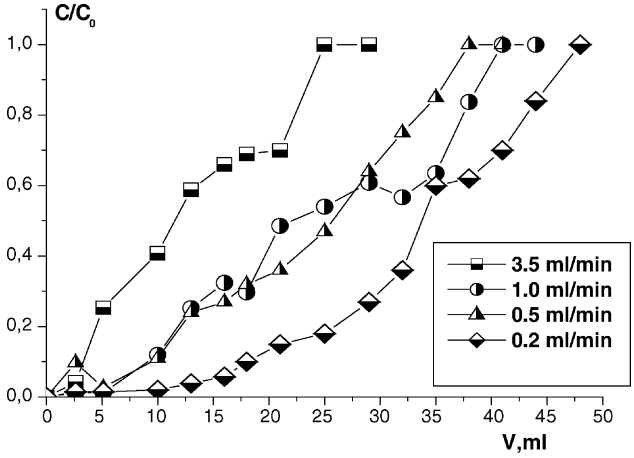


Fig. 5. The effects of the eremomycin frontal sorption velocity on forming stationary front.

Study of influence of eluate flow rate on elution curve shapes has also demonstrated the eremomycin dynamic desorption realized in regular regime. As it is seen in Fig. 6, the peak becomes more narrow and symmetrical with decrease of the eluent flow rate.

In the previous experiments, it has been shown that reversibility of the eremomycin sorption on the carboxylic exchanger to a considerable extent depends on degree of ionization of the antibiotic amino groups. Besides varying the pH value, this parameter can be controlled by varying the ionic strength value, too [9]. In a frontal ion-exchange process a chromatographic zone moves through the column and desorbed counterions moving in front of chromatographic zone interact with free sorption centers. If the pH gradient and ionic strength of eluent are not enough to suppress this interaction, the ions are capable of rebinding with sorbent. Such behavior in sorption system is observed when dynamic desorption of eremomycin is realized by 0.1 M ammonium acetate

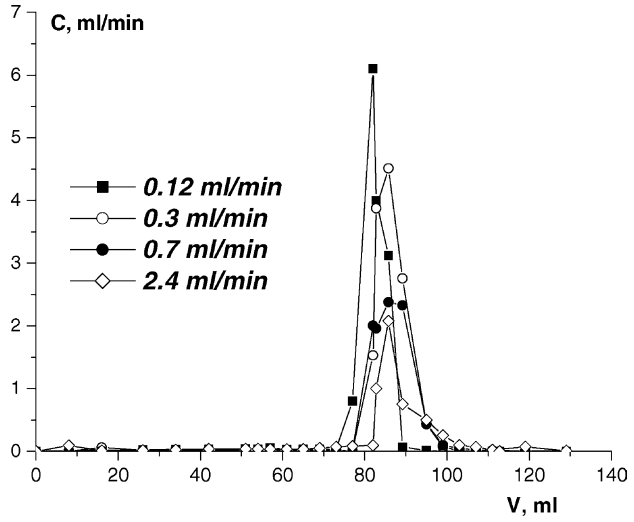


Fig. 6. Dependence of forming the eremomycin concentration front on the eluent velocity.

aqueous solution with pH 10.5. Since the pH value in front of the eremomycin concentration zone is less than initial pH of eluent and ionic strength of eluent is low enough, the displaced ions of eremomycin rebind with free sorption centers breaking the chromatographic zone into two zones. (Fig. 7a, curve 1). Shapes of both peaks permit to presume that the sorption dynamics in the system are in equilibrium. Earlier in the work, it has been shown that introducing 30% isopropyl alcohol to solution allows improving the eremomycin equilibrium sorption on the BDM sorbents [9]. It has been explained as that hydrophobic interaction in the transport sorbent pores is partly suppressed and ions better migrating into intraglobular pores bind with sorbent by cation exchange. In dynamic desorption, adding 30% isopropyl alcohol to eluent leads to increasing retention of the eremomycin in sorbent phase, because under such conditions the desorbed ions moving in front of concentration zone can diffuse easier into microglobular canals to bind with free carboxylic groups. As a result, the concentration front broadens (Fig. 7a, curve 2).

The character of the eremomycin concentration front principally changes with increase of the eluent ionic strength from 0.1 to 0.2 M. Under such ionic strength and the alkaline pH value any interaction of the desorbed ions with free sorption centers in front of chromatographic zone cannot be realized. That results in unity and sharpening the chromatographic zone (Fig. 7b, curve 1). When the 30% isopropyl alcohol is added to the eluent (Fig. 7b, curve 2) the concentration front also broadens by the reason described above and the elution front is not divided into two peaks.

Thus, it has been shown how the combination of physical and chemical conditions together with kinetic factor influence the shaping of the concentration front. Also it has been shown that the displaced molecules moving ahead of the concentration front interact with the vacant sorption centers. From this point of view it was important to study the dependence of the dynamic sorption on column size.

So the optimal conditions for desorption of eremomycicn from the BDM-12 have been established and elution front is demonstrated in Fig. 7b. On realizing desorption under these conditions but on a column 10 mm × 25 mm, eremomycin forms two peaks (Fig. 8, curve 1). The first wide peak corresponds to process of eremomycin being displaced by 0.2 M ammonium acetate with pH 6.9. That is precisely those conditions, which allow the eremomycin equilibrium and dynamic selective sorption to be conducted. Desorption of eremomycin in such conditions demonstrates destruction of the weak lateral connections in transport pores. The second peak corresponds to the process of eremomycin being displaced from the column by 0.2 M ammonium acetate with pH 10.5, in conditions of the eremomycin amino groups becoming uncharged. The same sorption system behavior is

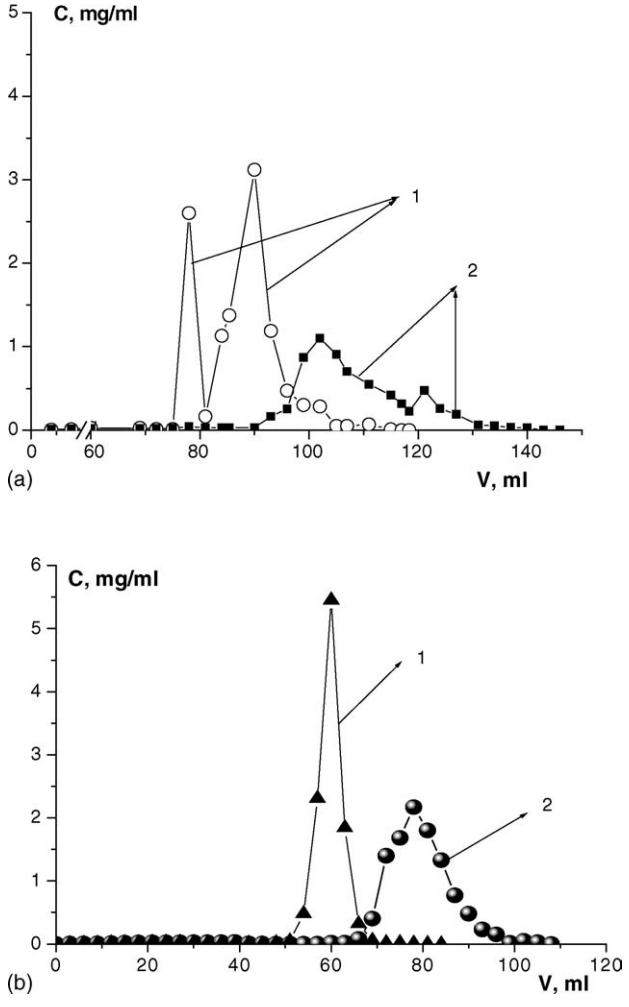


Fig. 7. Dependence of ionic strength of eluent on shaping the eremomycin elution front on BDM-12. (a) 1–0.1 M ammonium acetate, pH 10.5; 2–0.1 M ammonium acetate with adding 30% isopropyl alcohol, pH 10.5. The column size $D \times H = 10\,\text{mm} \times 70\,\text{mm}$; (b)1–0.2 M ammonium acetate, pH 10.5; 2–0.2 M ammonium acetate with adding 30% isopropyl alcohol, pH 10.5. The column size $D \times H = 10\,\text{mm} \times 70\,\text{mm}$.

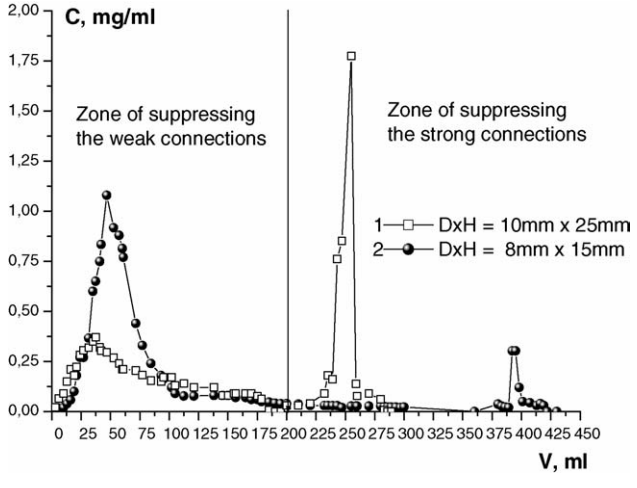


Fig. 8. The effects of column size on forming the eremomycin elution front on BDM-12. (a) $D \times H = 10\,\text{mm} \times 25\,\text{mm}$; (b) $D \times H = 8\,\text{mm} \times 15\,\text{mm}$. Zone of weak connections describes the process of eremomycin desorption by 0.2 M ammonium acetate with pH 6.9; zone of strong connections describes the process of eremomycin desorption by 0.2 M ammonium acetate with pH 10.5.

observed with decreasing the column size to 8 mm × 15 mm. Two concentration zones are obtained even more clearly (Fig. 8, curve 2). The first one is a zone of suppressing the weak connections by the 0.2 M ammonium acetate solution (pH 6.9). The other one is a zone of suppressing the strong ionic connections, which can be destroyed only by 0.2 M ammonium acetate solution with pH 10.5.

Thus, in this stage of work the effects of the column sizes on forming the concentration front has been studied. It has been established that the eremomycin ions redistribute in the bimodal structure of the sorbent. It has been shown that chromatographic zone sharpening is better on the column with bigger height to diameter ratio.

## 4. Conclusions

The study of the eremomycin equilibrium sorption by the carboxylic sorbents of BDM-group has shown that interactions in the sorption system have the polyfunctional nature. High selectivity and permeability of these sorbents provide high sorption capacity for absorbed antibiotic. The conditions for complete desorption of eremomycin from the sorbent phase can be achieved by varying the degree of ionization of the antibiotic amino groups.

The study of the sorption kinetics has shown the intradiffusion character of the eremomycin mass transfer on the carboxylic cation exchangers. The initial binding of the antibiotic to the sorbents results from electrostatical attraction and hydrophobic interaction in the interglobular space of sorbents. There non-exchange kinetics can be accompanied by the Donnan exclusion effect. The eremomycin transfer to the sorption centers is mainly impeded by the sorbents microglobular pores where the cation exchange limits the sorption process and predominates over other intermolecular interactions. Cation exchange kinetics is much slower than hydrophobic sorption kinetics. But eremomycin is maximally absorbed in the conditions when the cation exchange is the dominant factor in the polyfunctional sorption interactions.

The study of dynamic sorption has demonstrated that eremomycin redistributes in the bimodal sorbent matrix and can form two peaks. Optimization of the thermodynamics and kinetics sorption data allows the displaced antibiotic to form the unified concentration zone with sharp boundary.

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