Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1006 (2003) 121–126
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Optimization of experimental conditions for the preparative displacement chromatography of antitumor anthracycline antibiotics on carboxylic sorbents

A.Y. Toshchevikova, N.M. Ezhova, O.A. Pisarev*

Department of Biopolymers, Institute of Macromolecular Compounds, Russian Academy of Sciences, Bolshoy Pr. 31, St. Petersburg 199 004, Russia

Abstract

The physico-chemical conditions and the limits of the rates of mobile phases are determined when effective regimes of preparative chromatography in the conditions of sharpening the boundaries of chromatographic zones of anthracycline antibiotics are realized. The influence of pH on the equilibrium, kinetics and dynamics of sorption of anthracycline antibiotics (rubomycin, doxorubicin and carminomycin) on the carboxylic sorbents has been studied.

Keywords: Preparative chromatography; Displacement chromatography; Flow rate; Kinetic studies; pH effects; Adsorption; Optimization; Antibiotics; Anthracyclines; Rubomycin; Doxorubicin; Carminomycin

1. Introduction

Anthracycline antibiotics (Fig. 1) possess a high antitumor activity and are widely used in the chemotherapy of tumors [1]. Studying the equilibrium, kinetics and dynamics of sorption of anthracycline antibiotics is interesting both theoretically, from the point of view of understanding the regularity of sorption of organic substances having small molecular mass, and practically, to realize selective methods of bioseparation.

Production of anthracycline antibiotics is based on microbial biosynthesis and consequent multi-step extraction purification with the use of chlorine-containing organic solvents. Methods of extraction do not allow obtaining high yields of antibiotic preparations with purity grade satisfying modern pharmacopoeia requirements. The use of effective methods of preparative chromatography allows obtaining maximal yields of a desired bioactive compound with content up to 99% [2,3].

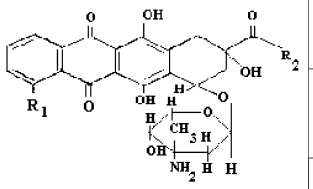
2. Experimental

Carboxylic sorbents BDM synthesized in the Institute of Macromolecular Compounds (St. Petersburg, Russia), are products of radical copolymerization of methacrylic acid and ethyleneglycol dimethacrylate. By varying the copolymer ratio and using a directed choice of copolymerization, it is possible to form permeable sorbents with a high level of polymer network stability and rigidity [4,5].

Distribution coefficients (Γ) were calculated using

*Corresponding author.
E-mail address: assa@imc.macro.ru (O.A. Pisarev).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00240-1



Antibiotic:	R_1	R_2	Mr	pI
Carminomycin	OH	CH_3	514	8,1
Rubomycin:	OCH_3	CH_3	528	8,2
Doxorubicin:	OCH_3	CH_2OH	544	8,4

Fig. 1. The structures of anthracycline antibiotics.

the ratio of antibiotic concentrations in stationary and mobile phases of chromatographic carrier.

The experiments on the study of sorption kinetics of anthracycline antibiotics were carried out at pH values 3.0, 7.0, 9.0 and at a temperature of 20 °C. Sorbent (30 mg) was placed in a glass vessel with 30 ml of 0.1 M solution of ammonium acetate with the required values of pH and antibiotic concentrations. The tightly closed vessels were placed on the mixing device. After certain intervals of time, aliquots for the analysis of antibiotic concentration were taken from solutions. Determination of the concentration of the antibiotic was carried out by spectrophotometric method at a wavelength of 495 nm. The total amount of the aliquots taken did not exceed 10% of the total amount of the solution. The aliquots were taken until equilibrium was achieved in the system. Determination of the saturation degree of the process was conducted using the formula:

$$F = \frac{m_t}{m_\infty}$$

where m_t is the quantity of sorbed antibiotic at moment of time (t) (mg); m_∞ is the quantity of sorbed antibiotic when the system reached equilibrium (mg); and t is the time of experiment (s).

The graphic dependences $F = f(\sqrt{t})$ were constructed from the results of each experiment (Fig. 2). On the linear site of this dependence, the coefficients of effective diffusion ($\bar{D}$) and mean time of sorption ($\bar{t}$) were calculated using the Boyd equations, which are correct for intradiffusion kinetic sorption from limited volume [6]:

$$\bar{D} = A \tan^2 \alpha \, d^2$$

$$\bar{t} = d^2 / 60\bar{D}$$

where A is the numerical coefficient depending on the form of sorbent particles ($A = \pi/144$ for spherical particles); d is the diameter of swollen sorbent particles (cm); tan α is the tangent of the inclination angle of the linear region of dependence $F = f(\sqrt{t})$, where F is the degree of sorbent saturation and t is the experiment time (min).

The intradiffusion mechanism of sorption was shown in experiments with interrupted phase contact; it was also determined from the linear character of dependence $F = f(\sqrt{t})$ up to a saturation degree of 0.3–0.4.

Studies on equilibrium and kinetics of sorption, as well as frontal saturation of the chromatography

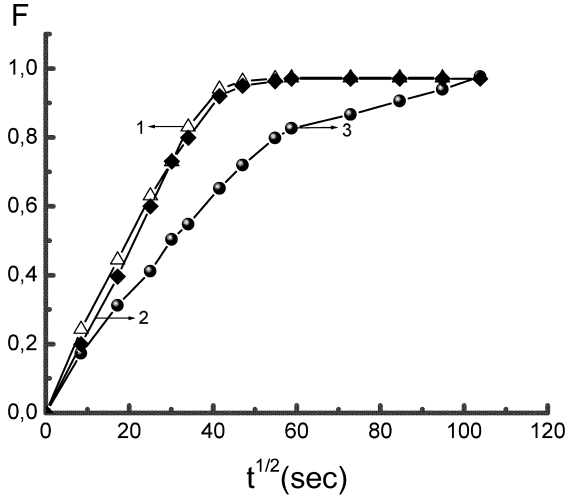


Fig. 2. Kinetic curves of carminomycin sorption on carboxylic sorbent BDM. 1 = pH 9.0; 2 = pH 7.0; 3 = pH 3.0.

columns were carried out at an antibiotic concentration of 0.6 mg/ml. This concentration corresponds to the antibiotic content in growth medium [7].

Mobile phase flow velocity was expressed as reduced flow-rate (ω), which did not depend on the geometrical properties of the column, and could be used to scale chromatography separations:

$$\omega = U/V$$

where ω is the reduced flow-rate in 1/s, U is the velocity of the mobile phase in ml/s and V is the column volume in ml.

3. Results

Fig. 3 shows the experimental data on the influence of pH on sorption equilibrium. The curve peak describes the mutual effect of resin ionization and antibiotic dissociation on sorbate distribution between solution and sorbent. At low pH, the distribution coefficient and pH increased simultaneously. It was connected to an increase in ionization of functional groups of the ion exchanger. Further increase in solution pH induced a shift of antibiotic dissociation equilibrium to primary formation of the undissociated form and, therefore, a decrease in ion–ion attraction of sorbent and sorbate. It should be noted that distribution coefficients remained high enough both at pH 2.5 (sorbent is not ionized and antibiotic molecules are dissociated totally) and 10.0 (sorbent is ionized and antibiotic molecules are not dissociated). It indicates the presence of sorbent–sorbate nonionic interactions in the system.

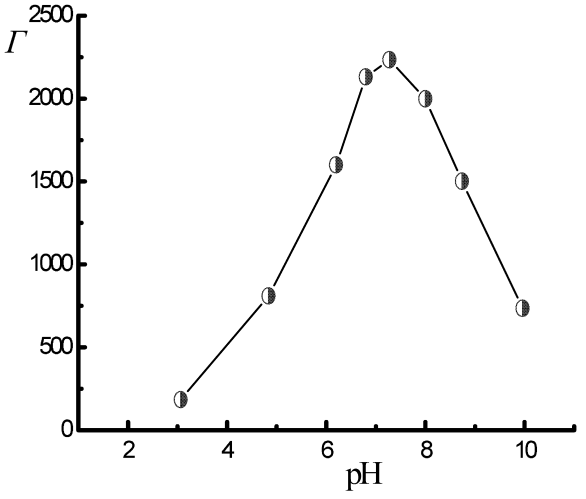


Fig. 3. The dependence of the distribution coefficient (Γ) on pH of equilibrium solution (0.1 M CH_3COONH_4, $C = 0.6$ mg/ml).

The study of the equilibrium and kinetics of sorption of antibiotic concentration has shown that absolute values of Γ strongly depend on medium acidity and reach the highest values in sorption in basic (pH 9.0) (Fig. 4a) and neutral (pH 7.0) (Fig. 4b) media. At these pH values, the value of Γ increases with increasing initial antibiotic concentration. This is explained by the fact that at basic pH values, ionogenic groups of a carboxylic sorbent are completely ionized, while antibiotic molecules are not dissociated. Therefore the pore space of the sorbent and its sorptional centers are most available for high antibiotic concentration at different types of interaction. At neutral pH values, all possible types of interaction are realized, especially ion–ion interactions, which are actually absent in the case of the sorption in basic medium. However, the value of these interactions is limited by the degree of ionization both of the antibiotic and of the sorbent. At acidic pH, antibiotic molecules are completely dissociated and a sharp decrease in absolute values of Γ (as compared with sorption from neutral and basic solution) as well as a decrease in Γ with increasing antibiotic concentrations are observed (Fig. 4c). This is associated with decreasing steric availability of interaction centers as well as with complete absence of ion–ion interactions with H^+-form of the sorbent.

Usually the chromatographer achieves the required difference between the distribution constant of the target substance and the corresponding values of undesirable impurities. Values of distribution coefficients during sorption of different anthracycline antibiotics in the same medium are used to determine a potential possibility of their separation by means of a value of the equilibrium constant of sorption selectivity ($K = \Gamma_2/\Gamma_1$). In real chromatographic processes, the possibility to separate substances by means of a column allows assessing the constant of kinetic sorption selectivity ($\tilde{K} = \bar{\Gamma}_2\bar{D}_2/\bar{\Gamma}_1\bar{D}_1$) more exactly, which takes into account both equilibrium and kinetic characteristics of sorption of the target component [8]. Increase of medium acidity strongly influences the values of effective diffusion coeffi-

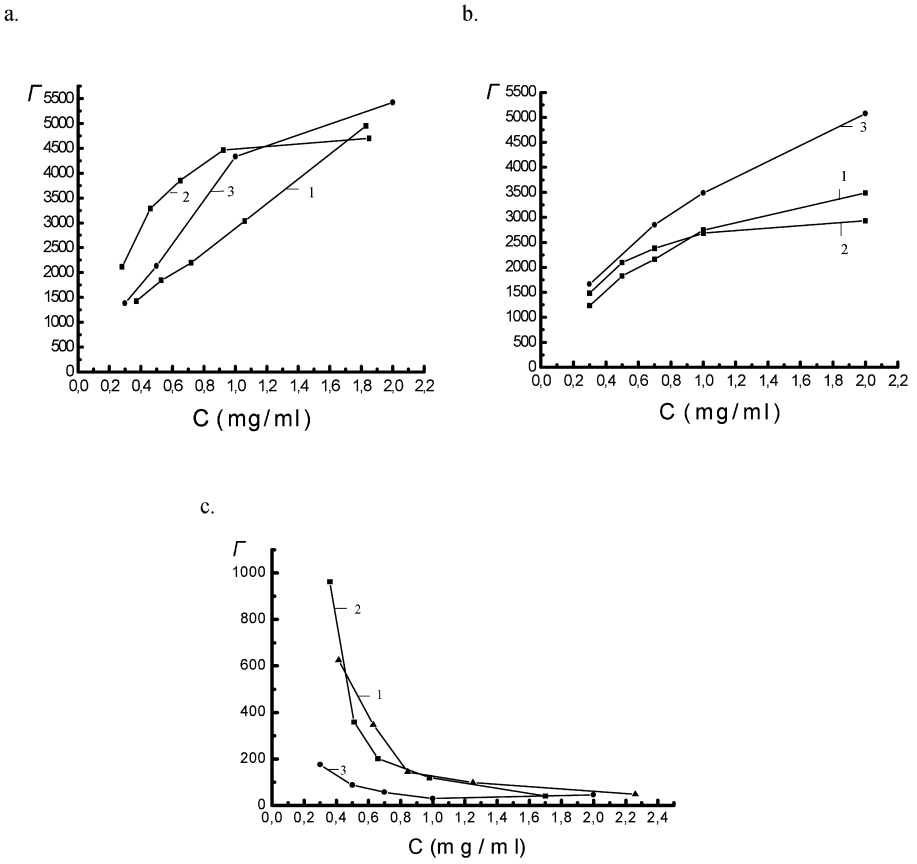


Fig. 4. The dependence of the distribution coefficient (Γ) on initial antibiotic concentration in solution. (a) pH 9.0; (b) pH 7.0; (c) pH 3.0; 1 = rubomycin; 2 = doxorubicin; 3 = carminomycin.

cient and mean sorption time. It should be noted that in basic and acidic media, the difference between the values of D leads to substantial differences between these constants.

Results of studying the sorption kinetic are shown in Table 1, while values of constants of sorption selectivity and constant of kinetic sorption selectivity for pairs of anthracycline antibiotics are shown in Table 2.

The maximum effective diffusion coefficients and, therefore, minimum mean sorption time were observed at high pH; under these conditions, anthracycline antibiotics were in the form of a neutral molecule. However, it is known that during sorption in acid (pH≤3) and basic (pH≥9) media, the

Table 1
Influence of pH on effective diffusion coefficients and mean time of sorption of anthracycline antibiotics

Antibiotic	pH	$\overline{D} \times 10^8$ (cm^2/s)	$\bar{t}$ (s)
Rubomycin	3.0	7.5	2007.8
	7.0	17.7	1375.7
	9.0	23.9	1317.7
Doxorubicin	3.0	9.8	1528.4
	7.0	17.7	1375.7
	9.0	15.6	2018.7
Carminomycin	3.0	5.6	2689.1
	7.0	17.7	1375.7
	9.0	40.7	773.8

Table 2
Values of constants of sorption selectivity and constant of kinetic sorption selectivity for pairs of anthracycline antibiotics

Antibiotic	pH	K	$\tilde{K}$
Doxorubicin/rubomycin	3.0	1.28	1.68
Doxorubicin/carminomycin	3.0	5.93	10.43
Rubomycin/carminomycin	3.0	4.63	6.20
Doxorubicin/rubomycin	7.0	1.92	1.92
Doxorubicin/carminomycin	7.0	1.66	1.66
Carminomycin/rubomycin	7.0	1.16	1.16
Doxorubicin/rubomycin	9.0	1.20	1.28
Carminomycin/doxorubicin	9.0	1.10	2.86
Carminomycin/rubomycin	9.0	1.32	2.24

aminoglycoside bond of the antibiotic molecule is hydrolyzed quickly and aglycon with pronounced cardiotoxicity is formed [1]. Since the distribution coefficients decreased quickly at high pH, optimization of selective carminomycin sorption with frontal column saturation was carried out at pH 6.8.

The effect of the mobile phase flow-rate on the character of sorbent saturation with antibiotic was determined from the form of curves of frontal sorption (Fig. 5). At $\omega = 5.2\times10^{-3}$ and $\omega = 15\times10^{-3}$ 1/s, curves of frontal sorption are symmetrical and inflection points are near the front midpoint (regular regime of sorption dynamics) (Fig. 5, curves 1, 2). The further increase in reduced flow-rate caused curve asymmetry and a shift of inflection points to lower volumes (Fig. 5, curves 3, 4). Thus, within the studied interval of mobile phase flow velocity, there was a transition from regular to irregular frontal chromatography. Under the conditions of frontal sorption studied, regular mode of chromatography was retained at significant flow-rates of mobile phase. For example, during desorption of the antibiotic using the laboratory column (volume 5.85 ml, $d = 1.4$ cm, $h = 3.8$ cm), the velocity of the mobile phase is 31.59 ml/h; this velocity will be 27 l/h when scaling this process to an industrial column having volume 5 l.

The use of a highly specific chromatographic carrier for selective sorption complicates the search for conditions of selective elution with sharp boundaries of chromatographic zone of the desired compound. Sharp boundaries of the chromatographic zone were not detected when antibiotic was dis-

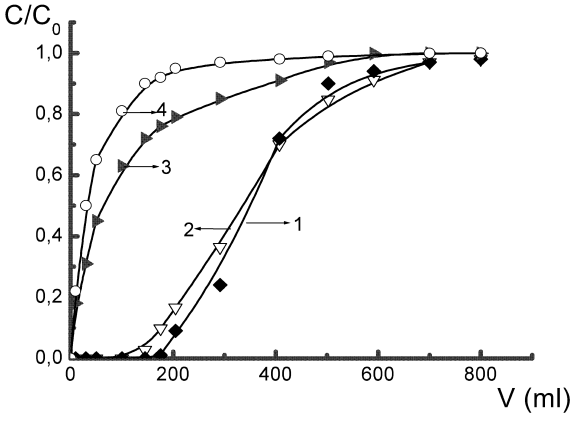


Fig. 5. Curves of carminomycin frontal sorption (0.1 *M* CH_3COONH_4, pH 6.8). (1) $\omega = 5.1\times10^{-3}$ 1/s, (2) $\omega = 15.0\times10^{-3}$ 1/s, (3) $\omega = 32.3\times10^{-3}$ 1/s, (4) $\omega = 50.0\times10^{-3}$ 1/s. *C* is the final concentration (mg/ml); C_o is the initial concentration (mg/ml); *V* is the volume of antibiotic solution passing through column (ml); ω is the reduced flow-rate (1/s).

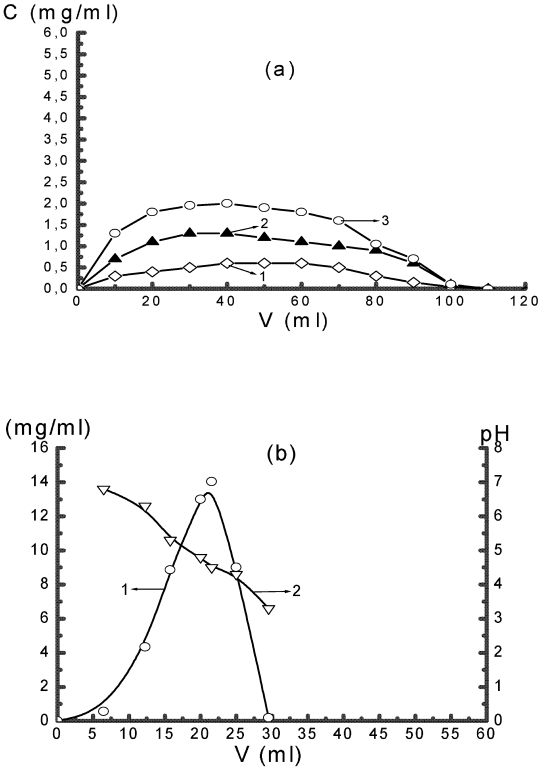


Fig. 6. Carminomycin desorption. (a) Eluents: (1) 90% isopropanol; (2) 1 *M* HC1; (3) 0.5 *M* NaCl; all in water; (b) Carminomycin desorption by 90% isopropanol (pH 1.5). Curve (1) describes the dependence of *C* on *V*; curve (2) describes the dependence of pH on *V*.

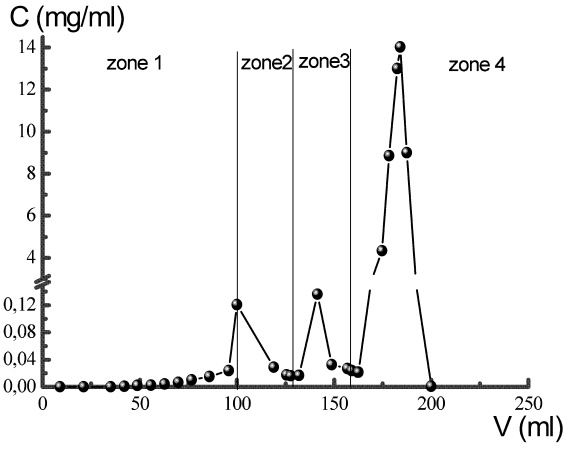


Fig. 7. The stages of chromatographic purification of anthracycline antibiotics. Zones 1=sorption on the carboxylic sorbent BDM (pH 6.8); 2=removal of the unbound material (pH 6.8); 3=desorption by eluent 70% isopropanol (pH 6.8); 4=desorption by eluent 90% isopropanol (pH 1.5).

placed by isopropanol or aqueous solutions of hydrochloric acid and sodium chlorate (Fig. 6a, curves 1–3). Maximum antibiotic concentration in eluate was 0.5–2.0 mg/ml; yield was 20–40%, narrow boundaries of chromatographic zone formed when a solution of hydrochloric acid in 90% isopropanol was used (Fig. 6b, curve 1). Probably, it is connected to a simultaneous rapid decrease in sorbent ionization and break of hydrophobic sorbent–sorbate interactions. When forming a narrow chromatographic zone, carminomycin was concentrated 22-fold compared to the initial solution, and acid used as eluent did not penetrate the antibiotic zone because of the regular character of desorption. Therefore, chromatographic columns yielded the most concentrated carminomycin fractions at a pH preventing acid hydrolysis of the target product (Fig. 6b, curve 2).

After studying the regularity of equilibrium, kinetics and dynamics of sorption and desorption of anthracycline antibiotics on carboxylic cation exchangers, we have found the optimal conditions and the sequence of stages of chromatographic purification (Fig. 7).

1. At the first stage, high selectivity sorption on carboxylic ion exchanger BDM takes place, and the solution has pH 6.8 (Fig. 7, zone 1).
2. At the second stage, removing of the unbound impurities is shown (Fig. 7, zone 2).
3. At the third stage, desorption of aglycone by suppression of hydrophobic interactions is shown (Fig. 7, zone 3).
4. At the fourth stage, selective desorption of the target product is demonstrated (Fig. 7, zone 4).

4. Conclusions

In this paper, it was shown that high-capacity frontal sorption of anthracycline antibiotics on the carboxylic cation exchanger BDM under the regular regime could be carried out at high reduced flow velocity of mobile phase ($\omega = 15 \times 10^{-3}$ 1/s). Total antibiotic elution could be carried out under the regular regime and at a high reduced flow velocity ($\omega = 15 \times 10^{-4}$ 1/s) when using eluent having the ability to break ion–ion and hydrophobic bonds of sorbate and sorbent. The flow velocity limits of desorbing solution with significant amounts of concentrated antibiotic in eluate were determined. The optimal conditions of sorption of the anthracycline antibiotics on carboxylic cation exchangers were found. Both the optimal eluent and conditions of complete desorption of the anthracycline antibiotics were found.

References

[1] G.F. Gauze (Ed.), The Antitumor Antibiotic Carminomycin and its Clinical Application, Meditsina, Moscow, 1980.
[2] G.V. Samsonov, O.A. Pisarev, A.T. Melenevsky, J. Pure Appl. Chem. 65 (1993) 2287.
[3] O.A. Pisarev, I.V. Kruchina-Bogdanov, N.V. Glazova, O.V. Bychenkova, Zh. Phys. Khim. (Russ.) 73 (1999) 1634.
[4] G.V. Samsonov, O.A. Pisarev, Zh. Prikl. Biokhim. Mikrobiol. (Russ.) 28 (1992) 5.
[5] N.M. Ezhova, S.G. Yunysheva, O.A. Pisarev, Zh. Prikl. Khim. (Russ.) 71 (1998) 991.
[6] A.Yu. Toshchevikova, O.A. Pisarev, Zh. Prikl. Biokhim. Mikrobiol. (Russ.) 38 (2002) 128.
[7] G.R. Gauze, L.R. Terekhova, T.S. Maksimova, Zh. Antibiotiki (Russ.) 20 (1975) 363.
[8] O.A. Pisarev, I.V. Kruchina-Bogdanov, N.V. Glazova, O.V. Bychenkova, Zh. Dokl. Akad. Nauk (Russ.) 362 (1998) 362.