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Role of the buffer in retention and adsorption mechanism of ionic species in reversed-phase liquid chromatography I. Analytical and overloaded band profiles on Kromasil-C_{18}

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Abstract

The influence of the pH, the concentration, and the nature of the buffer on the retention and overloading behavior of propranolol ($pK_a = 9.25$) on Kromasil-C_{18} was studied at $2.75 < pH < 6.75$, using four buffers (phosphate, acetate, phthalate, and succinate), at three concentrations, 6, 20, and 60 mM. The propranolol band profiles were recorded for three sample sizes, less than 1 μg and 375 μg (sample less concentrated than the buffer), and 7500 μg (band more concentrated than the buffer). Results showed that the buffer concentration, not its pH, controls the retention time of propranolol, in agreement with the chaotropic model. The retention factor depends also on the nature of the buffer, particularly the valence of the basic anion. At moderate loading, the band profiles are well accounted for by a simple bilangmuir model (no adsorbate–adsorbate interactions) with the monovalent anions $H_2PO_4^-$ (pH 2.75), HOOC–Ph–COO^- (pH 2.75), HOOC–CH_2–CH_2–COO^- (pH 4.16) and CH_3COO^- (pH 4.75), and by a bimoreau model (significant adsorbate–adsorbate interactions) with the bivalent anions $^-$OOC–Ph–COO^- (pH 4.75), $^-$OOC–CH_2–CH_2–COO^- (pH 5.61) and HPO_4^{2-} (pH 6.75). The isotherm were determined using the inverse method. The results show that both the saturation capacity and the equilibrium constant on the low-energy sites increase with increasing buffer concentration, a result similar to that observed with neutral salts. For bivalent anions, the adsorbate–adsorbate interactions are much stronger on the low than on the high energy sites. The density of high energy sites is lower and the equilibrium constant on the low energy sites are higher with bivalent than with univalent anions. These results are consistent with the formation of a propranolol–buffer (2:1) complex with bivalent anions.

Keywords: Retention mechanism; Adsorption isotherms; Buffer composition; Frontal analysis; Band profiles; Kromasil-C_{18} stationary phase; Salt effects; Ionic strength; Propranolol

1. Introduction

Because most compounds of interest in pharmaceutical and biomedical applications are ionizable, their retention mechanisms in RPLC have become of great interest. The addition of suitable concentrations of the proper salts and/or buffers into the mobile phase is essential to achieve their proper separations and accurate quantitation. However, in spite of the considerable insights brought about by investigations of the adsorption behavior of ionizable compounds at high concentrations [1–5], most studies of the retention of these ionizable compounds are made under analytical (i.e., highly dilute) conditions [6–12].

The influences of the pH, the buffer concentration, and the nature of the buffer on the adsorption process of ionizable compounds have attracted but limited attention. They are the topic of few systematic investigations. In most studies, these parameters are simply optimized for best selectivity or largest production rate. Models treating the effects of the pH [13] and the concentration of counter-anions [14] on the retention of ionizable compounds under linear conditions have been proposed and tested experimentally on C_{18}-bonded phases with large libraries of acidic and basic analytes (e.g., a series of β-blockers among which propranolol [15]). We

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have recently studied the adsorption behavior of this compound at a pH well below its pK_a (9.25) on a Kromasil column, using either an acetate buffer [16] or neutral salts [5]. We concluded that the presence of any ions in the mobile phase changes considerably its adsorption behavior and suggested that some interactions take place between the acetate counter-anion and the propranolonium cation and explain the rapid increase of hydrophobicity of the analyte when an acetate buffer is added to the mobile phase [16]. Upon addition of potassium chloride to the mobile phase, the adsorption isotherm evolves progressively from the bimoreau model at low salt concentrations (with adsorbate–adsorbate interactions in the monolayer) to the bilangmuir model at high salt concentrations (no such interactions) [5]. The adsorption behavior in presence of neutral salts depends not only on the solution ionic strength but on the nature of the anion. At a ionic strength of 200 mM, the band profile is anti-langmuirian with the bivalent anion sulphate (Na_2SO_4) but langmuirian with the monovalent anions KCl, KNO_3 or $CaCl_2$. The reasons for this difference remains unexplained.

In this work, we report on the influence of the pH, the buffer concentration, and the nature of the buffer used and the valence of its anion on the adsorption behavior of propranolol. We choose a pH range in which propranolol is present essentially as its cationic form ($2 \leq pH < 7$) and the Kromasil-C_{18} silica remains stable ($pH \leq 8$). In each case (pH, buffer nature, and concentration), injections were made using analytical, moderate, and high loadings, the last case sampling concentrations higher than the buffer capacity. The equilibrium isotherm was derived using the inverse and the perturbation methods.

2. Theory

2.1. Models of isotherm used

Previous studies suggested that the surfaces of many modern RPLC stationary phases are heterogeneous and consist in a few patches, often two rarely more than three, of relatively homogeneous surfaces (i.e., types of sites), having different adsorption energies. All the sets of equilibrium isotherm data measured for propranolol, under any of the experimental conditions ever used with C_{18}-bonded silica, are consistent with this description, showing that only two types of sites coexist for this compound. These data are best modeled by an isotherm equation that consists in the sum of two terms, each of them corresponding to the adsorption isotherm on a homogeneous surface. Depending on the experimental conditions, two such isotherm models were used, the Langmuir model, when no adsorbate–adsorbate interactions take place or when these interactions are negligible, the Moreau model [17] when these interactions are significant. The bimoreau model was found to describe the adsorption behavior of propranolol, whether the mobile phase was buffered or not [16], in the whole range of concentration of neutral salts considered. This model assumes that a different Moreau model applies to each of two types of patches, considered as homogeneous and acting independently:

$$q^* = q_{s,1} \cdot \frac{b_1 C + I_1 b_1^2 C^2}{1 + 2b_1 C + I_1 b_1^2 C^2} + q_{s,2} \cdot \frac{b_2 C + I_2 b_2^2 C^2}{1 + 2b_2 C + I_2 b_2^2 C^2} \quad (1)$$

where C and q^* are the liquid and the solid phase concentrations at equilibrium, respectively, and $q_{s,1}$, $q_{s,2}$, b_1, b_2, I_1 and I_2 are the monolayer saturation capacities, the low-concentration equilibrium constants, and the adsorbate–adsorbate interaction parameters on the sites of types 1 and 2, respectively.

The equilibrium constants b_1 and b_2 are associated with the adsorption energies $\epsilon_{a,1}$ and $\epsilon_{a,2}$, respectively, through the following classical equation [18]:

$$b_i = b_0 e^{\epsilon_{a,i}/RT} \quad (2)$$

where $\epsilon_{a,i}$ is the energy of adsorption, R is the universal ideal gas constant, T is the absolute temperature and b_0 is a preexponential factor that could be derived from the molecular partition functions in both the bulk and the adsorbed phases. b_0 is often considered to be independent of the adsorption energy $\epsilon_{a,i}$ [18].

The adsorbate–adsorbate interaction parameter, I, can be written as [17]:

$$I = \exp\left(\frac{\epsilon_{AA}}{RT}\right) \quad (3)$$

where ϵ_{AA} is the interaction energy (by convention, $\epsilon_{AA} \geq 0$) between two neighbor adsorbed molecules of A.

2.2. The inverse method of isotherm determination

The Inverse Method (IM) consists in adjusting the coefficients of an isotherm model in order to minimize the differences between a recorded experimental band profile and the profile calculated with the equilibrium-dispersive model of chromatography (see next section) and the isotherm model selected. The main advantage of the inverse method for isotherm determination is that it requires the measurement of only a few experimental overloaded band profiles [19–22]. Accordingly, the method is fast and requires little amounts of solvent and sample. This method was described previously [5]. It gives results that are in excellent agreement with those of FA [22].

2.3. Modeling of band profiles in HPLC

The overloaded band profiles of propranolol were calculated with the equilibrium-dispersive model (ED) of chromatography [23–25]. The ED model assumes instantaneous equilibrium between the mobile and stationary phases and

a finite column efficiency originating from an apparent axial dispersion coefficient, D_a, that accounts for the dispersive phenomena (molecular and eddy diffusion) and for the non-equilibrium effects that take place in a chromatographic column. The apparent axial dispersion coefficient is:

$$D_a = \frac{uL}{2N} \quad (4)$$

where u is the mobile phase linear velocity, L the column length, and N the number of theoretical plates or apparent efficiency of the column, measured under linear conditions, i.e., with a small sample size. In this model, the mass balance equation for a single component is written:

$$\frac{\partial C}{\partial t} + u\frac{\partial C}{\partial z} + F\frac{\partial q^*}{\partial t} - D_a\frac{\partial^2 C}{\partial z^2} = 0 \quad (5)$$

where q^* and C are the stationary and mobile phase concentrations of the adsorbate at equilibrium, respectively, t is the time, z the distance along the column, and $F = (1 - \epsilon_t)/\epsilon_t$ is the phase ratio, with ϵ_t the total column porosity. q^* is related to C through the isotherm equation, $q^* = f(C)$.

The mass balance equation for a single component (Eq. (5)) is valid in the present case, although the mobile phase is an aqueous solution of methanol and a buffer with an organic anion. Previous work [26] has shown that neither methanol nor the organic anions used are significantly retained while the neutral form of the acid has a retention factor that is always less than 0.1, because the corresponding perturbations are eluted with the hold-up time. It has been shown that when the additive has a retention factor which is more than one order of magnitude lower than that of the studied compound, competition is negligible and the chromatographic problem becomes a single-component problem [27].

2.3.1. Initial and boundary conditions for the ED model

At $t = 0$, the concentrations of the solute and the adsorbate in the column are uniformly equal to zero (except in staircase FA), and the stationary phase is in equilibrium with a stream of the pure mobile phase. The boundary conditions used are the classical Danckwerts-type boundary conditions [23,28] at the inlet and outlet of the column.

2.3.2. Numerical solutions of the ED model

The ED model was solved using the Rouchon program based on the finite difference method [23,29–31].

3. Experimental

3.1. Chemicals

The mobile phases used in this work were buffered aqueous solutions of methanol (40:60, v/v). Both water and methanol were of HPLC grade, purchased from Fisher Scientific (Fair Lawn, NJ, USA). The buffers were first prepared in pure water (all pH values reported in the text are those measured in pure water) and methanol was added thereafter to that solution to prepare the final mobile phase (see Table 1). The buffer concentrations given in the text are reported to the mobile phase mixture. Prior to their use, the solvents were filtered on an SFCA filter membrane, 0.2 μm pore size (Suwannee, GA, USA). Thiourea was chosen to measure the column hold-up volume. The solute studied was propranolol, an amino alcohol of structure $C_{10}H_7OCHOHCH_2NHCH(CH_3)_2$. It was injected under its protonated form, as the hydrochloride. Thiourea and propranolol; potassium acetate, potassium hydrogenphthalate, potassium dihydrogenphosphate and disodium succinate; 1 N hydrochloric acid, acetic acid 99.5% and succinic acid were all obtained from Aldrich (Milwaukee, WI, USA).

3.2. Columns

The 250 × 4.6 mm column used in this study (Kromasil-C_{18}, No. E6023) was given by the manufacturer (Eka Nobel, Bohus, Sweden, EU). The main characteristics of the packing material are summarized in Table 2. This column was one of the lot of ten columns previously used to test the column-to-column and batch-to-batch reproducibility under linear [32] and non-linear conditions [33,34]. The void volume of this column was derived from the average of the retention times of two consecutive thiourea injections (2.458 mL). The column porosity remained constant at 0.5916, whatever the buffer used and its concentration in the mobile phase (40:60, v/v). The porosity depends only on the methanol concentration of the mobile phase.

3.3. Apparatus

The overloaded band profiles were acquired using a Hewlett-Packard (now Agilent Technologies, Palo Alto, CA, USA) HP 1090 liquid chromatograph. This instrument includes a multi-solvent delivery system (volume of each tank, 1 L), an auto-sampler with a 250 μL sample loop, a diode-array UV detector, a column thermostat and a data station. Compressed nitrogen and helium bottles (National Welders, Charlotte, NC, USA) are connected to the instrument to allow the continuous operations of the pump, the auto-sampler, and the solvent sparging. The extra-column volumes are 0.058 and 0.93 mL as measured from the auto-sampler and from the pump system, respectively, to the column inlet. All the retention data were corrected for these contributions. The flow-rate accuracy was controlled by pumping the pure mobile phase at 23 °C and 1 mL/min during 50 min, from each pump head, successively, into a volumetric glass of 50 mL. The relative error was less than 0.4%, so that we can estimate the long-term accuracy of the flow-rate at 4 μL/min at flow rates around 1 mL/min. All measurements were carried out at a constant temperature of 23 °C, fixed by the laboratory air-conditioner.

Table 1
Preparation of the different buffers

Buffer	Acid solution	Base solution	Volume water (mL)	Volume acid (mL)	Volume base (mL)	pH	Volume MeOH (mL)	C_{Buffer} (mM)
Phosphate I	HCl 0.1 M	KH_2PO_4 0.1 M	0	56	300	2.75	237	50.6
			200	26	100		217	18.4
			270	14	30		209	5.7
Phosphate II	KH_2PO_4 0.1 M	NaOH 0.5 M	0	300	28	6.75	219	54.8
			200	100	7.8		205	19.5
			270	30	2		201	6.0
Phthalate I	HCl 0.1 M	$KH_5C_8O_4$ 0.1 M	0	226	375	2.75	400	37.5
			300	45	64		273	9.4
			540	25	22		391	2.2
Phthalate II	$KH_5C_8O_4$ 0.1 M	NaOH 0.1 M	0	300	109	4.75	273	44.0
			200	100	30		220	18.2
			270	30	7.5		205	5.9
Succinate I	$C_4H_6O_4$ 0.1 M	$Na_2C_4H_4O_4$ 0.1 M	0	250	99	4.16	233	60.0
			180	60	21		174	18.6
			235	20	6		174	6.0
Succinate II	$C_4H_6O_4$ 0.1 M	$Na_2C_4H_4O_4$ 0.1 M	0	51	253	5.61	203	60.0
			200	20	80		200	20.0
			270	7.5	25		202	6.4
Acetate	$C_2H_4O_2$ 0.1 M	$KC_2H_3O_2$ 0.1 M	0	175	175	4.75	233	60.0
			230	60	60		217	21.2
			310	20	20		205	7.2

The daily variation of the ambient temperature never exceeded ±1 °C.

3.4. Measurements of the overloaded band profiles of propranolol

Three types of injections of propranolol were made with the auto-sampler syringe (maximum volume 250 μl). 10 μl of a 0.1 g/L solution, 250 μl of a 1.5 g/l solution, and 250 μl of a 30 g/l solution were successively injected to record the analytical, moderately overloaded, and highly overloaded band profiles, respectively. These profiles were recorded at 310, 325, and 331 nm after injections of the 0.1, 1.5, and 30 g/l solutions, respectively. Segments of the elution profiles having between 500 and 1000 points were used to perform the IM calculations.

Table 2
Characteristics of the C_{18}-bonded Kromasil column used

Particle size (μm)	6
Pore size (Å)	110
Pore volume[a] (mL/g)	0.88
Surface area[a] (m^2/g)	314
Particule shape	Spherical
Total carbon (%)	20.0
Surface coverage (μmol/m^2)	3.6
Total porosity[b]	0.5916
Endcapping	Yes

[a] Data for the packing before derivatization.
[b] Data from injection of the non-retained thiourea compound in a methanol–water (40:60, v/v) mobile phase.

4. Results and discussion

4.1. Linear chromatography

Fig. 1 summarizes the retention factors of propranolol measured with all the buffers, pH and buffer concentrations investigated in this study. This includes one neutral salt (KCl), four buffers with a monovalent basic anion ($H_2PO_4^-$, CH_3COO^-, $HOOCC_2H_4COO^-$ and $HOOCC_6H_4COO^-$) and three buffers with a bivalent basic anion (HPO_4^{2-}, $^-OOCC_2H_4COO^-$ and $^-OOCC_6H_4COO^-$). These results call for the following three general comments.

(1) For all salts, except the phosphate buffer at pH 6.75, the retention factor of propranolol increases rapidly with increasing buffer concentration at very low concentrations to tend toward a limit at high concentrations. Despite the fact that only three data points were acquired, this behavior is consistent with the theory of chaotropicity [14]. This theory assumes that the counter-anions in the mobile phase form with the oppositely charged propranolol cation some ion-associated complexes. The fundamental cause of this association is the strong electrostatic interaction between these ions, causing the displacement of the surrounding water molecules. It follows that the apparent hydrophobicity of the analyte increases its affinity for the C_{18}-bonded stationary phase, hence its retention factor. When the counter-anion concentration exceeds largely that of the analyte, almost all the water-solvated propranolol cation is turned into the

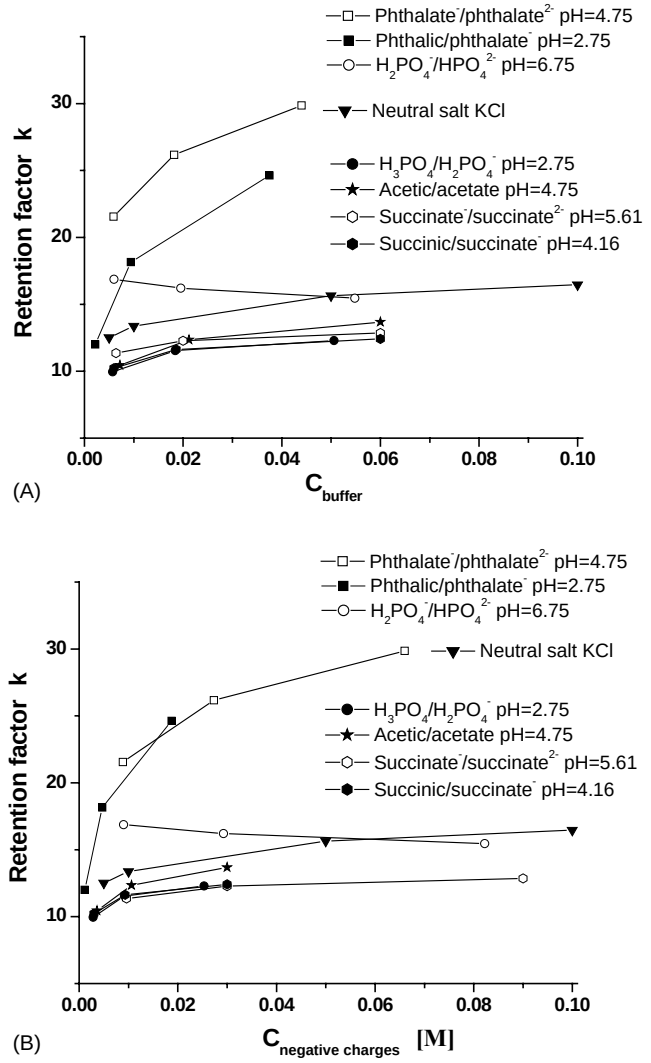

Fig. 1. (A) Evolution of the retention factor of propranolol as a function of the total buffer concentration for seven buffered mobile phase and one neutral salt. Note the increasing trend, except for phosphate buffer at pH 6.75. (B) Same as (A) except the axis scale represents the total negative charge of the buffer. Note the superposition of the curves for a same buffer, except phosphate.

ion complex and the retention factor measured is that of this complex. In the case of the phosphate buffer at pH 6.75, the converse result obtained suggests that the increase of the buffer concentration does not favour the formation of the ion-associated complex. This might be correlated to the low hydrophobicity of the bivalent hydrogenophosphate anion. Complementary and new information will be acquired by analyzing the mildly overloaded bands (see later).

(2) It is important to note that the retention factor of the propranolol cation depends hardly on the pH of the mobile phase. At the same pH, 2.75, the retention factor is about twice larger with the phthalate than with the phosphate buffer. Similarly, at the same pH, 4.75, the retention factor is almost three times larger with the phthalate than with the acetate buffer. This is probably because, in the pH range investigated, between 2 and 7, propranolol ($pK_a = 9.25$) is essentially under the cation form. Furthermore, the stationary phase has a high bonding density (3.60 μmol/m^2) and is end-capped, which much reduces its silanol activity under the experimental conditions selected.

(3) The retention factor is always larger in the presence of the bivalent than in that of the monovalent anion of a diacid. Obviously, this is in part because the abscissa scale in Fig. 1 gives the total buffer concentration in the mobile phase, not the total anion concentration. The concentration of the negative charge carrier is twice lower than the buffer concentration for monovalent buffers and 1.5 times larger for bivalent buffers. When this abscissa transform is made (see Fig. 1B), the curves for the diacids and monoacids (succinic and phthalic acids) measured at their half-neutralization pH (4.16 and 5.61 for succinic acid, 2.75 and 4.75 for phthalic acid) are superimposed. In other words, it seems that, for a given buffer, the essential factor that determines the retention of the propranolol cation is neither the pH nor the total buffer concentration, but the number of negative charges. This observation supports a retention mechanism based on the adsorption of ion-associated complexes. For a given number of negative charges, the second fundamental parameter is the hydrophobicity and hardness of the anion. The harder the anion (e.g., the smaller its size, the more poorly polarizable the anion with a limited charge delocalization), the lower the retention of propranolol. Based on our measurements, the hardness of monovalent anions increases in the following order: $HOOCC_6H_4COO^- \leq Cl^- \leq CH_3COO^- \leq HOOCC_2H_4COO^- \simeq H_2PO_4^-$. This order reflects well the global hydrophobicity of these anions.

4.2. Mildly overloaded band profiles

Figs. 2–8 show the experimental overloaded band profiles (dotted lines) recorded with the seven different buffers. The general effect of the buffer concentration can be seen on each figure. For all the buffers, the retention time of the band increases with increasing buffer concentration. For the phosphate buffer at pH 6.75, it is only at very low concentrations, below 0.01 g/L or 34 μM, that this elution order is no longer valid. The reversal of this order under linear conditions was reported in the previous section.

The best isotherm models for these different conditions were determined by IM using these experimental profiles. With buffers made of a neutral acid and a monovalent basic anion (Figs. 2–5), the heights of the elution profiles never exceed 0.10 g/L or 0.34 mM. In this range of concentrations, the product $C \times C$ is very small and the term IC^2 is negligible for any reasonable value of I (see Eq. (1)). Accordingly, no accurate estimate of the adsorbate–adsorbate parameters (I_1 and I_2 in the bimoreau model, Eq. (1)) could be

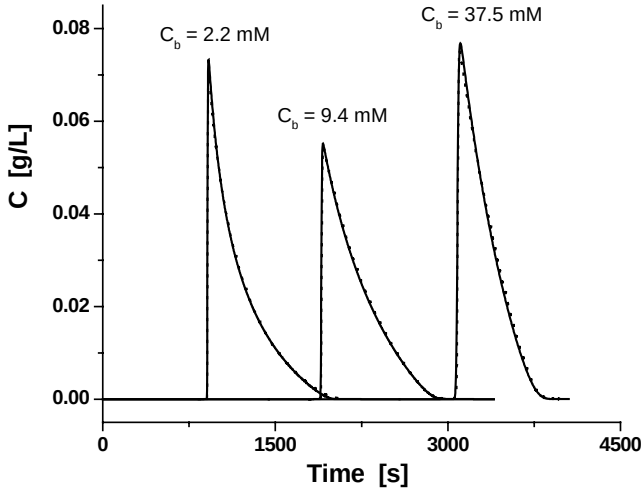

Fig. 2. Experimental (dotted lines) and best calculated (IM, solid lines) band profiles of propranolol after injection of 250 μL of a 1.5 g/L solution of propranolol chloride (mild overload) for three different buffered mobile phase (methanol–water, 40:60, v/v). Buffer: phthalate at pH 2.75. $T = 296$ K, flow rate 1 mL/min.

made because this term has no impact on the calculated band profiles. Furthermore, all the band profiles exhibit a front shock, followed by a large diffuse rear profile. Such profiles are typical of strictly convex upward isotherms. They show that, for propranolol concentrations below 0.34 mM, there are no adsorbate–adsorbate interactions in the stationary phase or that these interactions are negligible. Thus, the simple four parameters bilangmuir model was used, instead of the bimoreau model. The best profiles calculated with this model are in excellent agreement with the experimental profiles as shown by the agreement between the solid lines (best calculated profiles) and the dotted lines (experiments) in Figs. 2–5. The best values of the parameters are listed in Table 3. The standard deviations of these parameters are less than 5%.

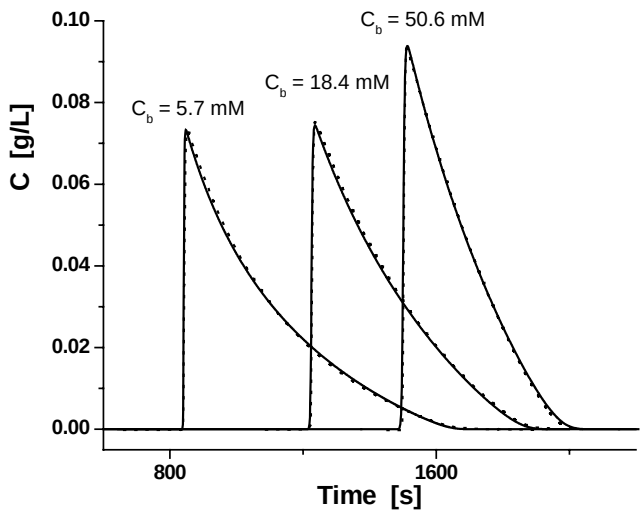

Fig. 3. Same as in Fig. 2, except buffer: phosphate at pH 2.75.

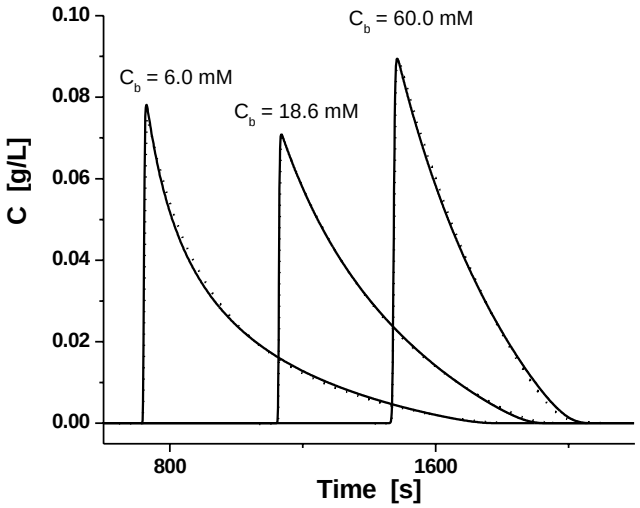

Fig. 4. Same as in Fig. 2, except buffer: succinate at pH 4.16.

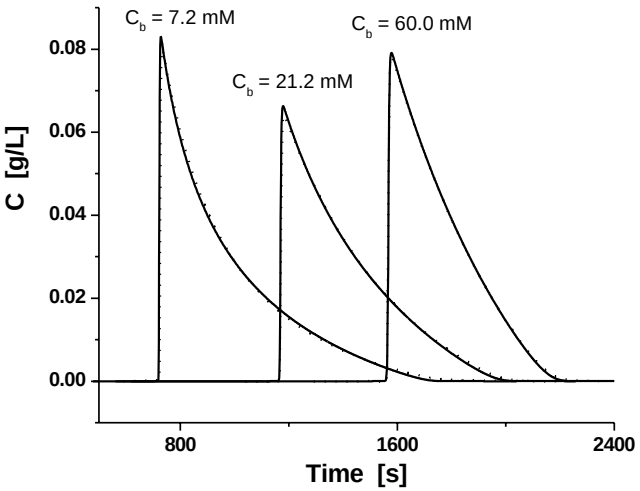

Fig. 5. Same as in Fig. 2, except buffer: acetate, pH 4.75.

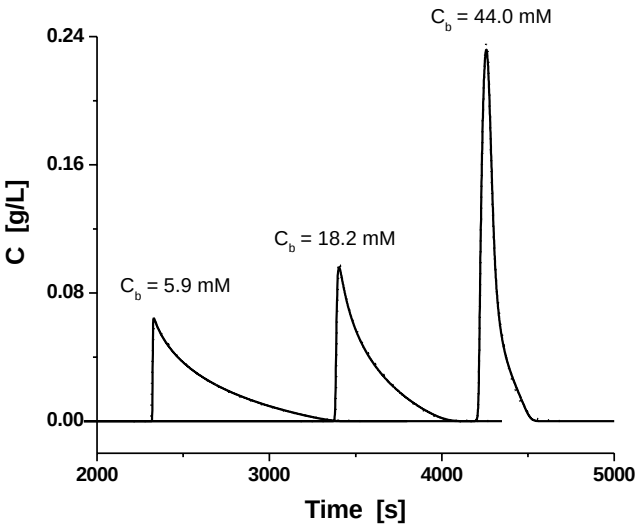

Fig. 6. Same as in Fig. 2, except buffer: phthalate at pH 4.75.

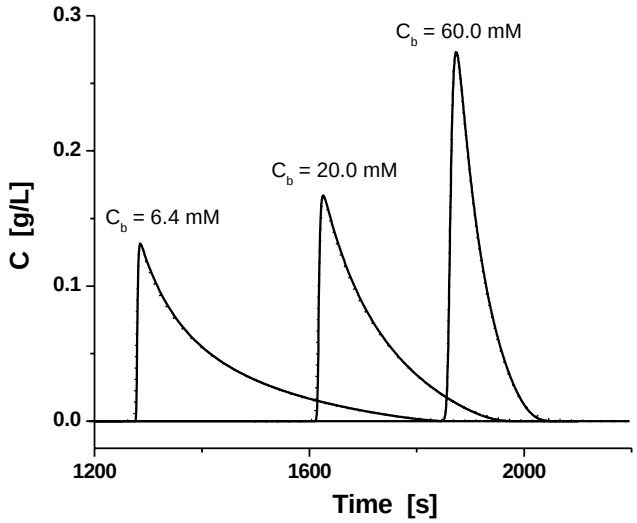

Fig. 7. Same as in Fig. 2, except buffer: succinate at pH 5.61.

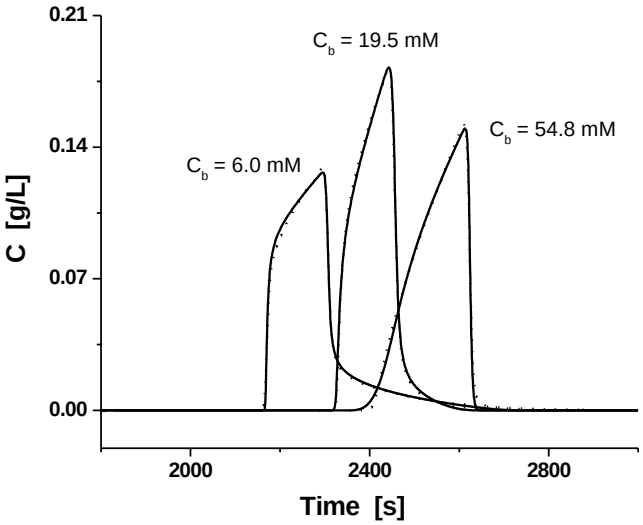

Fig. 8. Same as in Fig. 2, except buffer: phosphate pH = 6.75.

Some general conclusions can be drawn from these results (Table 3 and Figs. 2–5) regarding the evolution of the isotherm parameter with the buffer concentration. They are the same as those made in a previous report [5].

(1) Except for the succinate buffer at pH 4.16, the two saturation capacities increase with increasing buffer concentration. This is probably because the fraction of neutral propranol-buffer ion pair increases with increasing buffer concentration.

(2) The low-energy equilibrium constant, b_1, increases with increasing buffer concentration because the associated complex of the buffer ion and the propranolol cation is more hydrophobic and has a stronger affinity for the stationary phase than the solvated propranolol cations.

(3) The high-energy equilibrium constant, b_2, decreases with increasing buffer concentration, a fact for which there is no simple explanation. The higher b_2 values observed at low buffer concentrations is consistent with the stronger tailing of the corresponding bands in Figs. 2–5.

Table 3
Best isotherm parameters estimated by the inverse method (IM) for isotherm determination

Buffer	pH	C_{buffer} (mM)	$q_{s,1}$ (g/L)	b_1 (L/g)	I_1	$q_{s,2}$ (g/L)	b_2 (L/g)	I_2
Phosphate I	2.75	50.6	195	0.0444	0	4.45	2.01	0
		18.4	157	0.0384		3.03	3.46	
		5.7	142	0.0300		1.45	7.01	
Phosphate II	6.75	54.8	172	0.1040	11.5	0.76	5.93	1.65
		19.5	187	0.1020	6.73	0.40	11.0	0.33
		6.0	197	0.0684	29.8	0.93	11.8	0.33
Phthalate I	2.75	37.5	187	0.1391	0	2.68	3.36	0
		9.4	162	0.0780		2.12	6.45	
		2.2	106	0.0525		1.06	10.8	
Phthalate II	4.75	44.0	208	0.1870	2.59	0.23	13.3	0.26
		18.2	184	0.1410	4.55	1.50	7.74	0.09
		5.9	168	0.0758	17.2	1.90	9.80	0.65
Succinate I	4.16	60.0	201	0.0466	0	2.99	2.84	0
		18.6	196	0.0319		1.91	5.46	
		6.0	205	0.0216		0.73	14.7	
Succinate II	5.61	60.0	174	0.0940	2.12	0.83	1.96	<0.01
		20.0	170	0.0826	1.17	0.83	4.03	
		6.4	162	0.0654	0.47	0.70	7.86	
Acetate	4.75	60.0	163	0.0510	0	4.45	2.51	0
		21.2	136	0.0476		1.90	5.84	
		7.2	117	0.0357		0.96	11.3	

Optimization made on a band profile recorded after the injection of a 1.5 g/L solution of propranolol chloride during 15 s.

We observe also (Table 3) that the low-energy equilibrium constant, b_1, measured with the phthalate buffer is two to three times higher than with the other buffers (phosphate, succinate and acetate). This can be explaind by the higher hydrophobicity of the ion complex formed between the propranolol cation and the phthalate anion that contains a phenyl ring. Surprisingly, however, no such difference is observed for the high-energy equilibrium constant, b_2. The values of this constant are of the same order of magnitude for the different buffers. This suggests that the interactions that take place on sites 2 do not involve the hydrophobicity of the ionic complex. An ion-exchange mechanism would be inconsistent with the surface properties of the C_{18}-bonded material, however, the residual silanol groups of which being undissociated below pH 5.

When the buffer is made of a monovalent acid and a bivalent base (second phthalate, succinate and phosphate buffers, Figs. 6–8), the overloaded band profiles have clearly different shapes. For the same column loading and within the same range of total buffer concentration, the bands have a higher retention than with the corresponding neutral/monovalent buffer. As shown in the previous section, this not due to the higher pH. The bands are also much narrower and their height higher, reaching up to 0.3 g/L or about 1 mM. This implies that some adsorbate–adsorbate interactions take place in the adsorbed phase and that the bimoreau model should be preferred to describe the isotherm behavior under these conditions (Figs. 6–8). Note, for instance, that the bands recorded with the phosphate buffer at pH 6.75 have a diffuse front and a rear shock, a signature of anti-langmuirian behavior in the corresponding concentration range. The best bimoreau-isotherm parameters determined with IM are listed in Table 3. The comparison of the profiles calculated with this isotherm model (solid lines) and the experimental profiles (dotted lines) shows their excellent agreement (Figs. 6–8).

There are three important differences between the trends exhibited by the isotherm parameters obtained in this series and by those in the previous one.

(1) The low-energy equilibrium saturation capacity, $q_{s,1}$, is less affected by the buffer concentration. It slightly decreases (phthalate and succinate buffers) or even slightly increases (phosphate) with increasing buffer concentration. This is most probably due to the fact that, at all buffer concentrations, the buffer-ion/propranolol-cation form a neutral complex. The high-energy equilibrium saturation capacity, $q_{s,2}$, is always low (< 2 g/l).
(2) The low-energy equilibrium constant, b_1, is always higher ($\simeq$0.04–0.05 L/g) than with the corresponding buffer of the first series, which shows a larger hydrophobicity of the complex. The formation of a neutral complex that involves the bivalent basic anion and two propranolol cations could explain why the concentration in the adsorbed phase increases faster than the mobile phase concentration, which explains the observed anti-langmuirian behavior. No such difference is observed with the high-energy equilibrium constant b_2.
(3) Strong adsorbate–adsorbate interactions are found on the sites of type 1 ($0.5 \leq I_1 < 30$). Their intensity decreases in the order phosphate $\geq$ phthalate $\geq$ succinate. Weak adsorbate–adsorbate interactions take place on the high energy sites of type 2 ($0 \leq I_2 < 2$).

The large differences observed between the isotherm parameters of propranolol in the two buffers made at different pH's with the same diacid system illustrate the significantly different adsorption behavior of propranolol in these solutions. This difference is not due to the effect of a higher pH, which is set far from the pK_a of the compound studied (9.24), nor to the surface properties of the column that are not significantly modified in the pH range investigated. Instead, the formation of different ion complexes with widely different hydrophobicity explains the differences observed in the isotherm parameters:

$$[P^+ \cdots Cl^-] \Leftrightarrow P^+ + Cl^-$$

$$[P^+ \cdots B^-] \Leftrightarrow P^+ + B^-$$

$$[P^+ \cdots B^{2-} \cdots P^+] \Leftrightarrow 2P^+ + B^{2-}$$

Because the concentration of the buffer ions is much larger than that of propranolol, these reactions are widely shifted toward the left handside of these equations.

4.3. Highly overloaded band profiles

In the first two parts of this report, the retention of propranolol and the shape of its elution bands were studied at concentrations that were much lower than that of the buffer. Under such conditions, the injection of a 250 μL sample of a 1.5 g/L solution of propranolol prepared in the same buffer solution as the one used as the mobile phase causes the migration along the column of a single band of propranolol and of small perturbations of the buffer components. Because the solvated propranolol P^+ cation and the different possible complexes made of the ions in the buffer solution, $[P^+ \cdots Cl^-]$, $[P^+ \cdots B^-]$, and the possible $[P^+ \cdots B^{2-} \cdots P^+]$ are in equilibrium and exchange very rapidly, we observe a single band the retention of which is the average of the retention of each species pondered by its respective abundance at equilibrium. As usual in RPLC, there are no significant perturbations associated with the solvent molecules (MeOH and H_2O), the buffer system (B), the buffer co-cation (Na^+ or K^+) because these are too small to be detected at the wavelength used (325 nm). However, a perturbation associated with the co-anion of propranolol in the injected sample (chloride) should be detected. Chloride coexists as the solvated form Cl^-, the ion pairs $[Na^+ \cdots Cl^-]$ and $[K^+ \cdots Cl^-]$ that are all non retained, and the ion pair $[P^+ \cdots Cl^-]$ that is retained. According to the theory of perturbations [23], we should observe also perturbation peaks

for the chloride ion, although this ion could not be detected at 325 nm. Under linear conditions or with a moderate loading, we have always detected only the peak of propranolol because the buffer concentration is high compared to that of the sample so the concentration of the ion pair $[P^+ \cdots Cl^-]$ is small compared to that of the complexes $[P^+ \cdots B^-]$ and eventually $[P^+ \cdots B^{2-} \cdots P^+]$. Then, the perturbation associated with the chloride ion can be detected with a retention time close to the column hold-up time, at a suitable wavelength. It does not interfere with the propranolol band.

In this section, we consider the bands produced by the injection of a much larger sample of a 30 g/L (100 mM) solution of propranolol chloride prepared in the buffered mobile phase. The buffer capacity (6, 20 and 60 mM) is largely exceeded in the central region of the band for the first two buffer concentrations and it is still somewhat so for the third buffer concentration. In Fig. 9, for the sake of comparison, we show the profile obtained for the same sample in nonbuffered solutions of KCl. The overloaded band profiles recorded in the different buffer solutions are shown in Figs. 10–16.

We note first in these figures that, in the case of the monovalent ions (Figs. 10–13), the shape of the overloaded band profiles recorded with the highest buffer concentration (60 mM) is largely consistent with the strictly convex upward isotherm (Bi-langmuir) found in the previous section. The agreement is excellent in the eluate concentration range $0 \leq C \leq 1.5$ g/L, less satisfactory at higher concentrations. On the other hand, in the case of the bivalent anions (Figs. 14–16), the band profiles observed are markedly different, even in this concentration range. The band front is more diffuse and shock layers are observed on the desorption profiles. As suggested earlier, in Section 4.2, the choice of the bilangmuir model is no longer appropriate. The bimoreau model should be preferred in these cases.

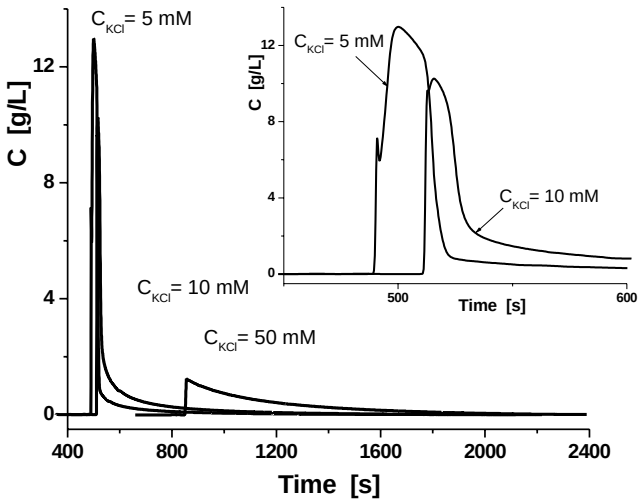

Fig. 9. Experimental band profiles of propranolol for an injection of 250 μL of a 30 g/L solution of propranolol chloride in mobile phases containing three different concentrations of the neutral salt KCl (methanol–water, 40:60, v/v). $T = 296$ K, flow rate 1 mL/min.

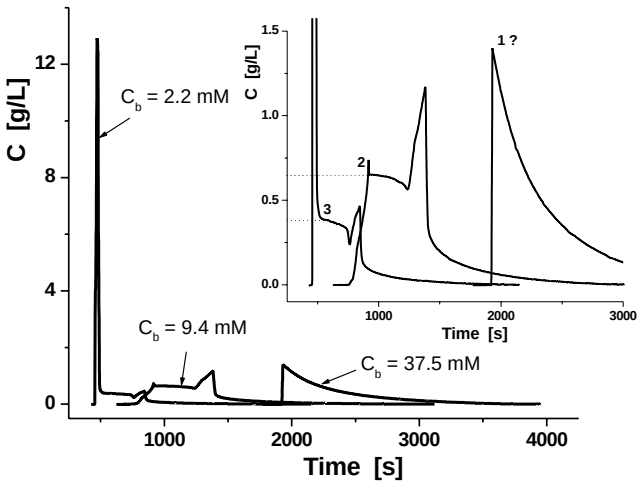

Fig. 10. Same as in Fig. 9, except the mobile phases contained no salt and were buffered with different concentrations of a phthalate buffer at pH 2.75.

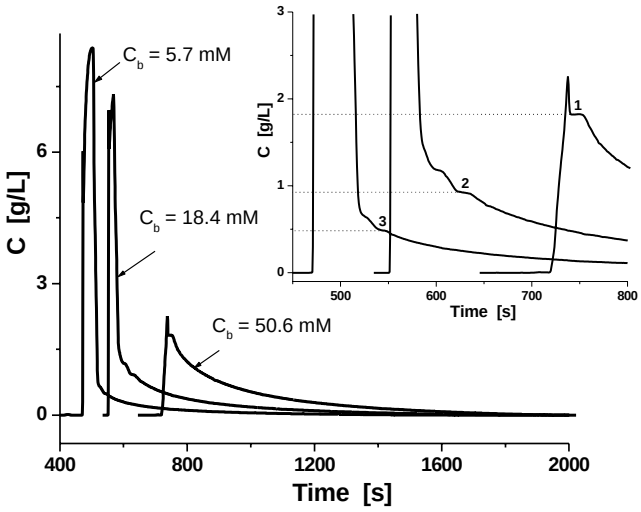

Fig. 11. Same as in Fig. 10, except phosphate buffer at pH 2.75.

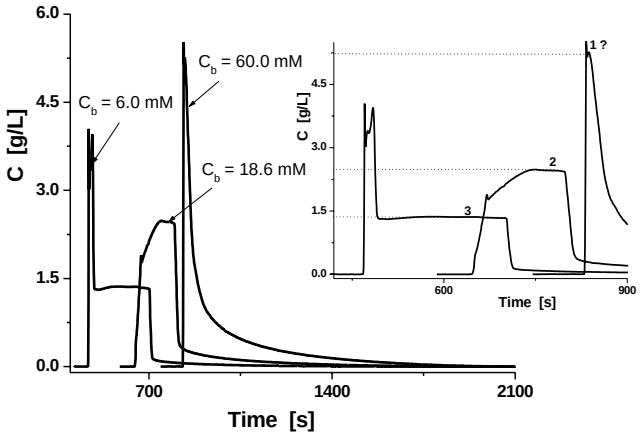

Fig. 12. Same as in Fig. 10, except succinate buffer at pH 4.16.

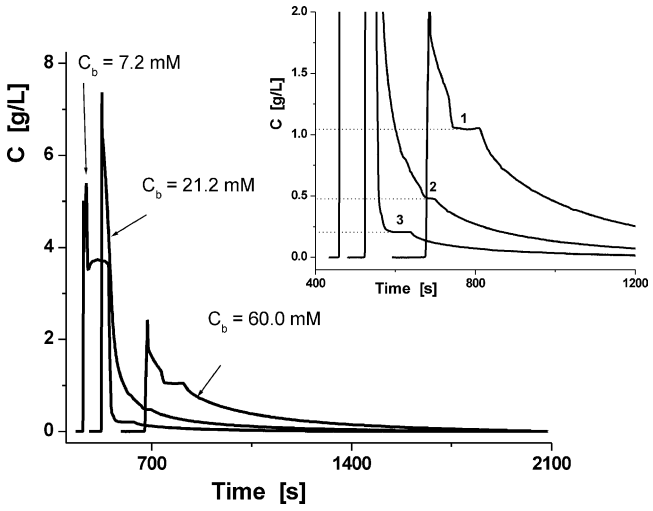

Fig. 13. Same as in Fig. 10, except acetate buffer at pH 4.75.

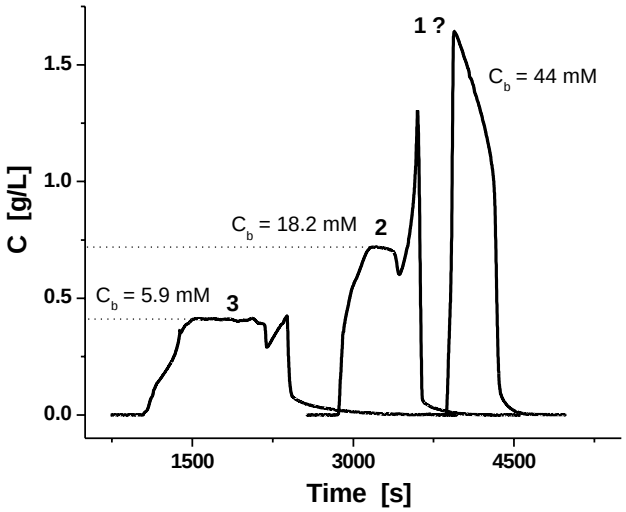

Fig. 14. Same as in Fig. 10, except phthalate buffer at pH 4.75.

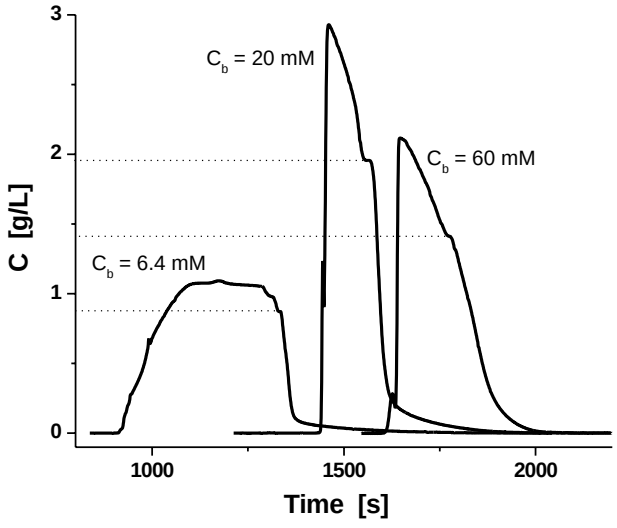

Fig. 15. Same as in Fig. 10, except succinate buffer at pH 5.61.

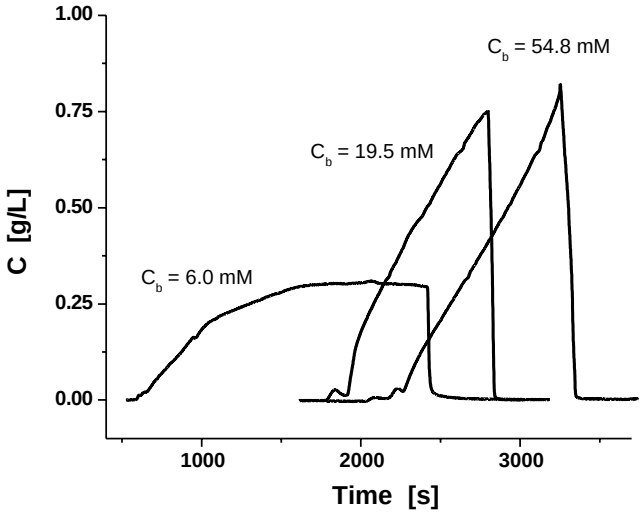

Fig. 16. Same as in Fig. 10, except phosphate buffer at pH 6.75.

Obviously, the same conclusion might have to be reached with the monovalent anions but at much higher propranolol concentrations, in which case adsorbate–adsorbate interactions might take place.

The concentrations of the chloride anion and the propranolol cation injected are sufficiently high in these experiments for the perturbations associated with the solvent molecules (methanol and water), the buffer molecules, and the buffer co-cation to be clearly detected. When propranolol chloride is injected into a mobile phase containing potassium chloride as the supportiong salt, the overloaded bands still have a smooth profile (Fig. 9). The perturbations associated with the two ions, propranolol and chloride, are related to the same retained ion pair, $[P^+ \cdots Cl^-]$, while both the solvated cation, P^+, and Cl^- are not retained. When a different anion is used, which is the case with all the buffers studied here, the overloaded band profiles exhibit several abrupt changes, many corresponding to discontinuities. Conventional chromatograms show the superimposition of the changes in UV absorption arising from all the perturbations occasioned by all the system components. For instance, the perturbations associated with the chloride anion, which exists in the system only as hydrated Cl^- and $[P^+ \cdots Cl^-]$, will elute with retention times than differ from the perturbation associated with propranolol that may exist as $[P^+ \cdots Cl^-]$, $[P^+ \cdots B^-]$, and probably $[P^+ \cdots B^{2-} \cdots P^+]$. The concentrations of these ion complexes may be comparable. Furthermore, we must consider the perturbation of the buffer itself, which may exist under its simple neutral form, as free solvated anions, and as ion complexes (in the case of the anions). The concentration of the complexes involving the buffer may be of the same order of magnitude as the concentration of the solvated buffer molecules.

The complex band profiles recorded when the buffer capacity is highly overloaded may be explained based on these considerations. In Figs. 10–13, we observe that the profile anomalies are related to the buffer concentration. However,

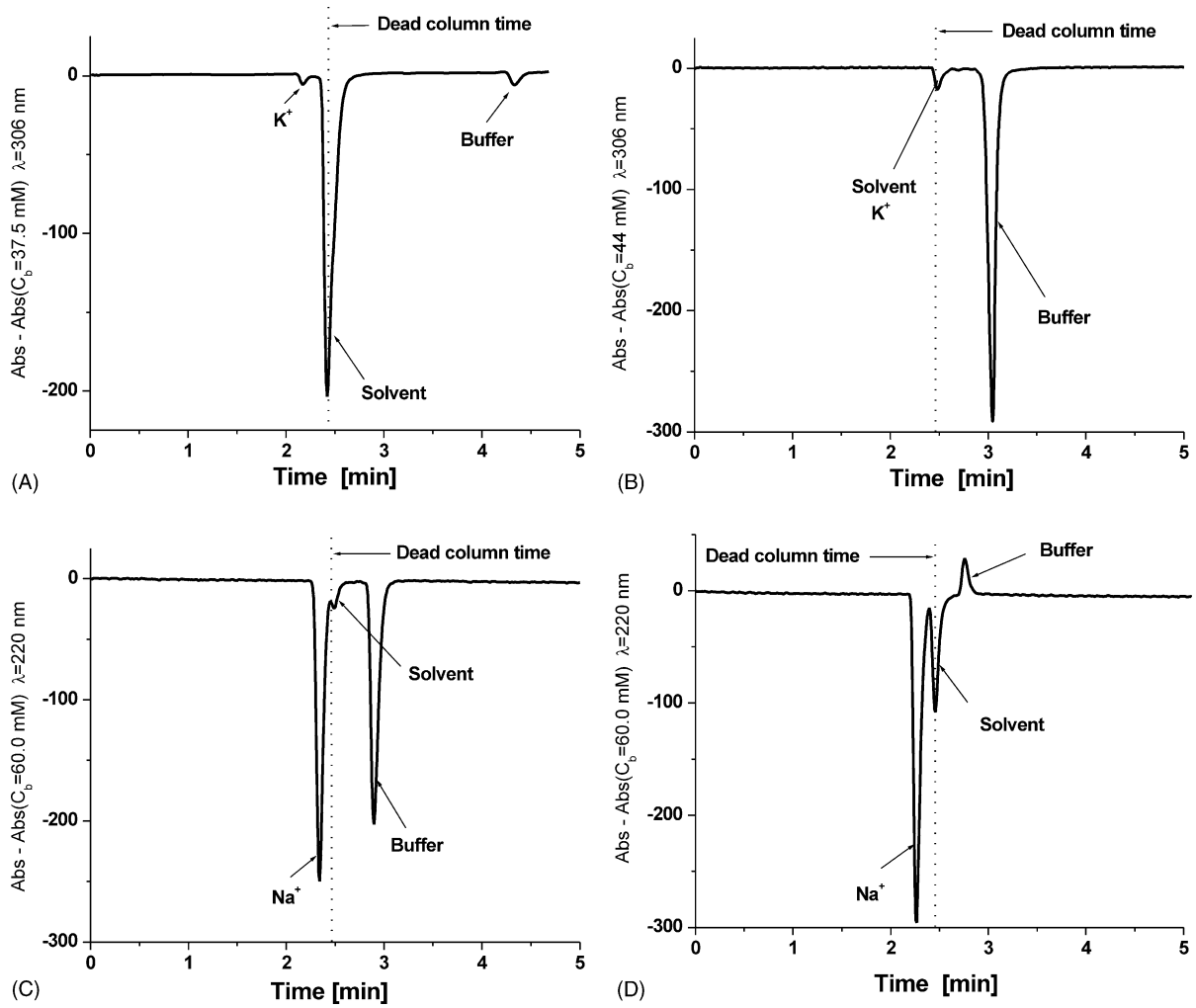

Fig. 17. Chromatograms showing the perturbations resulting from the injection of 30 μL of a pure methanol–water mixture (40:60, v/v) on concentration plateaus made of four different buffered mobile phases. Kromasil-C_{18} column. $T = 296$ K, flow rate 1 mL/min. (A) Phthalate 37.5 mM, pH 2.75. (B) Phthalate 44 mM, pH 4.75. (C) Succinate 60 mM, pH 4.16. (D) Succinate 60 mM, pH 5.61. The retention time of the buffer perturbations is always less than $2t_0$.

these anomalies cannot be interpreted as resulting from the competition for adsorption between the buffer and propranolol. Fig. 17 shows the recordings of perturbations made on equilibrium plateaus reached with eluents containing succinate or phthalate buffers at pH 4.16 and 5.61 for succinate, and 2.75 and 4.75 for phthalate. Three perturbations are always detected. They correspond to (1) the buffer which is slightly retained, but with a retention factor that was always less than 1.0; (2) the solvent, i.e., methanol and water which are unretained; and (3) the co-cation present in the buffer, i.e., Na^+ or K^+, which are excluded. On the other hand, the retention factor of propranolol was always larger than ten. So, no competition effect between these perturbations can be detected at the column outlet. However, perturbation signals associated with the buffer and the chloride ions could be at the origin of the profiles anomalies observed in Figs. 10–13 because these ions may form ion-complexes with the propranolol cation and these complexes are significantly retained on the Kromasil-C_{18} surface. For instance, the following retained complexes involving propranolol, the buffer and chloride may form with a succinate buffer at pH 5.61:

(1) Propranolol (P): $[P^+ \cdots Cl^-]$, $[P^+ \cdots B^-]$ and $[P^+ \cdots B^{2-} \cdots P^+]$.
(2) Succinate buffer (B): $[P^+ \cdots B^-]$ and $[P^+ \cdots B^{2-} \cdots P^+]$.
(3) Chloride (Cl): $[P^+ \cdots Cl^-]$.

To test these assumptions, perturbations were injected on three different concentration plateaus of a buffer and of the propranolol cation (Figs. 18–20). The buffer was an equimolar mixture of mono and di-sodium succinate, at pH 5.61, with a total buffer concentration of 12 mM. Three different concentrations of propranolol chloride were used, 1.2, 12, and 24 mM (equivalent to 1/10th, once, and twice the buffer concentration). The injection of 150 μL of a pure methanol:water solution caused five perturbations of the equilibrium plateau. As in Fig. 17, the main positive signal

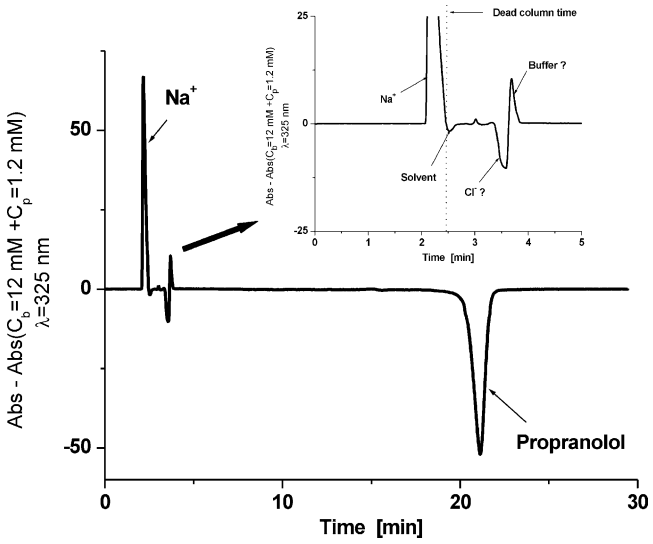

Fig. 18. Chromatogram showing the perturbations resulting from the injection of 150 μL of a pure methanol–water mixture (40:60, v/v) on a plateau made of a buffered mobile phase (succinate 12 mM, pH 5.61) in which was dissolved 1.2 mM of propranolol chloride. Kromasil-C_{18} column. $T = 296$ K, flow rate 1 mL/min.

appearing before the hold-up time in Fig. 18 is attributed to the co-cation of the buffer, Na^+. The solvent molecules (MeOH an H_2O) lead to a perturbation at about the column hold-up time. The three other perturbation peaks are due to the last three species involved, the propranolol cation, the chloride anion, and the buffer. The perturbation eluting at around 22 min is that of the plateau of propranolol, the most retained of these last three compounds. When very small amounts of propranolol chloride are dissolved in the buffered mobile phase (1.2 mM), propranolol exists almost only as one of the two ion complexes formed with the buffer, $[P^+ \cdots B^-]$ and $[P^+ \cdots B^{2-} \cdots P^+]$ because the complexation constant is larger with the buffer than with the chloride

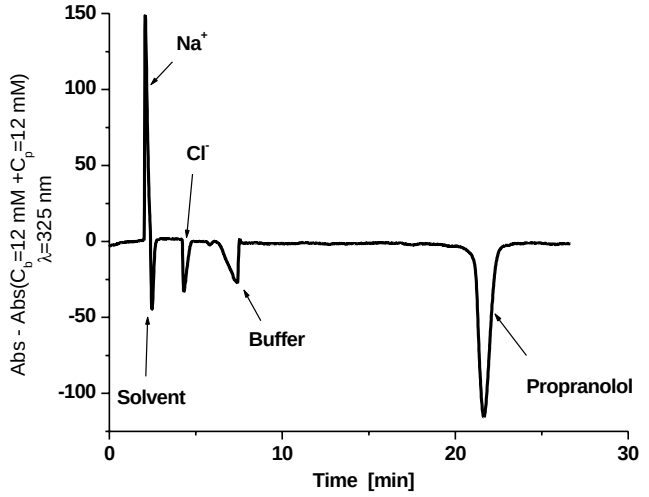

Fig. 19. Same as in Fig. 18, except that the concentration of propranolol in the mobile phase is ten times larger at 12 mM.

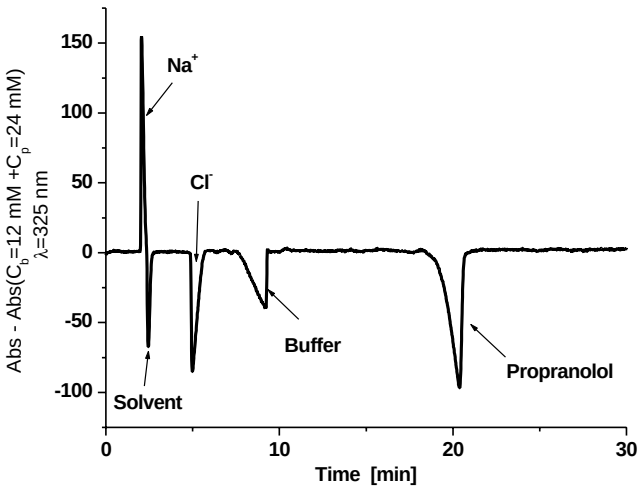

Fig. 20. Same as in Fig. 18, except that the concentration of propranolol in the mobile phase is 20 times larger at 24 mM.

anion. This conclusion is supported by the much shorter retentention time of the chloride perturbation at about 3.5 min. Also, since the succinate buffer is in large excess, it exists almost only as the free solvated ions, B^- and B^{2-}, which are not retained. This explains why the buffer perturbation peak has a short retention time, less than 4 min.

At a higher propranolol chloride concentration, 12 mM, the perturbation signals become higher (Fig. 19). The main effect of increasing the propranolol concentration, however, is to allow the formation of the complex $[P^+ \cdots Cl^-]$ at a significant concentration because the relative concentration of the buffer anions is lower and these anions cannot complex all the propranolol cations. As a result, the retention time of the perturbation signal of the chloride anion is larger, at about 4.5 instead of 3.5 min. In the same time, the concentration of free, solvated buffer anions in the solution is less and its perturbation peak is also more retained at 6.5 instead of 3.8 min.

These changes are similar but stronger when the concentration of propranolol chloride in the mobile phase is twice larger the buffer concentration (Fig. 20). The perturbations of the chloride and buffer ions have longer elution times (5.5 and 8 min, respectively). Because the relative abundance of $[P^+ \cdots Cl^-]$ is markedly increased, the retention time of the perturbation peak of propranolol drops down from 22 to 19 min.

Figs. 21–23 illustrate the result of similar perturbation experiments made with a buffer prepared with an equimolar mixture of neutral succinic acid and mono sodium succinate (pH 4.16). The perturbation peak of propranolol elutes earlier, at 10 to 15 min instead of more than 20 min at pH 5.61. This results from the two to three times lower values of the equilibrium constant at pH 4.16 than at pH 5.61 (Table 3). This result shows that the formation of the ion complex $[P^+ \cdots B^{2-} \cdots P^+]$ which is possible at pH 5.61, in addition to the complex $[P^+ \cdots Cl^-]$ that is always present in both buffers, contributes significantly to an increase of

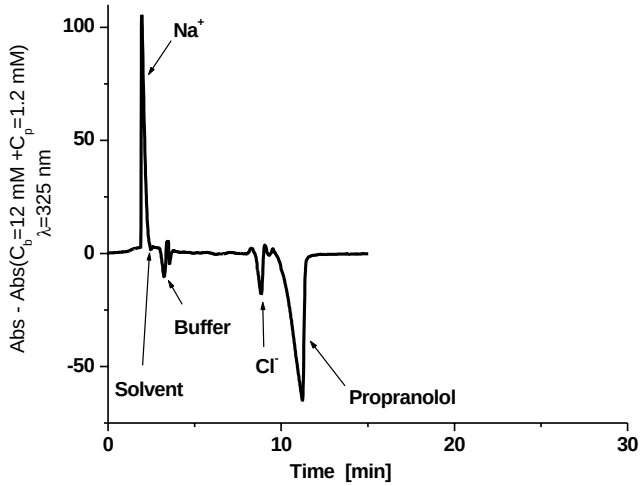

Fig. 21. Same as in Fig. 18, except that pH 4.16.

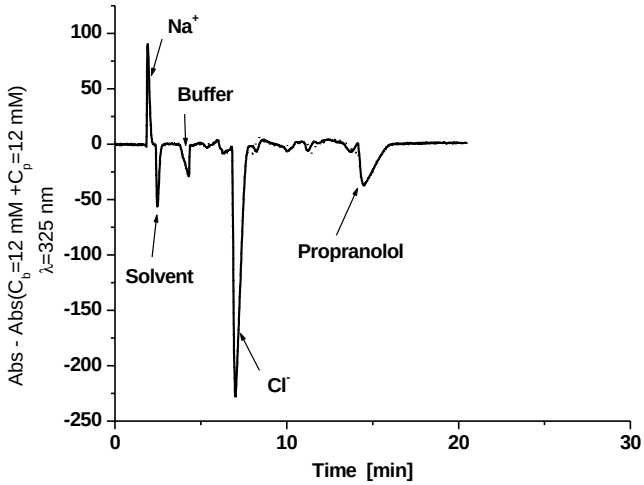

Fig. 22. Same as in Fig. 19, except that pH 4.16.

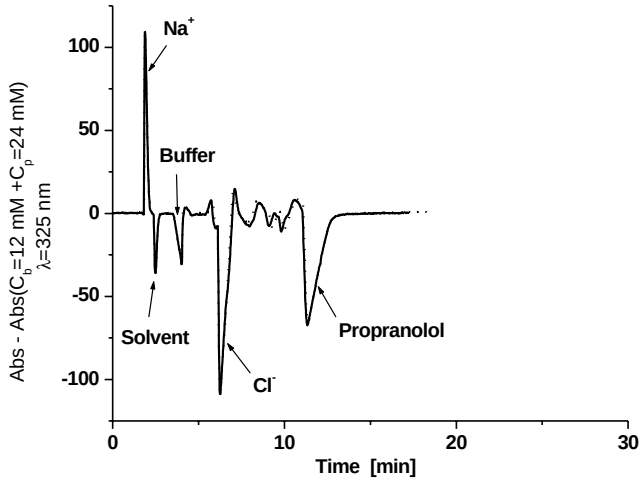

Fig. 23. Same as in Fig. 20, except that pH 4.16.

retention time. Note also that the retention time of the chloride perturbation is always larger at pH 4.16 because the concentration of the complex $[P^+ \cdots Cl^-]$ is higher at pH 4.16 than at pH 5.61 since, in the absence of 2:1 complex less propranolol is complexed with the buffer. The converse effect is observed for the buffer perturbation, in agreement with the lower proportion of buffer anions complexed with propranolol molecules.

In conclusion, the perturbation method confirms the existence of several ion-complexes that control the retention of propranolol when a salt or a buffer is dissolved in a methanol:water mobile phase. Both a 1:1 and a 2:1 propranolol–buffer ion complex can form when propranolol is dissolved in a buffer solution containing bivalent anions.

5. Conclusion

Strong evidence was presented that the use of buffers or even of simple neutral salts influences considerably the adsorption behavior of ionizable compounds such as the β-blocker propranolol. In this case, ion interactions between the protonated compound ($2 \leq$ pH ≤ 7, p$K_a = 9.25$) and the counter-anion present in the buffer or the salt solution determines the retention behavior. As reported by other authors, under linear conditions, the analyte retention increases with increasing counter-anion concentration in the mobile phase, in agreement with the prediction of the theory of chaotropicity [14]. However, we found one exception with a phosphate buffer at pH 6.75.

Measurements carried out in linear chromatography, isotherm data derived from overloaded band profiles, the shape of these profiles, and results of perturbation measurements show that the propranolol cation associates with monovalent and bivalent anions. In this last case, with succinate or phthalate basic buffers, a 2:1 propranolol:anion complex forms. Its formation explains the anti-langmuirian isotherm behavior that is detected in this case, at buffer concentrations that are lower than with monovalent anions. This result is consistent with the adsorption behavior of propranolol in a solution of the salt of a bivalent acid (SO_4^{2-}) that was described previously [5].

The manipulation of the buffer added to a mobile phase does not primarily allow the stabilization of the solution pH. It mostly interact with the analytes by forming ion complexes. The retention of ionizable compounds should not be merely seen as the retention of the solvated ion formed in the buffered solution. The retention observed experimentally depends on the specific equilibrium between the different associations involving the analyte and all the other ions in the solution. As a result, the valence, the polarisability, and the hydrophobicity of the anions used are fundamental factors in the understanding of the adsorption behavior of ions in RPLC systems.

The results described in this work seem to open several new challenging avenues of investigations. First, these

results confirm the importance of determining equilibrium isotherms when investigating retention mechanisms. The study of isotherms informs on the nature and energy of the interactions involved between the analytes and the stationary phase. Systematic investigations are now possible through the combination of the accurate determination of the functionality of the isotherm, by modeling of the FA data, and the rapid determination of the isotherm coefficients in any specific case, by the inverse method. This combination allows a rapid investigation of the influence of the important parameters affecting the retention mechanisms. Second, our results suggest that the realm of ion-pair chromatography extends farther into RPLC that what was generally accepted until now. Definitive conclusions on this point will be warranted, however, only after further systematic investigations regarding the extension of these results to numerous other basic compounds, in a wide range of pK_a values. Finally, if the results reported here are sufficiently general, they will facilitate the selection of the best buffer for the analysis of mixtures of related basic compounds, by providing a reliable tool and new criteria to affect retention and separation.

Acknowledgements

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