Extent of the influence of phosphate buffer and ionic liquids on the reduction of the silanol effect in a C18 stationary phase

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Abstract

The presence of anionic free silanols in the silica-based stationary phases gives rise to broad and asymmetrical peaks when cationic basic compounds are chromatographed using hydro-organic mobile phases. The addition to the mobile phase of a reagent with ionic character prevents the access of analytes to the free silanols, improving the peak shape. The silanol activity can be affected by the buffer concentration and mobile phase pH, factors that are not always considered sufficiently in the literature. In this work, the chromatographic behaviour of three basic β-adrenoceptor antagonists (acebutolol, nadolol and timolol), using mobile phases containing acetonitrile, was examined at different phosphate buffer concentrations (5 to 50 mM) and mobile phase pH (2 to 8), in the absence and presence of three imidazolium-based ionic liquids (1-ethyl-, 1-butyl- and 1-hexyl-3-methylimidazolium chloride). All factors were evaluated through both the retention and peak shape. The imidazolium cations can block the access of cationic analytes through electrostatic interaction with the anionic silanols, or association with the alkyl chains bound to the stationary phase. In previous reports, the protection mechanism was demonstrated to be directly related to the cation size. The studies in this work reveal that the effectiveness of the mobile phase additive as silanol blocker also depends on the concentration of the buffer anion and the protonation degree of the silanols on the stationary phase. Increasing amounts of phosphate at low pH give rise to increasing retention times. Also, the peak shape is improved, which indicates the influence of phosphate on blocking the activity of free silanols. However, the benefits obtained by the combined effect of buffering the mobile phase at low pH and the use of a bulky additive are lost at pH > 6.

Keywords: Reversed-phase liquid chromatography; Silanol blocking; Ionic liquids; Buffer concentration; Mobile phase pH

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1. Introduction

Peak tailing has been a persistent problem in reversed-phase liquid chromatography (RPLC), using silica as column support. At the usual working pH range of typical RPLC columns ($2 < pH < 8$), anionic silanols (which are present as a consequence of steric problems in the derivatisation of silica supports) introduce additional ion-exchange interactions in the chromatographic process [1–4]. The interaction with residual silanols is severe in the case of basic compounds, which is especially relevant if we take into account that most compounds of pharmaceutical interest contain basic nitrogen in their structure. As a result, the retention of cationic basic compounds increases, and due to the slow kinetics of this process, tailed and broad peaks are obtained, affecting the selectivity and peak resolution.

The use of impure silica in column manufacturing was highlighted as the origin of the high activity of silanols, even at low pH. These phases are known as type-A, and attracted numerous investigations focused on reducing the silanol activity [5]. Two decades later, the use of high purity silica (> 99.99 %), which allowed an increase in the average surface coverage, was introduced as a great advance to reduce peak tailing [6]. The key to this higher performance was a fully hydroxylated silica surface [7]. These phases are identified as type-B, and to date, are widely used in RPLC. However, although peak symmetry has considerably improved by minimizing the number of residual silanols, most modern type-B phases have not satisfactorily eliminated the peak tailing. As Fourier transform infrared spectroscopy studies evidenced, even fully hydroxylated silica has a small population of residual silanols [8].

To reduce or supress the silanol activity, several strategies have been addressed. The most extended consist of protonating (at least partially) the residual silanols by buffering the pH at acidic values, or masking the silanol interaction through the addition to the mobile phase of ionic (cationic or anionic), or even non-ionic reagents, able to be adsorbed on the stationary phase. The latter practice has shown very satisfactory in terms of peak symmetry with bulky

reagents added to the mobile phase, as is the case of room temperature ionic liquids (RTILs) or amines [9,10]. Other strategies, such as an increased ionic strength in the mobile phase, or the addition of a more retentive buffer cation (e.g. potassium) are less common [1].

The evaluation and understanding of the minimization of the silanol effect is important to improve the chromatographic behaviour of basic compounds. The observed retention is explained by a mixed mechanism that combines a reversed-phase interaction of the positively charged solute with the alkyl-bonded stationary phase and electrostatic interaction with the anionic silanols. The presence of additives in the mobile phase usually modifies this mechanism by introducing, to the separation environment, additional equilibria between analytes and additives, either adsorbed on the stationary phase or dissolved in the mobile phase. The global process has positive consequences on the peak profile, and consequently, on the chromatographic resolution. However, other factors such as buffer concentration and pH should be also considered.

Regardless of the selected strategy, the evaluation of the silanol activity and the contribution of the different parameters that help to reduce it is not straightforward. Approaches based on mathematical models that consider different retention sites for solutes on the stationary phase [11,12], or the design of experimental tests based on the use of basic probe compounds [13,14], have tried to offer some light on this topic, but not always with consistent conclusions.

In this work, the chromatographic behaviour of three basic compounds was examined at different buffer concentrations and mobile phase pH, in the absence and presence of three imidazolium-based RTILs (1-ethyl-, 1-butyl- and 1-hexyl-3-methylimidazolium chloride). The influence of these factors on the silanol activity of a C18 stationary phase, under different experimental conditions, was evaluated through the observation of the changes in the retention and peak shape, the latter expressed as the ratio of the slopes of the straight-lines obtained by plotting the values of the peak half-widths versus the corresponding retention

time. The study of the influence of the pH on the silanol activity revealed new information on the mechanism of protection of residual silanols, when mobile phase additives of different size are introduced in the separation support.

2. Experimental

2.1. Reagents

Three β-adrenoceptor antagonists were used as probe compounds: acebutolol, nadolol (commercialised as a mixture of isomers), and timolol (all from Sigma, St. Louis, MO, USA). The pK_a of these drugs is ≥ 9 [15], which means that at the working pH range of the mobile phases (2–8), they are mainly cationic. Stock solutions of approximately 100 μg/ml of the drugs were prepared in a small amount of acetonitrile, with the aid of an Elmasonic IT-H ultrasonic bath from Elma (Singen, Germany), and diluted with water. The solutions remained stable during at least two months at 4 ºC. The concentration of the injected solutions was 20 μg/ml. Uracil (Acros Organics, Geel, Belgium) was used as dead time marker.

The mobile phases contained acetonitrile (Scharlab, Barcelona, Spain) and one of the following RTILs: 1-ethyl-3-methylimidazolium chloride (EMIM·Cl), 1-butyl-3-methylimidazolium chloride (BMIM·Cl), and 1-hexyl-3-methylimidazolium chloride (HMIM·Cl) (Sigma). Based on previous experience [16,17], the concentration of acetonitrile was fixed at 10 % (v/v) as reference value. The addition of bulky imidazolium cations to the mobile phase yields a significant decrease in the retention of the β-adrenoceptor antagonists up to column saturation is reached, where minimal changes in retention are observed [17]. In this work, the three RTILs were added to the mobile phase at a fixed concentration of 10 mM. The concentrations of acetonitrile and RTILs were selected to guarantee the elution of the assayed drugs in practical analysis times.

The acetonitrile-water mobile phases were first buffered at pH 3 with sodium dihydrogen phosphate (Sigma) at concentrations varying from 5 to 50 mM, to which HCl was added. Next, the mobile phases were buffered at pH ≈ 2, 3, 4, 5 6, 7 and 8 (measured in the aqueous portion of the mobile phase), using a constant concentration of 10 mM sodium dihydrogen phosphate. Sodium hydroxide (Panreac, Barcelona) was used to get pH values above 4. The drug solutions and mobile phases were filtered through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA, USA), and degassed in an ultrasonic bath. Nanopure water (Barnstead, Sybron, Boston, MA) was used throughout.

2.2. Apparatus and columns

An Agilent instrument (Waldbronn, Germany), equipped with an isocratic pump (Series 1100), an autosampler (Series 1260), a thermostated column compartment (Series 1260) set at 25 ºC, and a UV-visible wavelength detector (Series 1100), was used. Acebutolol and nadolol were monitored at 254 nm, and timolol, at 300 nm. An HPChemStation (Agilent, B.02.01) assisted in data acquisition. The mathematical treatment was carried out with Excel (Microsoft Office 2010, Redmond, WA, USA). The chromatographic peaks were integrated with MICHROM, based on the polynomially modified Gaussian model. This software allowed the measurement of peak half-widths with big accuracy. [18].

A Zorbax Eclipse XDB (Agilent) column with the following characteristics was used: 150 mm × 4.6 mm i.d., 5 μm particle size, 10 % carbon load, 180 m^2/g surface area, and 80 Å pore size. A 30 mm C18 precolumn was connected before the injector to protect the analytical column from the mobile phase. The flow-rate was 1 ml/min. Duplicate injections of 20 μl were carried out.

3. Results and discussion

Recent reports have indicated that bulky additives do not interact directly with residual silanols by direct association, but give rise to a sort of coating on the stationary phase, forming a layer with a positive charge oriented towards the mobile phase that avoids solute access to the silanol sites [9,16,17]. This is the case of $BMIM^+$ and $HMIM^+$, which are adsorbed on the C18 bonded stationary phase due to their hydrophobic character, depending on their alkyl chain length [19]. Instead, small additives (as $EMIM^+$) may be able to block the residual silanols by direct electrostatic interaction, being this protection mechanism less effective (the peak shape is poorer). The constitutive anion of a given RTIL may have a very different effect compared to the associated cation [19]. When the anions of the RTILs are strongly adsorbed on the stationary phase, such as tetrafluoroborate or hexafluorophosphate, the interaction (attraction) of cationic solutes with these anions can be more important than the interaction (repulsion) with the adsorbed cations in the RTIL. This results in an increased retention, which depends on the concentration of the RTIL in the mobile phase. In contrast, those anions with low affinity for the stationary phase (such as chloride) gives rise to short retention times, since the repulsion of the cationic solutes from the cationic stationary phase by adsorption of the RTIL is dominant. According to its close position to chloride in the Hofmeister series [19], a similar behaviour should be expected for phosphate used as buffer in the mobile phase.

This work examines the influence of the concentration of phosphate buffer and pH in the mobile phase on the retention and peak shape of related basic probe compounds with different polarity, eluted with mobile phases containing acetonitrile, in the absence and presence of a fixed concentration (10 mM) of the RTILs EMIM·Cl, BMIM·Cl and HMIM·Cl, which are capable of blocking the activity of residual silanols on silica-based chromatographic columns. Based on previous experience with a larger group of β-adrenoceptor antagonists, analysed

with RPLC using diverse RTILs [16,17,20], it was decided to make the study with only three representative compounds of the group (acebutolol, nadolol and timolol).

β-Adrenoceptor antagonists are commercialised and used for the treatment of various cardiac diseases [21]. The octanol-water partition coefficients (log $P_{o/w}$) are 1.17, 1.83 and 1.98 for nadolol, acebutolol and timolol, respectively [15], and have a strong basic character due to the presence of an aromatic ring attached to a side alkyl chain possessing secondary hydroxyl and amine functional groups. These features make them useful to evaluate the silanol effect on basic compounds using C18 columns and additives [20,22,23].

3.1. Influence of the buffer concentration on the silanol activity

3.1.1. Changes in retention

The influence of the buffer concentration on retention has been studied by several authors. In a few reports, the buffer concentration was varied to investigate its effect not only on the retention, but also on the peak shape and column overloading, for different types of solutes and chromatographic modes [14,24,25]. Ammonium buffers were used to estimate the contribution of the silanol activity on the retention of different types of solutes, based on a mathematical model that assumed the existence of two independent and additive interaction sites in the stationary phase: the hydrocarbon chains and the residual silanols. This two-site model, originally proposed by Sokolowski and Wahlund [26], and further developed by Horváth and co-workers [27,28], was applied to investigate the contribution of solute-silanol interactions on the retention for a group of benzylamines in polybutadiene coated zirconia and octadecyl silane columns [11], and on the separation of a group of RTILs by hydrophilic interaction liquid chromatography [29]. More recently, the two-site model has been applied to estimate the effect of several amine additives added to the mobile phase at varying

concentration, and fixed buffer content, on the overall separation of a group of β-adrenoceptor antagonists [10].

The predictions of the two-site model are based on the values of the intercept and slope obtained for the linear relationships between the logarithm of the retention factor (*k*) for the studied solutes, and the logarithm of the concentration of buffer or mobile phase additive. Since the model was defined for cationic buffers and additives, the slope varies between zero if the reversed-phase process governs the retention, and –1 when the electrostatic interaction with the cationic reagents is dominant. In contrast, the retention factors of the three β-adrenoceptor antagonists studied in this work increased slightly at increasing concentration of phosphate buffer (see *k* values in Table 1), using mobile phases containing 10 % acetonitrile at pH 3, where the $H_2PO_4^-$ species is dominant. The log *k* vs. log [buffer] relationships would then yield plots with positive slope. This trend remained in the presence of 0.01 M BMIM·Cl and HMIM·Cl, and in lesser extent for EMIM·Cl (see also Table 1).

The behaviour of the cationic solutes may be the result of the combination of the following interactions:

(i) The hydrophobic association of solutes with the alkyl-bonded stationary phase.

(ii) The association of the solutes to the anionic silanols.

(iii) With RTILs, the repulsion of the solutes by the RTIL cations adsorbed on the alkyl chains of the stationary phase (which get positive charge).

(iv) In spite of the rather weak adsorption of phosphate ion on the alkyl chains, the adsorbed amount may give rise to attraction of cationic solutes.

A comprehensive report on the expected interactions can be found in Ref. [9]. As commented, the trend in the presence of increasing phosphate buffer, which is anionic, is opposite to the behaviour observed with amine buffers. Since the retention increases at increasing buffer concentration, interaction (iv) may be primarily responsible of the observed

behaviour. Interaction (i) and (ii) should also cooperate to the retention of solutes. Meanwhile, interaction (iii) would give rise to an opposite effect, reducing the retention. The combined effects can explain the behaviour in the presence of the three RTILs. A detailed study of the changes in peak shape, described in the next section, will confirm this conclusion.

3.1.2. Changes in peak shape

The effect of the buffer concentration at pH 3 on the silanol activity, revealed by the peak shape, was checked using the half-widths plots tool, whose validity to compare the behaviour of basic drugs eluted with different types of columns and mobile phase additives has been demonstrated in previous reports [10,16,20,22,30]. The half-width plots are described by the following equations:

$$A = m_A t_R + A_0 \quad (1)$$

$$B = m_B t_R + B_0 \quad (2)$$

where A and B are the left and right half-widths, which should be measured at 10 % peak height to avoid baseline noise, m_A and m_B are the slopes of the linear correlations for the left and right half-widths, and A_0 and B_0 represent the extra-column contributions to the peak broadening, respectively. One of the most interesting applications of this tool is the characterisation of a chromatographic system based on the parameters calculated from Eqs. (1) and (2): the sum of slopes ($m_A + m_B$) represents the peak broadening rate inside the column, whereas the ratio (m_B/m_A) indicates the asymmetry of peaks eluting at a time where the extra-column contribution is non-significant.

Fig. 1 depicts the half-widths plots for the set of probe compounds, eluted with the mobile phases without additive and in the presence of HMIM·Cl, at three assayed phosphate concentrations (see also Fig. S1 in the Supplementary material). The most remarkable characteristic is that in the absence of additive (Figs. 1a-c), the slope of the right half-width

(*B*) was significantly larger than for the left half-width (*A*), which indicates the formation of tailing peaks. The peak shape improved at increasing buffer concentration, as observed by the smaller angle between the *A* and *B* plots. The improvement in peak symmetry was significantly larger in the presence of HMIM·Cl, without apparent influence of the buffer (Figs. 1d-f). The behaviour of EMIM·Cl was similar to that obtained in its absence, and the behaviour of BMIM·Cl was similar to that obtained with HMIM·Cl (results not shown).

The parameters obtained from the fitting of the half-widths plots are shown in Table 2. As observed, an increasing buffer concentration in the range 5 to 50 mM resulted in more symmetrical peaks without an additive and in the presence of EMIM·Cl. The changes in peak shape were especially remarkable in the range 5 to 30 mM. Meanwhile, at any buffer concentration, the peaks obtained in the presence of BMIM·Cl and HMIM·Cl were always symmetrical (i.e. the slopes ratios were close to unity).

In previous work, the adsorbed amount of several RTILs with imidazolium cations was found to increase with the hydrophobicity of the alkyl-bonded chain of the ILs [19]. Thus, it can be expected that the adsorbed amount of EMIM·Cl on the stationary phase should be smaller compared to BMIM·Cl and HMIM·Cl. This creates a separation environment closer to that obtained without additive, which would explain the more intense influence of buffer on the peak shape. In contrast, the symmetrical peaks obtained in the presence of BMIM·Cl and HMIM·Cl at any buffer concentration indicate an effective column protection by coating the surface of the C18 stationary phase with the RTIL.

3.2. Influence of the pH on the silanol activity

Silanols are weakly acidic, with average protonation constants ranging from ca. log K = 4.5 to ca. 7, depending on the type of silica [31]. Therefore, the degree of ionisation of silanols varies with the mobile phase pH, inducing changes in the cationic solute-silanol

interaction. At higher pH, changes in retention can be also observed depending on the solute pK_a. Besides, close to pH 7.5, the phosphate system losses an additional hydrogen ion, giving rise to HPO_4^{2-}. For the three studied β-adrenoceptor antagonists with pK_a ~ 9, the cationic species is dominant at the usual working pH range of reversed-phase columns (2 < pH < 8). This means that the only relevant acid-base equilibria in this pH range is that of silanols and phosphate. An increase in the pH should induce higher retention due to the interaction of the cationic drugs with the anionic silanols, which results in poorer peak shape. In the presence of salts of RTILs with a poorly adsorbed anion, such as chloride, it should be expected that the retention and peak shape should not change considerably with the mobile phase pH (at constant concentration of the buffer), due to the effective column protection. It should be also noted that RTILs have been indicated to exhibit minimal influence on the pH of the mobile phase when used as additives [32], with the exception of some imidazolium-based RTILs with tetrafluoroborate anion [33].

In order to check this behaviour, mobile phases at fixed buffer concentration of 10 mM without additive, or containing 10 mM EMIM·Cl, BMIM·Cl or HMIM·Cl, were prepared in the pH range 2–8. The changes in retention factor at varying pH are depicted in Fig. 2 (see also Fig. S2 in the Supplementary material). As observed, the retention of the β-adrenoceptor antagonists increased slightly between pH 2 and 6, and strongly at pH > 7, especially in the presence of HMIMCl (Fig. 2d). The relative increase in the retention factors for the studied solutes calculated as:

$$k' = \frac{k_{\mathrm{pH8}} - k_{\mathrm{pH2}}}{k_{\mathrm{pH2}}} \qquad (3)$$

are given in Table 3. In Eq. (3), k_{pH2} and k_{pH8} are the retention factors for acebutolol, nadolol and timolol obtained at pH 2 and 8, respectively.

Figure 3 shows the effect of pH on the peak asymmetry, expressed as m_B/m_A. It is observed that this notably deteriorates between pH 6 and 8 when the deprotonation of silanols becomes significant, even in the presence of the RTILs. It was not possible to obtain the slope of the half-width plots at pH > 7, due to a split peak for nadolol in the presence of the RTILs. The results reveal that the stationary phase is probably not completely covered by the cation of the imidazolium-based RTILs and this protection does not hinder the access of the cationic solutes to the residual silanols at high pH.

It is interesting to note that the m_B/m_A ratio is practically constant between pH 2 and 6. The average values in this range were 2.5 in the absence of additive, and 1.63, 1.25 and 1.00 in the presence of EMIM·Cl, BMIM·Cl and HMIM·Cl, respectively. The relative increase in the m_B/m_A ratio calculated as:

$$(m_A/m_B)' = \frac{(m_A/m_B)_{pH7} - (m_A/m_B)_{pH2\text{-}6}}{(m_A/m_B)_{pH2\text{-}6}} \quad (4)$$

was 0.32 in the absence of additive, and 0.38, 0.50 and 1.82 in the presence of EMIM·Cl, BMIM·Cl and HMIM·Cl, respectively. Therefore, the effective peak shape improvement of RTILs depends on the pH value. When silanols are deprotonated, this effect is lost.

4. Conclusions

The evaluation and understanding of the minimization of the silanol effect is important to improve the chromatographic behaviour of basic compounds. The observed retention is explained by a mixed mechanism that combines a reversed-phase interaction of the positively charged solutes with the alkyl-bonded stationary phase and electrostatic interaction with the anionic silanols. The presence of additives in the mobile phase, such as RTILs, usually modifies this mechanism by introducing additional equilibria to the separation environment (mainly repulsion of the cationic solutes by the RTIL cations adsorbed on the alkyl chains of

the stationary phase and attraction of the analytes by the constitutive RTIL anion). The global process has positive consequences on the peak profile, and consequently, on the chromatographic resolution. However, other factors such as the presence of buffer (and its concentration), and pH should be also considered.

The results obtained in this work reveal that the adsorption on the stationary phase of the phosphate anion, added to buffer the pH, may have an important role in the separation process. Increasing amounts of phosphate at low pH give rise to increasing retention times. Also, the peak shape is improved, which indicates the influence of the phosphate adsorbed on the stationary phase on blocking the activity of residual silanols in the C18 column. The observed effect (increased retention time, and improved peak symmetry) is minimal if the RTIL cation is largely adsorbed on the stationary phase (case of $BMIM^+$ and $HMIM^+$), whereas it is significant in the absence of RTIL and presence of an RTIL experiencing small adsorption ($EMIM^+$). At pH > 6, the column seems to be more effectively protected using an RTIL cation able to directly access to the silanols to establish electrostatic interaction ($EMIM^+$).

The observed effects upon changes in the pH of the mobile phase at fixed buffer concentration, in the absence and presence of the studied RTILs, revealed that the stationary phase does not seem to be completely covered by the imidazolium cations: the retention of the β-adrenoceptor antagonists increased at increasing pH and the peak symmetry deteriorated, especially for the RTILs associated to bulky cations ($HMIM^+$). These results suggest that the capability of a mobile phase additive as silanol suppressor depends on the non-protonated/protonated silanols ratio on the stationary phase. Thus, the benefits obtained by the combined effect of buffering the mobile phase at low pH and addition of a bulky reagent ($BMIM^+$ or $HMIM^+$) are lost at pH > 6.

It should be aware that as with most mobile phase additives, it is very difficult to run LC-MS. In spite of this, in recent time, the interest in ionic liquids used as additives in liquid chromatography has remarkably increased, finding many applications.

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FIGURE CAPTIONS

Fig 1. Half-width plots (left, *A* (●), and right, *B* (○) peak regions), for the set of β-adrenoceptor antagonists, obtained with mobile phases containing 10 % acetonitrile without additive (a–c), and in the presence of 10 mM HMIM·Cl (d–f), at different NaH_2PO_4 concentrations: (a,d) 5 mM, (b,e) 10 mM, and (c,f) 30 mM.

Fig. 2. Retention factors for nadolol (▲), timolol (■) and acebutolol (●), using aqueous mobile phases containing 10 % acetonitrile in the absence of additive (a), and presence of 10 mM EMIM·Cl (b), 10 mM BMIM·Cl (c), and 10 mM HMIM·Cl (d), at different pH values buffered with 10 mM NaH_2PO_4.

Fig. 3. Plots of peak asymmetry expressed as m_B/m_A versus the pH: without additive (●), and with EMIM·Cl (○), BMIM·Cl (■), and HMIM·Cl (□).

Figure 1

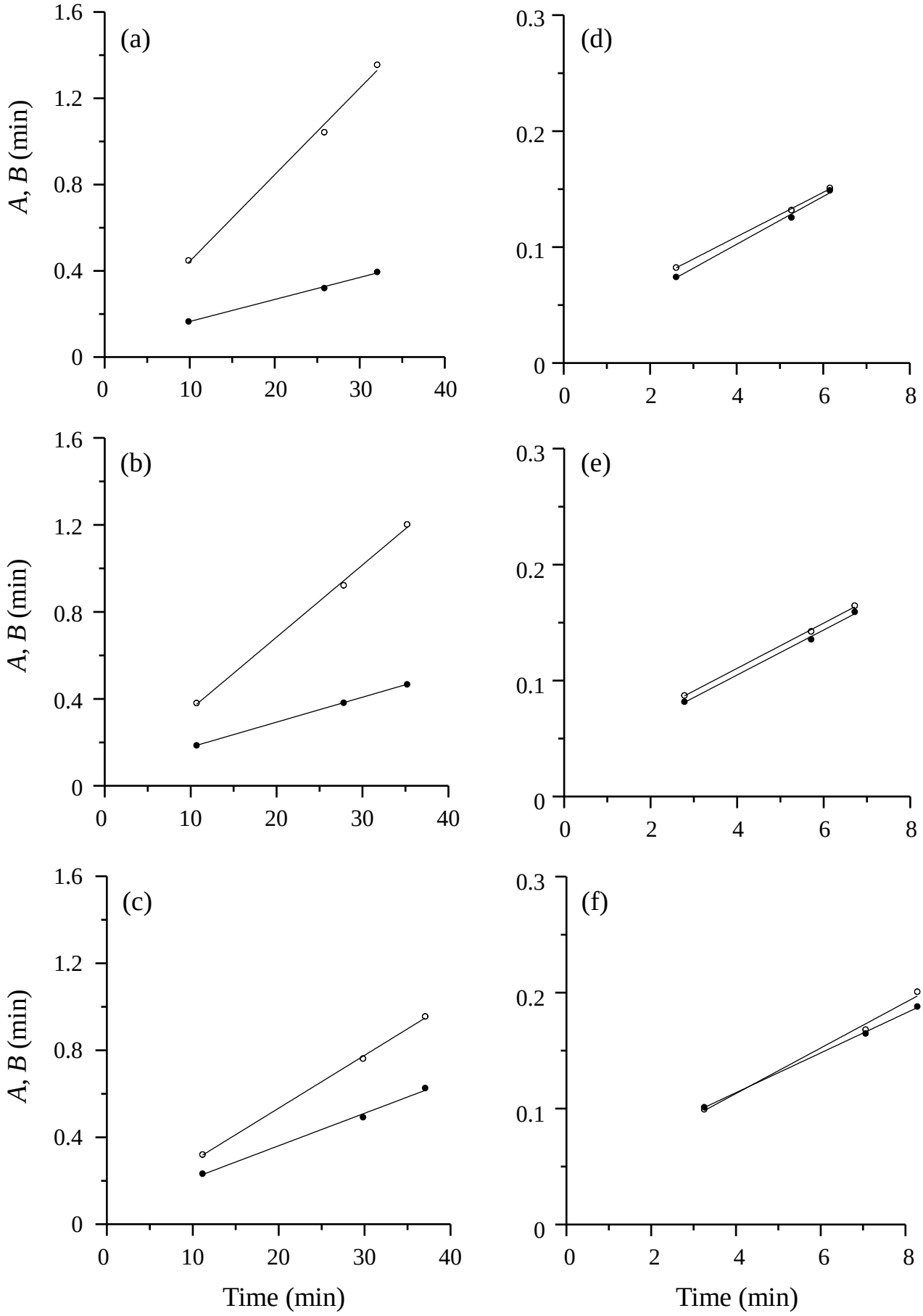

Figure 1

Figure 2

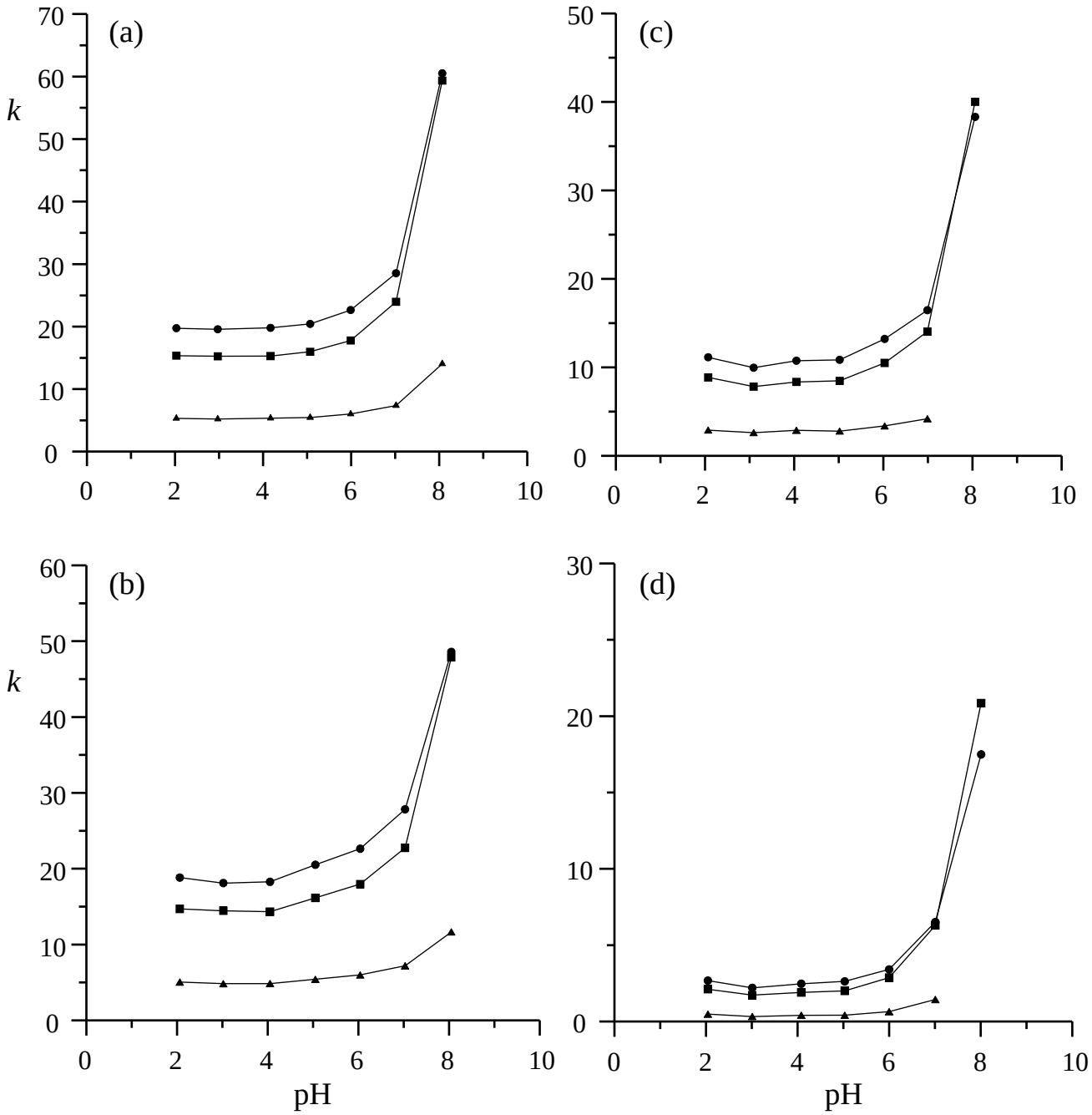

Figure 2

Figure 3

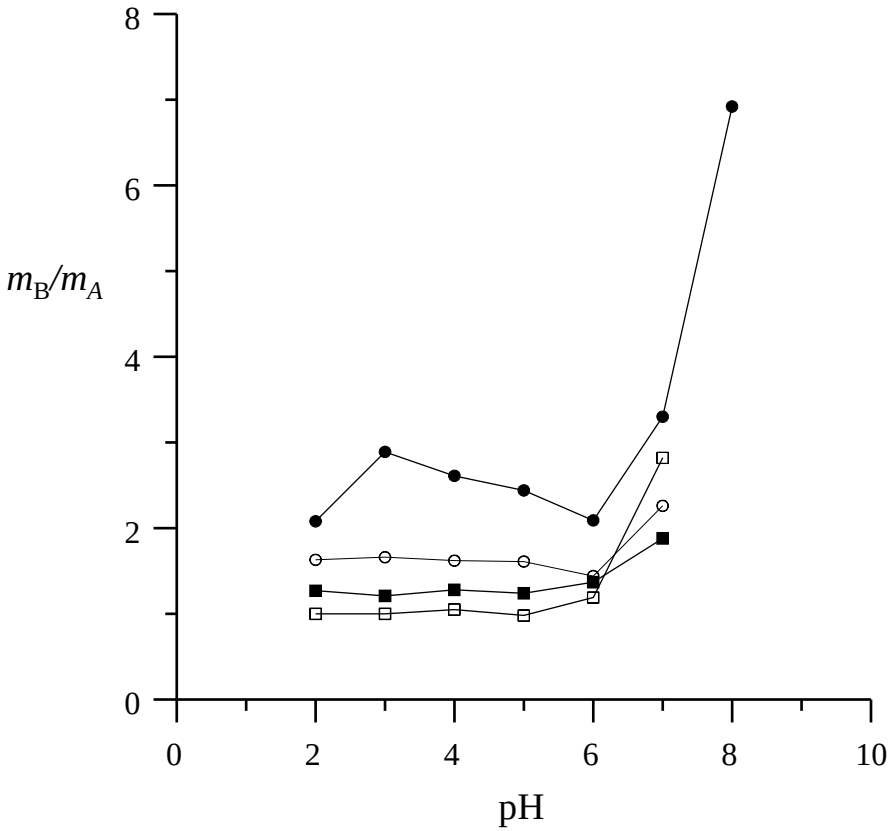

Figure 3

Tables

Table 1. Retention factors for the assayed β-adrenoceptor antagonists at increasing buffer concentration, in the presence and absence of 10 mM RTILs and 10 % acetonitrile.

	NaH_2PO_4 (mM)	Nadolol	Timolol	Acebutolol
Without additive	5	4.7	13.9	17.5
	10	5.2	15.1	19.3
	30	5.4	16.2	20.4
	50	5.7	16.9	21.3
EMIM·Cl	5	4.8	14.6	18.3
	10	4.8	14.4	18.0
	30	5.0	15.0	18.6
BMIM·Cl	5	2.1	6.6	8.4
	10	2.5	7.5	9.5
	30	2.8	8.5	10.6
HMIM·Cl	5	0.5	2.0	2.6
	10	0.6	2.3	2.9
	30	0.9	3.1	3.8

Table 2. Half-widths plots parameters for the assayed β-adrenoceptor antagonists at increasing buffer concentration, in the absence and presence of 10 mM RTILs and 10 % acetonitrile.[a]

	Without additive		EMIM·Cl (10 mM)		BMIM·Cl (10 mM)		HMIM·Cl (10 mM)	
NaH_2PO_4 (mM)	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A
5	0.050	3.94	0.053	3.19	0.037	1.17	0.040	0.93
10	0.045	2.89	0.039	1.65	0.037	1.21	0.039	1.00
30	0.039	1.63	0.037	1.42	0.036	1.16	0.037	1.14
50	0.038	1.39						

[a] m_A and m_B are the slopes of the half-widths plots (see Eqs. (1) and (2)).

Table 3. Relative increase in the retention factors for the assayed β-adrenoceptor antagonists at varying pH under different conditions, calculated using Eq. 3.

Additive	Nadolol	Timolol	Acebutolol
Without additive	1.6	2.9	2.1
EMIM·Cl	1.3	2.2	1.6
BMIM·Cl	ND[a]	3.5	2.4
HMIM·Cl	ND[a]	8.8	5.5

[a] ND: Non determined. Split peak

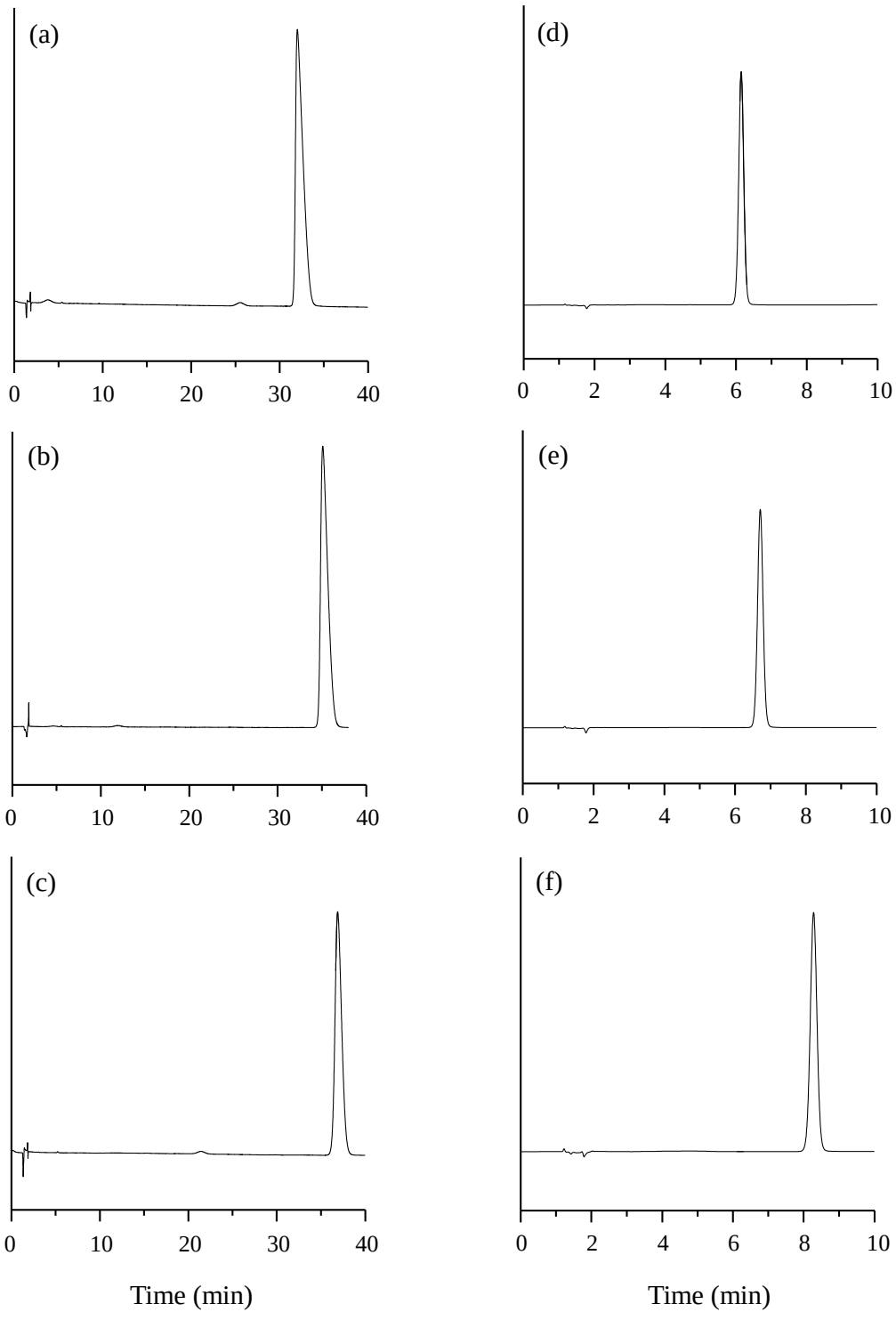

Fig. S1. Chromatograms for acebutolol obtained using aqueous mobile phases containing 10% acetonitrile without additive (a-c) and presence of 10 mM HMIM·Cl (d-f) at different NaH_2PO_4 concentrations and pH 3: (a,d) 5 mM, (b,e) 10 mM, and (c,f) 30 mM

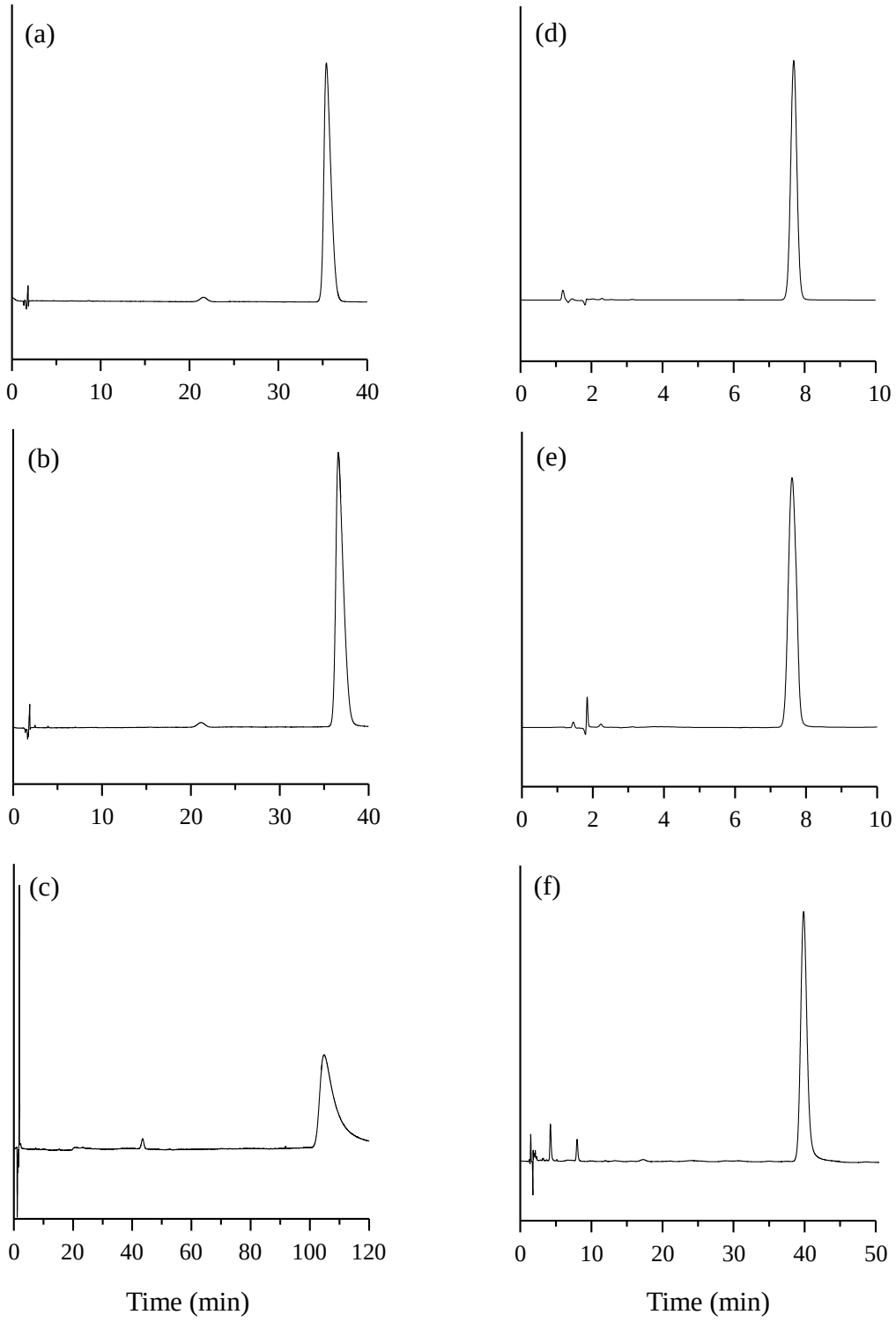

Fig. S2. Chromatograms for acebutolol obtained using aqueous mobile phases containing 10% acetonitrile without additive (a-c) and presence of 10 mM HMIM·Cl (d-f) at different pH values: (a,d) 2, (b,e) 5 and (c,f) 8.