ELSEVIER

Journal of Chromatography A, 964 (2002) 179–187

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Effect of the counter-anion type and concentration on the liquid chromatography retention of β-blockers

Alan Jones[1], Rosario LoBrutto[2], Yuri Kazakevich*

Seton Hall University, Department of Chemistry and Biochemistry, 400 South Orange Avenue, South Orange, NJ 07079-2694, USA

Received 25 September 2001; received in revised form 17 April 2002; accepted 17 April 2002

Abstract

Analysis of β-blockers, basic pharmaceutical compounds with pK_a values greater than 8.5, in reversed-phase HPLC can sometimes be challenging in terms of selection of the mobile phase pH, buffer concentration, and acidic modifier. The effect of the type and concentration of various mobile phase additives on the reversed-phase HPLC retention of these compounds was studied. HPLC analysis was performed at a mobile phase pH of 3 ensuring the protonation of the β-blockers. It was found that at increasing perchlorate anion concentration at a constant mobile phase pH the retention factor for all β-blocker compounds studied increased to varying degrees. The relative increase in the retention was attributed to ion interaction with the anionic mobile phase additive. Similar trends were observed when other types of inorganic salts such as NaH_2PO_4, $NaPF_6$, $NaBF_4$, and CF_3CO_2Na were employed. Differences in selectivity of the β-blockers were obtained at a constant pH and an equimolar concentration of the different additives throughout the whole concentration range studied.

Keywords: Solvation; Pharmaceutical analysis; Beta-blockers

1. Introduction

β-Adrenoceptor blocking drugs are important substances of therapeutic value in the treatment of cardiovascular disorders. β-Blockers can be used in the treatment of hypertension, angina pectoris, arrhythmia and congestive heart failure [1,2]. Several high-performance liquid chromatography (HPLC) methods have been developed for analysis of β-blockers due to the great interest in the pharmaceutical industry [3–6].

Rapado-Martinez et al. used a micellar mobile phase for the chromatographic analysis of β-blockers [7,8]. A 0.15 *M* sodium dodecyl sulfate (SDS) mobile phase containing propanol and 0.01 *M* NaH_2PO_4 at pH 3 was used on a C_{18} column. Secondary equilibria related to the analyte inclusion in the micelles shifted the retention of β-blockers. Retention factors were found to decrease at lower concentrations of SDS and higher concentrations of alcohol in the mobile phase.

Basci et al. however concluded that mobile phase additives such as alkyl sulfonates and organic amines were not essential for the adequate separation of

*Corresponding author. Tel.: +1-973-761-9042; fax: +1-973-761-9772.

E-mail address: kazakeyu@shu.edu (Y. Kazakevich).

[1]Current address: Schering-Plough Research Institute, Union, NJ, USA.

[2]Current address: Merck Research Laboratories, P.O. Box 2000, RY818-C220, Rahway, NJ 07065, USA.

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00448-X

β-blockers [9]. The retention factors of β-blockers with a phosphate buffer–acetonitrile mobile phase at pH 3.0 were studied on a C_{18} column in the presence of different organic amines (diethylamine, tetraethylammonium, *N*,*N*-dimethyloctylamine, etc.) and in the presence of alkyl-sulfonates (pentanesulfonate, hexanesulfonate, heptanesulfonate, etc.) as well as without these mobile phase additives. Alkyl-sulfonates were used as ion-pairing reagents while the organic amines were used as a masking agent for residual silanols. Mobile phases containing only buffer and organic solvent gave similar or better separation than most of the mobile phase additives studied.

Shen et al. developed a reversed-phase HPLC method employing ion-pairing for the analysis of β-blockers in human plasma [10]. Using a Hypersil-ODS microbore column they found that through the increased addition of SDS anion at a constant mobile phase pH of 2, the retention of β-blockers reached a maximum. Further increase of the SDS anion concentration led to a gradual decrease in the retention factor. The authors suggested that an ion-exchange desolvation mechanism is responsible for different retention maxima of the solutes.

Thompson et al. studied the effect of various polarizable anions on the chiral separation of four optical isomers of aminoindanol on a Crownpak CR crown ether column [11]. Significant increases in retention factor were obtained with mobile phases containing nitric acid, trifluoroacetic acid and perchloric acid. However the separation factors for both pairs of enantiomers were the same regardless of the type of anion employed.

Machida et al. found that changes in retention for hydrophobic amino compounds could be obtained by the addition of highly polarizable counter-anions: $H_2PO_4^-$ Cl^-, Br^-, NO_3^-, I^- [12]. Using a crown ether silica based column, they had shown that increased selectivity and retention of alanine-β-naphthylamide and 1-(1-naphthyl)ethylamine enantiomers could be achieved at a constant mobile phase pH of 2.0, 15% methanol and a fixed concentration (500 m*M*) of the potassium salts. The capacity factors were the greatest with iodine and the least with dihydrogenphosphate.

Ishikawa and Shibata successfully performed a chiral separation of propanolol on a Chiralcel OD-R [based on cellulose tris(4-methylbenzoate)] column under reversed-phase conditions [13]. It was found that the resolution of the propanolol enantiomers could be enhanced by the addition of perchloric acid and/or sodium perchlorate. Other types of anions such as PF_6^-, BF_4^-, and $CCl_3CO_2^-$ were selected as additives to the mobile phase and gave similar or greater resolution of the enantiomers.

In a previous paper we described the retention behavior of several basic compounds as a function of the concentration of chaotropic mobile phase additives in the mobile phase at low pH [14]. The increasing order of retention was shown to agree with the theory of chaotropicity [15].

It was shown that the retention factor increased for protonated basic compounds due to their electrostatic interaction with the perchlorate and trifluoroacetate anions. This in turn led to increased hydrophobicity of the ion-associated complex. Solvation shell of the basic analyte is disrupted due to formation of the ionic complex therefore causing an increase of apparent analyte hydrophobicity. A desolvation parameter was derived through mathematical modeling and correlated to the experimental data [16].

Our study focuses on the retention behavior of basic pharmaceutical compounds, β-blockers, as a function of type and concentration of mobile phase additive. The effect of chaotropic mobile phase additives, such as $NaClO_4$, NaH_2PO_4, $NaBF_4$, CF_3CO_2Na, and $NaPF_6$ on the retention factor were studied and the desolvation parameters were determined.

2. Experimental

2.1. Apparatus

A Hewlett-Packard (HP) 1100 HPLC system (Hewlett-Packard, Wilmington, DE, USA) was used for the chromatographic analysis. Chromatograms were processed using HP ChemStation software. The column used was a Zorbax Eclipse XDB-C_{18}, 150× 4.6 mm I.D., particle diameter 5 μm (Hewlett-Packard). Mobile phase pH was measured with a Fisher Scientific Accumet pH meter 15 (Denver Instrument, USA). The pH meter was calibrated with buffer solutions of 1.00, 2.00, 4.00, and 7.00.

2.2. Chemicals

Perchloric acid (redistilled), trifluoroacetic acid (spectrophotometric grade), *o*-phosphoric acid (analytical grade) and acetonitrile (HPLC grade) were obtained from Aldrich (Milwaukee, WI, USA). Inorganic salts sodium perchlorate, sodium hexafluorophosphate, sodium tetrafluoroborate, sodium trifluoroacetate, and sodium dihydrogenphosphate were also obtained from Aldrich. The β-blockers nadolol, acebutolol, atenolol, labetalol, metoprolol, pindolol, alprenolol, and propanolol were purchased from Sigma (St. Louis, MO, USA).

2.3. Chromatographic conditions

Chromatographic analysis was performed under isocratic conditions at a flow-rate of 1.0 ml/min. UV detection of the β-blockers was done at 225 nm. The eluent composition was aqueous–organic (70:30). The pH of the aqueous phase was held constant at 3.0 by adjustment with phosphoric acid for the experiments utilizing the following inorganic salt additives: sodium hexafluorophosphate and sodium tetrafluoroborate. The pH of the aqueous phase was held constant at pH 3.0 by adjustment with phosphoric acid, trifluoroacetic acid and perchloric acid for experiments utilizing sodium dihydrogenphosphate, sodium trifluoroacetate and sodium perchlorate, respectively. Counter-anion concentrations were increased by addition of the salt of the respective anion. β-Blocker solutions were prepared at 0.1 mg/ml concentrations.

Column void volume was determined to be 1.38 by the injection of deuterated acetonitrile in a neat acetonitrile eluent [17].

3. Results and discussion

β-Blockers are typically secondary amines. The β-blocker structures and their pK_a values [18] are shown in Fig. 1. The analytes were studied at a mobile phase pH of 3.0 to ensure complete protonation of all analytes. Protonation occurs on the secondary nitrogen group of these compounds.

Figs. 2 and 3 show the influence of perchlorate anion concentration on retention of several β-blocker compounds. The analytes chosen were atenolol, nadolol, acebutolol, metoprolol, labetalol, and propanolol. The concentration of perchlorate anion in the aqueous portion of the mobile phase was increased by the addition of sodium perchlorate. The pH of the aqueous portion was held constant at 3.0. For all of the compounds studied a characteristic sharp increase in retention factor is seen at the low concentration region. The retention factor plateaus as the concentration of perchlorate is increased above 10 m*M*.

The retention factor increase for β-adrenergic compounds is in agreement with the theory of chaotropicity [15]. Acidic counter-anions in the mobile phase may cause the disruption of the primary and secondary solvation shell [19] of protonated species in solution. The perchlorate anion has a negative charge dispersed about the entire ion. Through electrostatic interaction the perchlorate anion is attracted to the positively charged β-blocker. Ion interaction occurs between the two oppositely charged molecules, which displaces the surrounding water molecules as the two ions approach each other. As a result of this desolvation the apparent hydrophobicity increases therefore increasing the analyte affinity for the stationary phase.

In a previous paper [16] mathematical description of the effect of this solvation–desolvation equilibria on the HPLC retention factor was given. The resulting Eq. (1) relates the HPLC retention factor with the counter-anion concentration in the mobile phase:

$$k = \frac{k_s - k_{us}}{K[A^-] + 1} + k_{us} \quad (1)$$

where $[A^-]$ is the concentration of chaotropic anion, k is the analyte retention factor, K analyte desolvation parameter, k_s and k_{us} are the analyte limiting retention factors for completely solvated and completely unsolvated forms, respectively.

Assuming the absence of analyte–analyte interactions and thermodynamic equilibrium of the chromatographic system, desolvation parameter K indicates the slope of the retention dependence in the low counter-anion concentration region. The upper dashed line in Fig. 4 illustrates the limit of the desolvated retention factor when the analyte is completely desolvated. A larger value for the con-

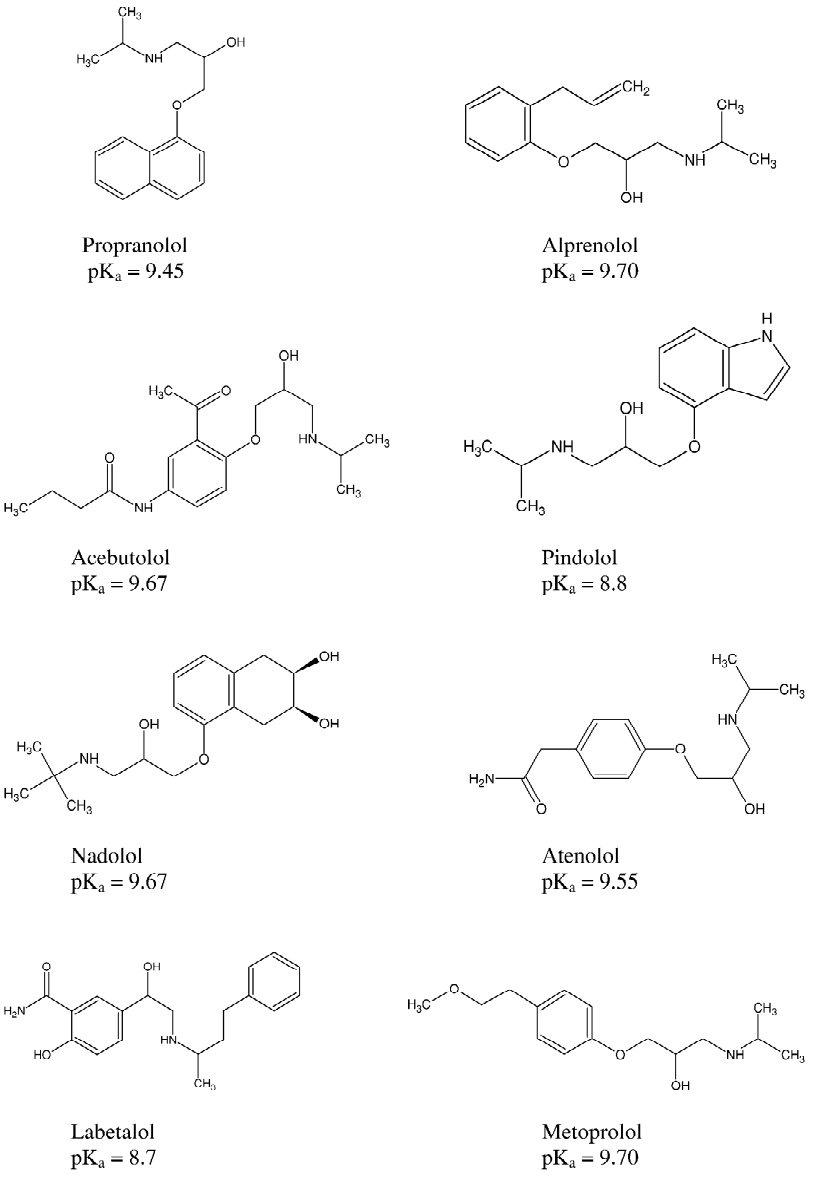

Fig. 1. pK_a of β-blockers obtained from Ref. [18].

stant K indicates the analyte nears complete desolvation at a low concentration of chaotropic counteranion resulting in a sharper knee in the curve.

3.1. Effect of different mobile phase additives

There are several mobile phase additives that can affect retention behavior of amines. Guo et al. had shown the influence of phosphoric, trifluoroacetic and heptafluorobutyric (HFBA) acids on the retention and resolution for a mixture of basic peptides of similar size but containing varying number of positively charged groups [20]. It was found that the hydrophobic mobile phase additive HFBA induced the greatest change in the retention time of the peptides and the magnitude of the retention increase

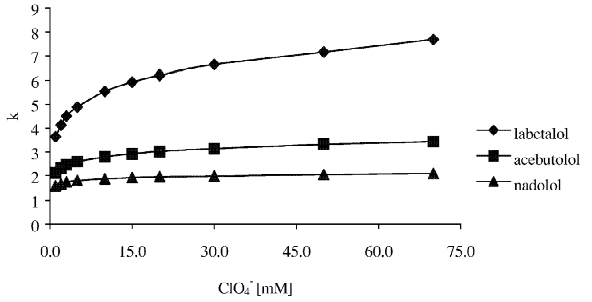

Fig. 2. Dependence of the retention factors for labetolol, acebutolol, and nadolol versus the concentration of perchlorate counter-anion in the mobile phase. Chromatographic conditions: column: Zorbax Eclipse XDB-C_{18} (150×4.6 mm), mobile phase: aqueous adjusted with perchloric acid and/or sodium perchlorate (pH 3.0)–acetonitrile (70:30), flow-rate: 1 ml/min, detection: UV at 225 nm.

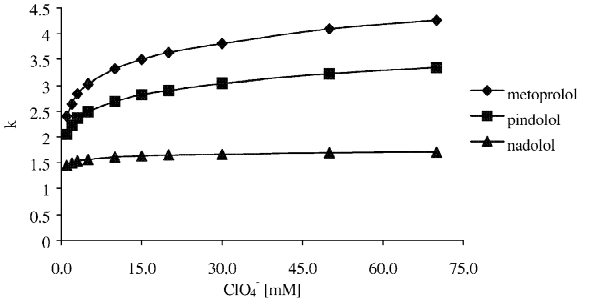

Fig. 3. Dependence of the retention factors for metoprolol, pindolol, and nadolol versus the concentration of perchlorate counter-anion in the mobile phase. Chromatographic conditions as in Fig. 2.

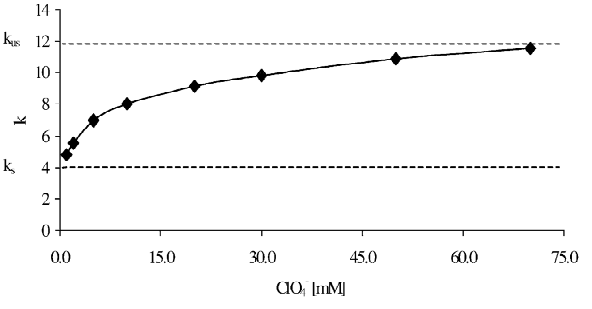

Fig. 4. Dependence of the retention factors for propanolol versus the concentration of perchlorate counter-anion in the mobile phase. k_s and k_{us} are the limiting retention factors for solvated analyte in the absence of counter-anion and for completely desolvated analyte, respectively.

depended on the number of positively charged residues in the peptide. Iskandarani et al. tried several tetraalkylammonium and alkylsulfonate salts as mobile phase additives in separating amino acids and peptides [21]. The alkylsulfonate anions successfully separated peptides that would otherwise show no retention without the mobile phase additive.

The effect of hexafluorophosphate, tetrafluoroborate, trifluoroacetate, dihydrogenphosphate and perchlorate anion species on the β-blocker retention was investigated. Retention increases were observed for the β-blocker compounds using a Zorbax Eclipse XDB-C_{18} column with an aqueous–acetonitrile (70:30) mobile phase composition at increasing concentration of each of the respective anions at a constant pH of 3.0.

A comparison of the acebutolol retention dependencies on counter-anion concentration for different counter-anions is shown in Fig. 5. Regardless of the counter-anion employed in the mobile phase at increasing counter-anion concentration, a consequent increase in the retention for all β-blockers was observed. At each concentration studied the greatest retention increase was obtained when hexafluoro-

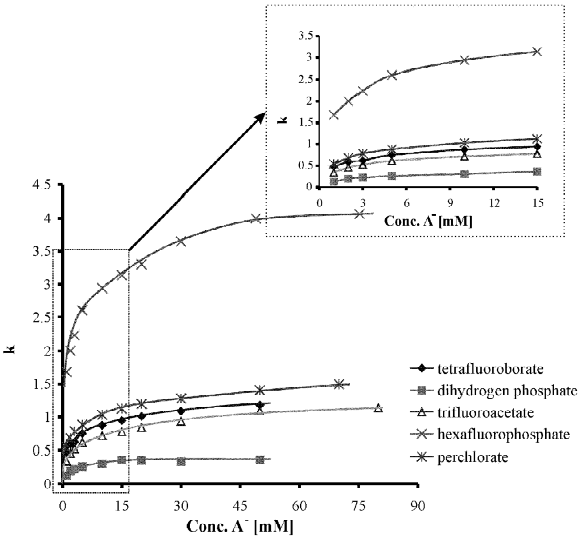

Fig. 5. Plot of the acebutolol retention factors versus counter-anion concentration in the mobile phase for different counter-anions used. Chromatographic conditions: column: Zorbax Eclipse XDB-C_{18} (150×4.6 mm), mobile phase: aqueous (pH 3.0)–acetonitrile (70:30), flow-rate: 1 ml/min, detection: UV at 225 nm.

phosphate was employed and the lowest when dihydrogenphosphate was employed. The differences observed in the analyte retention maybe attributed to the extent of anion solvation. The hydration of ions differs from ion to ion. The anion that was least capable of being solvated and had a greater propensity for ion interaction led to greatest disruption of the analyte solvation and consequently the increase of the analyte hydrophobicity.

Dihydrogenphosphate anion in an aqueous environment is highly solvated due to its hydrogen bonding capabilities. Trifluoroacetate anion is capable of acting as a hydrogen bond donor and acceptor however is not as polar as dihyhydrogenphosphate since its negative charge is more delocalized as a result of the electron withdrawing fluorine atoms. Tetrafluoroborate has four electron withdrawing groups further delocalizing the negative charge and may act only as a very weak hydrogen bond acceptor. Perchlorate has four electron withdrawing oxygen atoms. However greater changes in retention were observed with perchlorate as opposed to BF_4^-. This maybe attributed to the charge density in which the charge is more delocalized in perchlorate than tetrafluoroborate since the central atom, Cl, has a greater atomic radius than B. PF_6^- had shown the greatest effect on the desolvation of the protonated β-blockers. This anion has six strong electron withdrawing groups further delocalizing the charge and hence being the least solvated anion of the series studied. An interesting solubility relationship occurs between hexafluorophosphate and perchlorate such that low solubility of hexafluorophosphate salts correspond to low solubility of perchlorate salts [22] hence indicating similar properties in their solvation.

Fig. 6 represents an overlay of the chromatograms for six β-blockers analyzed at an equimolar concentration of the different inorganic additives. It is

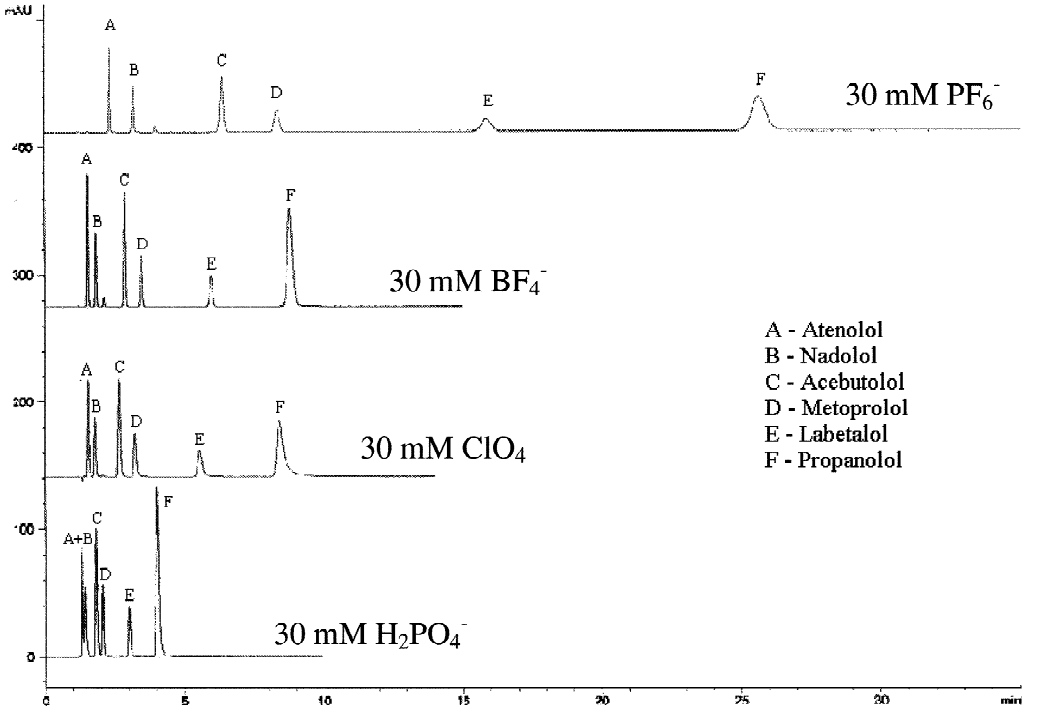

Fig. 6. Chromatograms of a mixture of β-blockers with different inorganic anions in the mobile phase. Chromatographic conditions as in Fig. 5.

shown that an increase in selectivity can be obtained solely by changing the type of inorganic additive employed.

In the derivation of the Eq. (1) we assumed an existence of the following equilibrium:

$$[\text{Analyte}_{\text{solvated}}] + [\text{A}^-_{\text{solvated}}] \Leftrightarrow [\text{Analyte}^+ \cdots \text{A}^-]$$

Ionic interaction of the protonated basic analyte with the counter-anion leads to their mutual desolvation and formation of an ion-associated complex. The reversed-phase HPLC retention of that complex is dependent not only on the degree of analyte hydrophobicity but also on the strength of the ionic interactions and the properties of particular counter-anion used. Figs. 5 and 6 reveal the significant effect of the type and amount of counter-anion on the retention of the same analyte.

Desolvation parameter K in Eq. (1) is essentially the equilibrium constant of the solvation–desolvation process. To a certain extent this constant represents the stability of the ion-associated complex. In Table 1 the values are given for the desolvation parameters and limiting retention factors that were calculated from the experimental dependencies shown in Fig. 5.

The desolvation parameters of each β-blocker with the different inorganic counter-anions except for dihydrogenphosphate were essentially on the same level. The dihydrogenphosphate counter-anion showed a noticeable difference from all other counter-anions employed. This suggests that the stability of ion-associated complexes of dihydrogenphosphate with studied analytes is three to five times more stable than for other counter-anions.

In contrast to the stability of ion-associated complexes, the limiting retention factors are significantly higher for hexafluorophosphate, perchlorate and tetrafluoroborate than for trifluoroacetate and especially for dihydrogenphosphate. There is a greater than 10-fold increase in the analyte retention for all analytes when hexafluorophosphate is employed instead of dihydrogenphosphate.

While the general effect of the counter-anion concentration is similar for all mobile phase modifiers (Langmuir-type isotherm) the most significant retention change occurs in a region between 0 and 10 mM. At higher concentrations of counter-anions their apparent hydrophobicity and charge delocalization are probably the most important factors affecting analyte retention.

The effect of these counter-anions on the retention of protonated basic compounds is of practical importance because of their potential for improvement towards HPLC method development. The retention increase caused by the chaotropic counter-anion can induce significant changes in selectivity.

Table 1
Desolvation parameter of β-blockers with different inorganic anions

	Atenolol	Nadolol	Acebutolol	Metoprolol	Labetalol	Propanolol
k_{us}						
PF_6^-	0.722	1.495	4.410	6.514	14.096	24.762
ClO_4^-	0.248	0.531	1.600	2.293	5.039	8.748
BF_4^-	0.168	0.418	1.305	1.843	4.170	6.714
CF_3COO^-	0.162	0.399	1.290	2.378	3.292	4.057
$H_2PO_4^-$	0.000	0.083	0.380	0.529	1.283	2.047
K						
PF_6^-	0.283	0.138	0.095	0.072	0.059	0.050
ClO_4^-	0.203	0.166	0.095	0.078	0.070	0.049
BF_4^-	0.211	0.139	0.108	0.094	0.075	0.077
CF_3COO^-	0.272	0.124	0.062	0.028	0.152	0.012
$H_2PO_4^-$	0.430	0.526	0.465	0.459	0.498	0.534

Calculations were performed using MathCad optimization of Eq. (1): $k = \dfrac{k_s - k_{us}}{K[\text{A}^-] + 1} + k_{us}$.

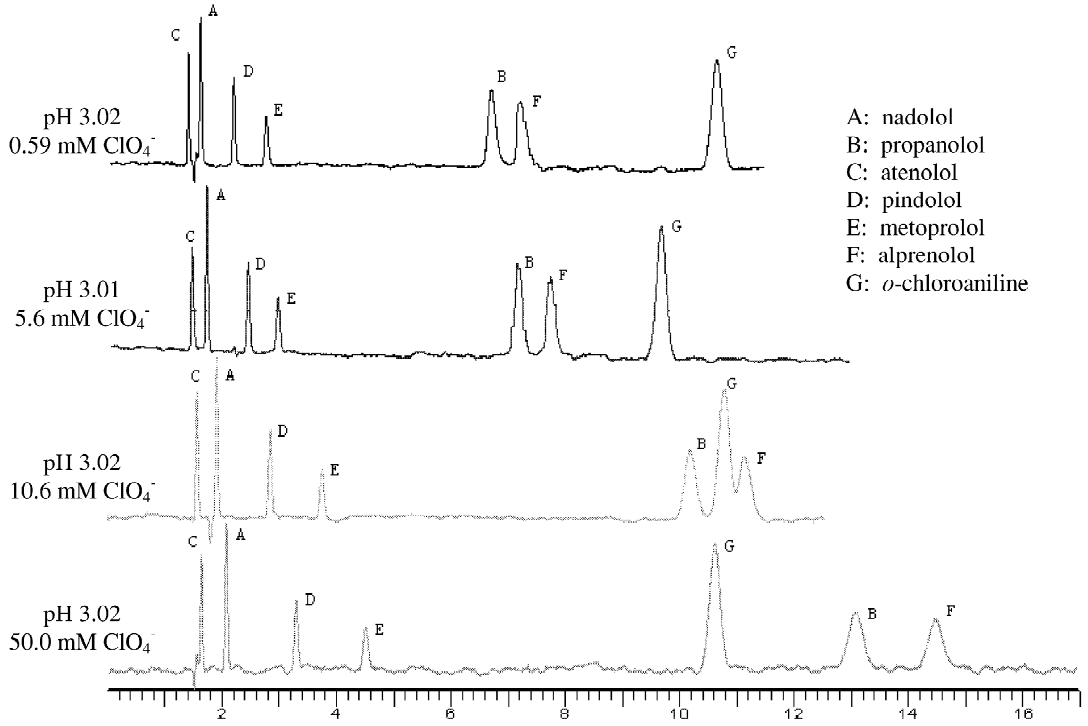

Fig. 7. Chromatograms of a mixture of β-blockers and *o*-chloroaniline analyzed at constant pH and increasing perchlorate concentration. Chromatographic conditions as in Fig. 2.

Fig. 7 illustrates the influence of the chaotropic counter-anion concentration on the selectivity. At 0.59 m*M* concentration of perchlorate anion, all of the compounds are resolved. Atenolol, nadolol, pindolol, and metoprolol elute fairly close to one another and also close to the void volume. The increase of the retention for all compounds except for *o*-chloroaniline is observed upon the addition of 5.6 m*M* ClO_4^- to the mobile phase. The pK_a of *o*-chloroaniline is 2.64 indicating that at the mobile phase pH studied the compound was not completely in the ionized form. Ion association between *o*-chloroaniline and perchlorate therefore does not occur considerably and no consistent retention increase is observed. Further increase of the perchlorate concentration in the mobile phase led to the corresponding increase of the retention of all β-blockers, while the retention of *o*-chloroaniline remained essentially unchanged. This is in accordance with the chaotropic effect [16], which is applicable for fully protonated analytes, while partially protonated species are not affected by the counter-anion concentration. The increase of the salt concentration in the mobile phase, thus, led to significant changes in the selectivity and elution order of the studied analytes.

4. Conclusions

The effect of the type and concentration of different inorganic mobile phase additives on the reversed-phase HPLC retention of β-blockers was studied. These analytes are fully protonated at low pH and chaotropic counter-anions in the mobile phase disrupt the surrounding water molecules and increase the relative analyte hydrophobicity.

Effect of the counter-anion concentration in the mobile phase for all studied analytes was found to be well described by the general equation derived previously [16]. Analysis of the equation parameters calculated from experimental retention data show similar effect of all types of counter-anions studied on the analyte solvation–desolvation equilibria.

The significant difference in the analyte retention was observed when different counter-anions were applied. Specific properties of the counter-anion employed, such as apparent hydrophobicity and charge delocalization, are thought to play a major role on the retention of the ion-associated complex.

The use of chaotropic anions for a chromatographic separation may be beneficial for method development strategies. Application of a chaotropic species to a mobile phase could induce changes in selectivity and resolution leading to faster and more rugged methods without the need of changing the column type and/or the addition of hydrophobic ''ion pairing'' reagents.

References

[1] R.G. Shanks, Trends Pharm. Sci. 5 (1984) 405.
[2] W. Chavey, Am. Fam. Physician 62 (11) (2000) 2453.
[3] M.T. Saarinen, H. Siren, M.-L. Riekkola, J. Chromatogr. B 664 (1995) 341.
[4] M.I. Maguregui, R.M. Alonso, R.M. Jimenez, J. Chromatogr. B 674 (1995) 85.
[5] P.M. Harrison, A.M. Tonkin, A.J. McLean, J. Chromatogr. 339 (1985) 429.
[6] A.G. Gonzalez, M.A. Herrador, A.G. Asuero, Int. J. Pharm. 123 (1995) 149.
[7] I. Rapado-Martinez, M.C. Garcia-Alvarez-Coque, R.M. Villanueva-Camanas, J. Chromatogr. A 765 (1997) 221.
[8] I. Rapado-Martinez, M.C. Garcia-Alvarez-Coque, R.M. Villanueva-Camanas, Analyst 121 (1996) 1677.
[9] N.E. Basci, A. Temizer, A. Bozkurt, A. Isimer, J. Pharm. Biomed. Anal. 18 (1998) 745.
[10] J. Shen, S. Wanwimolruk, C.T. Hung, A.R. Zoest, J. Liq. Chromatogr. 14 (4) (1991) 777.
[11] R.A. Thompson, Z. Ge, N. Grinberg, D. Ellison, P. Tway, Anal. Chem. 67 (1995) 1580.
[12] Y. Machida, H. Nishi, K. Nakamura, J. Chromatogr. A 830 (1999) 311.
[13] A. Ishikawa, T. Shibata, J. Liq. Chromatogr. 16 (4) (1993) 859.
[14] R. LoBrutto, A. Jones, Y.V. Kazakevich, H.M. McNair, J. Chromatogr. A 913 (2001) 173.
[15] Y. Hatefi, W.G. Hanstein, Proc. Natl. Acad. Sci. USA 62 (1969) 1129.
[16] R. LoBrutto, A. Jones, Y.V. Kazakevich, J. Chromatogr. A 913 (2001) 189.
[17] Y. Kazakevich, H.M. McNair, J. Chromatogr. Sci. 31 (1993) 317.
[18] F. Barbato, G. Caliendo, M.I. La Rotonda, P. Morrica, C. Silipo, A. Vittoria, Farmaco 45 (6) (1990) 647.
[19] J.C. Burgess, Ions in Solution: Basic Principles of Chemical Interactions, Ellis Horwood, 1988.
[20] D. Guo, C.T. Mant, R.S. Hodges, J. Chromatogr. A 386 (1987) 205.
[21] Z. Iskandarani, R.L. Smith, D.J. Pietrzyk, J. Liq. Chromatogr. 7 (1) (1984) 111.
[22] J. Van Wazer, Phosphorous and its Compounds, Chemistry, Vol. 1, Interscience, New York, 1958.