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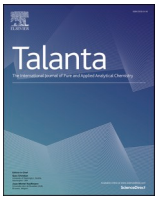

Mixed-mode chromatography of mixed functionalized analytes as the homologues of benzalkonium chloride. Application to pharmaceutical formulations☆

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ABSTRACT

In this work, a retention behavior based on mixed-mode reversed-phase (RP)/hydrophilic interaction liquid chromatography (HILIC) was observed for benzalkonium chloride (BAK) using a core-shell column functionalized with biphenyl groups. Although in the literature, the U-shaped retention was reported for polar compounds in mixed functionalized phases, in the present work, the behavior was dependent upon the chemical structure of the analyte with mixed functionality (ammonium group, a benzyl group and an alkyl chain) and on the high selectivity of the chromatographic column. The bimodal retention was observed for the four BAK homologues using a content of acetonitrile from 65 to 95% in the mobile phase. The data were adjusted to polynomial equations which allow for modeling and predicting the U-shaped retention. The salt concentration (50 and 100 mM), anion (formate and acetate) and cation (ammonium and triethylammonium) of the salt, pH (4 and 5) in the mobile phase were studied in order to understand their influence on the two retention modes. Significant electrostatic interactions were involved in the two retention modes, especially with a content of acetonitrile higher that 90%. Linear relationships between the retention factors of the four homologues were found in a wide range of %acetonitrile when the salt and triethylamine concentration, pH and nature of salt were changed. The differences found on the retention of the homologues, when increasing the alkyl chain length, were more significant in the RP mode due to predominant hydrophobic interactions. A pH decrease and a salt concentration increase caused a retention decrease for both modes. A decrease on ~~of~~ the retention was observed when acetate anion was replaced by formate anion. The different order of the polynomial equations according to the used mobile phase confirmed its relevant role in the interactions with the analytes and stationary phase. A mobile phase was selected (85% acetonitrile, pH 4 and 100 mM ammonium formate) for the BAK determination in cutaneous, otic and ophthalmic formulations with different active pharmaceutical ingredients and excipients. Low sample volume (500 μL) and short analysis time (<5 min) were some of the advantages of the proposed method. In addition, good analytical performance ($R^2 > 0.999$, % RSD <4.5% for intra-day precision and <5.8% for inter-day precision, and recoveries in the 92–105% range) was obtained.

1. Introduction

In recent years, new developments in liquid chromatography, beginning with the new chromatographic columns and modes have become increasingly important. Consequently, the models of retention, especially for the ionizable compounds, are under study [1,2].

Hydrophilic interaction liquid chromatography (HILIC) has been a good choice for the analysis of polar and ionizable compounds when compared to reversed-phase liquid chromatography (RP-HPLC) or ionic chromatography (IC). In HILIC, an aqueous-organic mobile phase (with high concentration of organic solvent) and a polar stationary phase are used. Like in the non-aqueous normal phase, the higher the organic solvent, the more retained analytes will be [3]. Type and content of organic modifier, pH, type and concentration of salt and

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functionalization of stationary phase are some of parameters that have to be taken into account. Although many of the parameters have already been studied in other types of chromatography such as ion-pairing liquid chromatography (IP-HPLC), the HILIC behavior needs new models of retention to explain the experimental data [4].

In pharmaceutical and environmental fields, mixtures of neutral, basic, acidic and highly polar compounds can be separated by multi-component methods. Columns with different functionalization (hydrophobic alkyl chain, exchange ionic moiety, polar groups) provided mixed-mode separations with excellent selectivity [5]. Using the mixed-mode chromatography for the separation of polar and ionizable analytes [6] or of mixtures of polar and neutral compounds [7], a dual retention behavior was described. The retention (measured as retention time or as retention factor) was examined according to the content of organic modifier (frequently acetonitrile) in the mobile phase. Two different behaviors were observed: 1) for high percentages of acetonitrile, the retention was increased with the increasing of acetonitrile proportions, showing a HILIC retention; 2) for lower acetonitrile percentages, the retention increase occurred when acetonitrile decreased, following a reversed-phase retention. The plot of the retention versus % acetonitrile gives a U-shaped retention behavior in which the minimum (the % acetonitrile that provides the lowest retention) is the union between the two studied zones. The U-shaped retention was also observed with a HILIC column and some analytes, such as surfactants. Thus, Hammer et al., 2018 [8] reported, according to the acetonitrile percentage, hydrophobic and hydrophilic interactions in the retention of alkyl sulfonates, alkyl carboxylates and alcohol ethoxylates using a HILIC column functionalized with diol groups and an alkyl chain. U-shaped retention was also observed by Bell and Jones, 2005 [9] using pentafluorophenylpropyl phases for the retention of quaternary ammonium salts with short alkyl chains (bretylium salt). The retention using high percentages of the organic solvent has been explained by ion-exchange mechanisms between the analyte and the ionized silanol groups.

Surfactants are a group of compounds with a high production volume, which can be found in numerous consumer products and some pharmaceutical products. Cationic surfactants are one of the types with differentiated properties, such as biocides and preservatives. Benzalkonium chloride (BAK) is a cationic surfactant with four main homologues depending on the alkyl chain length (C12, C14, C16 and C18). It is widely used in ophthalmic formulations (74% of the products marketed in Europe) and also in nasal and otic formulations [10]. BAK is a regulated biocide substance [11] and, in the European Union, its use as a preservative in cosmetic products [12] and for wood [13] is also regulated. The analytical literature of BAK covers from its industrial synthesis [14–16] to commercial products and environment [17–19]. Regarding pharmaceutical analysis, although the Handbook of Pharmaceutical Excipients establishes levels for total BAK content, the determination of individual homologues is of great interest because of the different biocidal properties of the homologues (C12 against yeast and fungi, C14 and C16 against gram-positive and gram-negative bacteria respectively). The reference methodology of the US Pharmacopeia for BAK analysis in pharmaceutical formulations, is based on high performance liquid chromatography with ultraviolet detection.

Reversed-phase mode using cyanopropyl [14–16,20,21], octadecyl columns [18,22–29] or titania-based column [17] and mobile phases containing a buffer at acidic pH were the employed chromatographic conditions for BAK determination. However although the reference method of US Pharmacopeia employes a cyano column, several authors have reported reproducibility and column stability issues [22], especially with ophthalmic formulations with several active ingredients and multiple excipients, when injected at high concentration [26]. On the other hand, the use of alternative phases to traditional C18, such as phenyl phases, have been gaining acceptance in the analysis of polar and aromatic compounds in pharmaceutical analysis because of their selectivity due to their capacity to establish π-π interactions. Nevertheless, as far as we know, phenyl phases have not been employed for the analysis of BAK homologues in pharmaceutical analysis.

During several decades, the conventional packed columns with porous particles were the columns used in HPLC. Lately other columns were developed such as monolithic columns, which provide a good resolution with low pressures in the chromatographic system [1]. Another type of new columns is based on the core-shell technology, which allows analyzing small and large molecules with narrow peaks and moderate pressures. The particle structure (a solid core coated with a porous shell) determines its chromatographic behavior. Core-shell columns have a more homogeneous particle size distribution and an improved packing of the particles with respect to packed particles columns with fully porous [30]. For this reason, the contribution to the Eddy dispersion term in the Van Deemter equation decreases. Moreover, the dead volume is lower in core-shell columns than in packed columns with fully porous particles, decreasing in this way the contribution to the longitudinal diffusion term in the Van Deemter equation. Consequently, a lower band broadening and narrow peaks are achieved [31]. Therefore, core-shell columns can be considered as an alternative for the analysis of compounds such as cationic surfactants which usually show broadening of peaks.

The present work has two objectives: i) to examine the retention of mixed-mode chromatography (HILIC and RP) on a core-shell column with biphenyl groups when the analytes (homologues of BAK) are mixed functionalization (benzyl group, cationic group (ammonium) and alkyl chain). The behavior was not observed on reversed-phase columns using the same mobile phase containing water:acetonitrile mixtures with salts at an adjusted pH in aqueous phase. Specific models of retention for surfactants had to be developed. ii) to determine four BAK homologues in pharmaceutical formulations with different active pharmaceutical ingredients (API) and excipients. To the best of our knowledge, the BAK chromatographic behavior was not examined in any core-shell column with biphenyl groups. Furthermore, the methods proposed so far have not ensured the determination of the most hydrophobic homologues in pharmaceutical formulations.

2. Material and methods

2.1. Instrumentation and reagents

The chromatographic system used (Waters, Milford, MA, USA) was comprised of a 2690 Alliance Separations module, a UV–visible diode array detector (Waters996 PDA) and the Empower Pro software. The tested analytical columns were Kinetex Biphenyl (150 × 3.0 mm, 5 μm) obtained from Phenomenex and Nova Pak CN HP (150 × 3.9 mm, 4 μm) from Waters.

The BAK homologues were N-benzyl-N-dimethyl-N-dodecylammonium chloride (C12-BAK, ≥99%) from Sigma-Aldrich; N-benzyl-N-dimethyl-N-tetradecylammonium chloride dihydrate (C14-BAK, ≥99%) from Aldrich; N-benzyl-N-dimethyl-N-hexadecylammonium chloride (C16-BAK, 95%) from Sigma; N-benzyl-N-dimethyl-N-octadecylammonium chloride monohydrate (C18-BAK, 90%) from Aldrich. The aqueous phases were prepared using ammonium acetate and formate, (≥99%) from Fluka Analytica, glacial acetic acid, (+99.99%) from Aldrich, formic acid (98–100%) from Riedel de Haën and triethylamine (TEA), (≥99%) from Merck. The solvents were acetonitrile (Carlo Erba) and water Milli-Q quality. Aqueous phases were filtered through 0.22 μm nylon membranes (Millipore, Darmstadt, Germany) before use.

2.2. Preparation of standards and real samples

Stock standard solutions of each BAK homologue were prepared at 2.0 mg/mL concentration in a water:acetonitrile mixture (1:1). Working solutions containing the four homologues were prepared at 0.1 mg/mL concentration of each homologue and employed for the retention study. All solutions were stored at 4 °C in glass vials.

Five kinds of commercial pharmaceutical formulations with different applications and active pharmaceutical ingredients (APIs) and excipients were analyzed (Table 1). For the analysis, 500 μL of sample were mixed with 500 μL of mobile phase into an injection vial for 5 min in an ultrasound bath (Ultrasons-H, J.P.Selecta, Spain). Samples with concentration over the linearity limit, were diluted accordingly with mobile phase.

2.3. Chromatographic conditions

Twelve mobile phases were employed in the retention study with mixtures of acetonitrile: aqueous phase ranging from 60:40 to 95:5 (v/v) at a 0.7 mL/min flow-rate. The temperature of analysis was kept at 25 °C and the injection volume was 10 μL. The composition of the aqueous phases (type of buffer, concentration of buffer and pH) is shown in Table 2. Retention factors were calculated from hold-up time and retention time of the analytes. The hold-up times were determined by the first disturbance upon injection in the range from 1.109 min to 0.915 min [9].

Under all tested conditions (Table 2) the separation of the four homologues was achieved with the exception of 95% ACN using ammonium acetate at pH 5. For values lower than 70% ACN, employing ammonium acetate at pH 4, the BAK analysis is not recommended due to the increase of baseline noise, low sensitivity and asymmetry of peak.

For the BAK determination in pharmaceutical samples, the Kinetex Biphenyl column and isocratic mobile phase composed of 85% acetonitrile and 15% of 100 mM ammonium formate at pH 4, allowed the separation of BAK homologues in less than 5 min. The monitoring of the signal between 200 and 400 nm, led to confirming identification and also to check the peak purity. The quantification was performed at 210 nm in order to achieve maximum sensitivity.

2.4. Validation of the method

The method was validated in terms of linearity, accuracy, precision and limits of detection and quantitation according to ICH guidelines (ICH Q2 R1) [32]. The linearity of the calibration curves was calculated using eight concentration levels (0.0025, 0.005, 0.075, 0.01, 0.025, 0.05, 0.075, and 0.1 mg/mL) by triplicate analysis. The limit of detection (LOD) and quantitation (LOQ) was 3 and 10 $S_{y/x}/b$ respectively, "$S_{y/x}$" being the residual standard deviation and "b" the slope of the calibration curve.

The precision of the chromatographic system was evaluated at two concentration levels (0.01 and 0.1 mg/mL) of standard solutions. For intra-day precision, eight replicates of each level were injected; for inter-day precision, eight replicates were analyzed on three consecutive days.

The precision of the method was evaluated using the ophthalmic formulation B, which contains tetryzoline hydrochloride as API (Table 1). Since the formulation only contained C12-BAK and C14-BAK homologues, it was spiked with the C16-BAK and C18-BAK homologues (0.070 mg/mL). The precision of the method, expressed as the relative standard deviation (RSD), was evaluated measuring eight replicate samples the same day (intra-day precision) and eight replicate samples on three consecutive days (inter-day precision).

The accuracy of the method was evaluated regarding the recovery assay at three concentration levels. Thereby, 0.015, 0.035 and 0.05 mg of each homologue were added to 500 μL of sample of ophthalmic formulation (B), preparing three replicates at each concentration level. For the A, C, D, E formulations, the recovery was obtained adding 0.035 mg of each homologue to 500 μL of sample.

Table 1
Pharmaceutical formulations (types, API and excipients) in which BAK was analyzed.

Type of formulation	Active pharmaceutical ingredient (API)	Excipient
Cutaneous (A)	Ethanol	Benzalkonium chloride
Ophthalmic (B)	Tetryzoline hydrochloride	Boric acid, sodium borate, sodium edetate, benzalkonium chloride and purified water
Ophthalmic (C)	Hamamelis water	Boric acid, sodium borate, benzalkonium chloride, glycerol and purified water
Ophthalmic (D)	Polymyxin B sulfate, neomycin sulfate, gramicidin	Sodium chloride, ethanol 96%, benzalkonium chloride, propylene glycol, poloxamer 188, sodium hydroxide or sulfuric acid by adjusted pH, purified water
Otic (E)	Ciprofloxacin	Benzalkonium chloride, sodium acetate trihydrate, glacial acetic acid, mannitol, sodium edetate, sodium hydroxide or hydrochloric acid (adjust pH) and purified water

Table 2
Aqueous phases employed for studying of the BAK homologues retention.

Code	Type of buffer		Concentration (mM)	pH
	Cation	Anion		
AF50_4	Ammonium	Formate	50	4
AF50_5	Ammonium	Formate	50	5
AF100_4	Ammonium	Formate	100	4
AF100_5	Ammonium	Formate	100	5
AA50_4	Ammonium	Acetate	50	4
AA50_5	Ammonium	Acetate	50	5
AA100_4	Ammonium	Acetate	100	4
AA100_5	Ammonium	Acetate	100	5
TEAF50_4	TEA	Formate	10	4
TEAF50_5	TEA	Formate	10	5
TEAF100_4	TEA	Formate	15	4
TEAF100_5	TEA	Formate	15	5

3. Results and discussion

3.1. BAK homologues separation using a core-shell column with biphenyl groups: dual retention

A core-shell column with biphenyl groups was tested for the separation of the four BAK homologues. Besides its structure, the functionalization of the column could be suitable for the present proposal due to the potential interactions π-π between the biphenyl group of stationary phase and the benzyl group of BAK. Moreover, a rotation of 45°, which was reported for the biphenyl group, could have impact on its selectivity [33]. On the other hand, cation-π interactions between the quaternary ammonium group (charged positively) and the aromatic moieties of the stationary phase could contribute to the separation [34].

Our results revealed a dual retention for four homologues in all tested conditions. Fig. 1 shows the U-shaped retention for the four homologues for the selected mobile phase (mixture of acetonitrile and aqueous phase containing 100 mM of ammonium formate at pH 4, AF100_4). In the range from 95% to 80% of acetonitrile, the higher concentration of organic solvent, the higher the retention of analytes, the same as in the case of the HILIC retention. For %ACN lower than 80% the retention increased when the concentration of organic solvent decreased, corresponding to the reversed-phase retention. Consequently, it was a case of mixed-mode retention. The minimum of retention was found at 85% ACN for C18-BAK and at 80–85% ACN for the other homologues. In the literature, a minimum of 85% acetonitrile with U-shaped retention has also been reported in the analysis of anionic and non-ionic surfactants using a mixed-mode column [8].

If the experimental retention data (ln k) are plotted against the percentage of organic solvent, a deviation of the quadratic model (a typical RP retention) was obtained according to other works [6] which examined U-shaped retention. The best correlation was obtained for the fourth-order polynomial equation (correlation coefficients

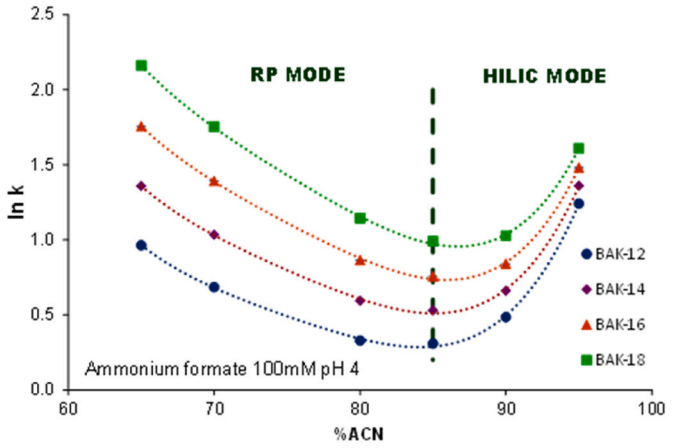

Fig. 1. Effect of acetonitrile percentage on the retention of the four BAK homologues. Aqueous phase: 100 mM ammonium formate at pH 4.

0.9990–0.9995) describing the dependence of the Neperian logarithm of retention factor (k) upon the acetonitrile percentage (φ) (Table 1S)

$$\ln k = c_0 - c_1\varphi + c_2\varphi^2 - c_3\varphi^3 + c_4\varphi^4$$

Concerning the resolution, the separation of the four BAK homologues until baseline was obtained under all tested conditions but with differences in the peak symmetry (especially for the peaks corresponding to C16-BAK and C18-BAK). An increase of peak tails starting from 70% ACN (in RP zone) was observed, whereas in the HILIC zone, there were no significant changes of symmetry according to the acetonitrile content. It is worth noting that in previous studies carried out under similar conditions employing a cyanopropylsilica column, which is the used column by the reference method of US Pharmacopeia, the separation of the homologues was only achieved for low ACN percentages ($\leq$60%).

The elution order was from C12-BAK to C18-BAK in both retention modes. Other authors observed, using HILIC columns for the surfactant analysis that the order of elution of the homologues was also from the shortest to longest alkyl chain [8]. In RP, the hydrophobic interactions are predominant, which are most related to the size of the alkyl chain. In the HILIC mode the retention is influenced by hydrophilic properties of the head group that determines the degree of hydration of surfactants and electrostatic interactions with silanol groups.

3.2. *Effect of the mobile phase variables on the BAK retention*

The separation of four BAK homologues was carried out in acidic medium in order to increase the protonation of the silanol groups of the stationary phase and thus minimize the electrostatic interaction of the ammonium group of BAK with the free silanol groups. The retention at pH 4 and 5 and 100 mM ammonium formate (AF100_4 and AF100_5) was examined within the range of acetonitrile between 95% and 60–65%. In Fig. 1AS, the data of C12-BAK and C18-BAK homologues were plotted because they showed the most different behavior among the studied homologues.

The changes for each studied parameter (pH, salt concentration and type of anion and cation) were studied. A retention decrease with the pH decrease was found for all homologues in the two zones being more pronounced for C18-BAK and especially on RP retention mode. Although the pH was controlled in aqueous phase, the effective pH of the water: acetonitrile mixtures changes according to the % acetonitrile. Thus, the higher the values of acetonitrile, the higher the pKa for the additives of the mobile phase and for the silanol groups [35]. However, the assayed pH in the mobile phase had no influence on pKa of BAK which had a permanent positive charge. A linear relationship was established between the retention factors of the four homologues at pH 4 and pH 5 in the range of ACN from 90 to 65% (Fig. 2A). This means that, the same interactions are acting in both retention zones (HILIC and RP) for the four homologues. However, the data for 95% ACN showed a good correlation between the homologues under the tested conditions but a different linear relationship (lower slope, see black dots in Fig. 2A) that using other acetonitrile percentages. The lower protonation of free silanol groups at 95% ACN could cause a higher contribution of the ionic interactions than under the other conditions.

In addition to the pH control, the ammonium formate concentration in the aqueous phase played an important role on the BAK retention. A decrease in retention was observed when the concentration increased for both retention zones of the U-shaped curve at two pH values (Fig. 2AS (AF100_5 and AF50_5) and 2BS (AF100_4 and AF50_4). In general, the effect of salt concentration can be explained by the ionic interactions of BAK with the silanol groups of the stationary phase (cation exchange sites) in competition with the salt cation of the mobile phase. In addition to electrostatic interactions, in HILIC zone an increase of salt concentration could increase the eluting strength of mobile phase due to salting-out effect in the immobilized water layer on the stationary phase [36–39]. In this case, a linear relationship was also established between the retention factors of the four homologues at 100 mM and 50 mM in the range of ACN from 90 to 60–65% (Fig. 2B). This fact confirmed the existence of ionic interactions between the silanol groups, the salt and the analytes. Similarly, using a 95% ACN the retention factors showed a different linear relationship (see black dots in Fig. 2B). If the hydrophilic interaction was predominant, the salting-out effect was more pronounced (a higher slope) at 95% ACN.

Changes were observed in the degree of polynomial with the pH at 100 mM of ammonium formate (AF100_4 and AF100_5) (fourth-order equation at pH 4 and fifth-order equation at pH 5). However, there were no changes with pH (fifth-order equation) at 50 mM (AF50_4 and AF50_5) (Fig. 1BS).

Ammonium formate and acetate were compared under the same conditions of salt concentration, pH and %ACN. Retention times using acetate were greater than using formate anion. For the two retention zones (90%–60% ACN), an excellent linear relationship was found between the retention factors obtained using the formate and acetate anions (Fig. 2C). The anion may act as an ion-pairing reagent for BAK. In the RP retention, the salt anion (counter ion) was proven to play an important role in the analysis of other quaternary compounds, decreasing also the retention when acetate anion was replaced by formate anion [40]. In the present work, an acetate concentration of 100 mM led to values of retention equivalent to a formate concentration of 50 mM in the RP mode (Fig. 3S).

The effect of the cation type was also studied using triethylamine and adjusting pH with formic acid (TEAF10_4, TEAF15_4). TEA is an additive of the mobile phase, reported in the literature for BAK analysis [39]. The tested TEA concentrations were lower (10 and 15 mM), since higher values could be difficult to remove after the analysis and to condition the behavior of the analytical column. The obtained results (Fig. 4AS) showed a dual retention with a minimum between 85% and 80%, depending on the homologues. As could be expected, the higher the TEA concentration, the lower the BAK retention. The four homologues were separated under all tested conditions, although with a slight asymmetry of the peaks. Experimental data were adjusted to fifth-order polynomial equations (correlation coefficients 0.9999). As it occurs in the case of ammonium cation, the retention factors at two tested concentrations showed a good linearity (Fig. 2D) but in a wider range of ACN (95%–60%) which include the 95% ACN. It is interesting to note that the slopes of the linear plots have very similar values (1.20–1.24) but a more in-depth study would be necessary to distinguish the contribution of ionic interaction from other interactions.

The comparison between the two tested cations (ammonium and triethylammonium) is more difficult that in the case of the assayed anions due to differences of the concentration employed. However, the plot of ammonium formate (50 mM) and TEA (15 mM), both adjusted at pH 4

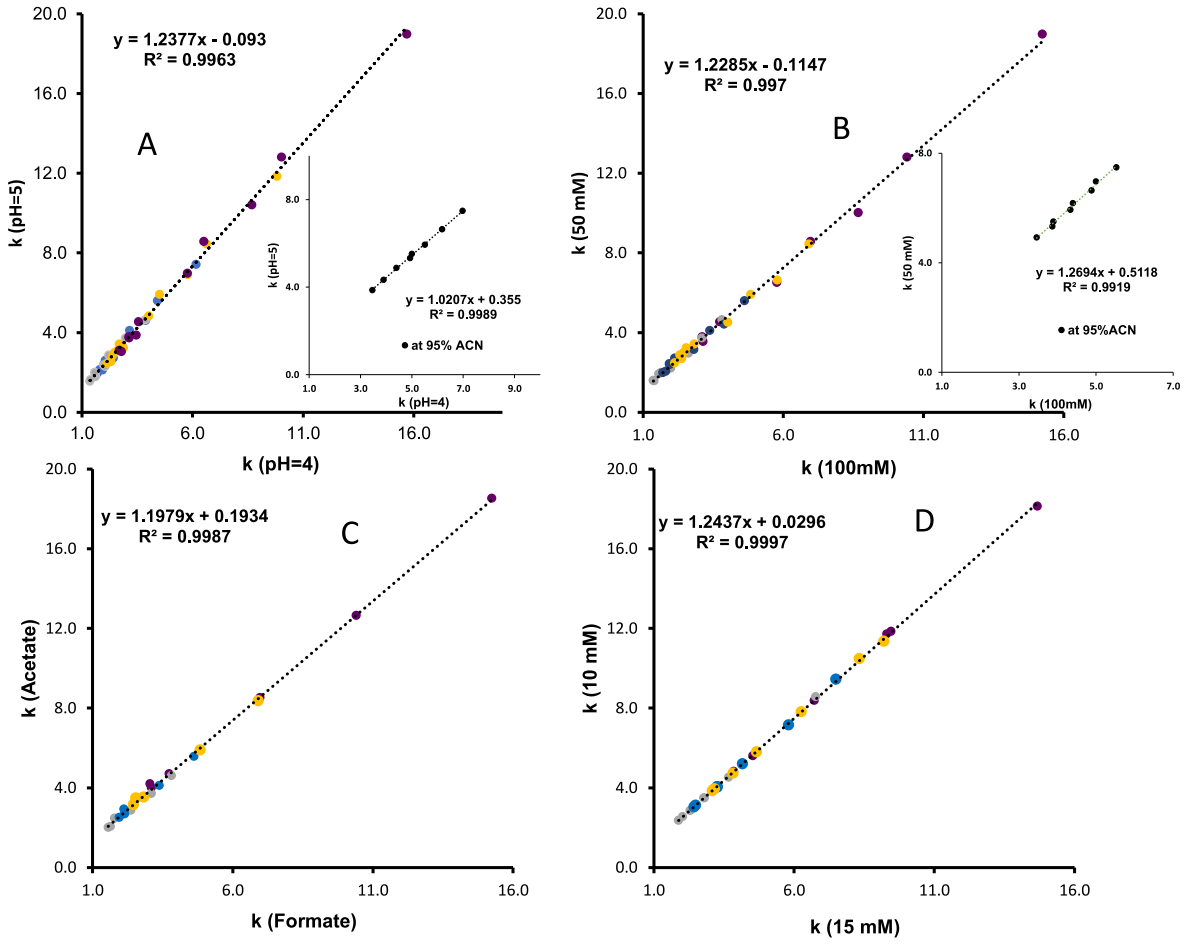

Fig. 2. Relationships between retention factors (k) of four homologues at the tested different conditions. A) k (pH = 4) versus k (pH = 5) in the ACN range from 90% to 65% using ammonium formate (50 and 100 mM) Insert figure: Fig. 2A at 95%ACN. B) k (100 mM) versus k (50 mM) in the ACN range from 90% to 60% using ammonium formate at pH4 and pH5. Insert figure: Fig. 2B at 95%ACN. C) k (formate) versus k (acetate) at 100 mM and pH = 5 in the ACN range from 90% to 65%. D) k (15 mM) versus k (10 mM) of TEA at pH 4 in the ACN range from 95% to 60%. [BAK-12 (grey dots) BAK-14 (blue dots), BAK-16 (yellow dots) and BAK-18 (purple dots)]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with formic acid (AF50_4, TEAF15_4), showed some marked similarities (Fig. 4SB). In the RP area, the retention was equal except for the extreme points, contrary to what was expected because of the lower TEA concentration. These results showed that TEA was very effective in controlling the silanol groups in the RP mode. In the HILIC zone, greater retention was observed using TEA since other hydrophilic contribution could occur. Cation-π interactions between the salt cation and stationary phase could occur. As with silanol groups, the competition for the biphenyl groups would also condition the retention.

3.3. Validation of the method. Determination of the BAK homologues in pharmaceutical samples

Although the separation was practically achieved at baseline under all tested conditions, the minimum of retention (85% acetonitrile) with a higher concentration of ammonium formate and lower pH levels (100 mM and pH 4) was chosen for BAK determination because it provided the lowest retention time and the best symmetry of the chromatographic peaks. Due to the sensitivity of chromatographic methods, the sample treatment was hardly necessary; therefore, a single dilution with the mobile phase in the injection vial allowed for a minimum manipulation of the sample. Another advantage of the method was the low volume of sample employed (500 μL) since the volume of commercial samples is usually a few milliliters. Samples with concentration over the linearity limit, must be previously diluted with mobile phase.

The values obtained in the validation of the method are shown in Table 3. As it can be observed, excellent linearities were obtained with coefficients of determination (R^2) higher than 0.9993 for all homologues, meeting the acceptance criterion (≥0.990) []. Moreover, the

Table 3
Values obtained in the method validation (LOD, LOQ, system precision and precision and recoveries in real formulation).

Parameter of validation	C12-BAK	C14-BAK	C16-BAK	C18-BAK
Determination Coefficient	0.9998	0.9998	0.9993	0.9997
LOD (μg/mL)	0.7	1.4	1.2	1.1
LOQ (μg/mL)	1.3	3.9	3.7	3.3
Intra-day precision (%RSD)				
0.01 mg/mL	2.8	2.3	2.6	3.7
0.10 mg/mL	0.9	0.8	1.1	1.4
Inter-day precision (%RSD)				
0.01 mg/mL	4.0	4.6	5.3	5.5
0.10 mg/mL	1.2	1.2	1.5	2.3
Real formulation (B)				
Concentration (mg/mL)	0.056	0.027	0.070*	0.070*
Intra-day precision (%RSD)	2.4	1.9	3.9	4.5
Inter-day precision (%RSD)	5.0	4.6	5.8	5.7
Recovery: %R (%RSD)				
Spiked amount				
0.015 mg	97 (0.5)	94 (1.8)	97 (5.5)	93 (7.3)
0.035 mg	102 (1.5)	98 (1.6)	101 (5.2)	92 (4.1)
0.050 mg	105 (0.7)	103 (1.5)	98 (1.0)	94 (1.9)

*concentration level spiked to sample.

linear range (from 0.005 to 0.100 mg/mL) was wider than those reported in the literature.

Since the methods for BAK analysis in the literature were designed as routine methods, LODs and LOQs were not usually calculated. However, in the present work both limits were calculated due to their inclusion in the ICH guidelines. As shown in Table 3, LODs varied from 0.7 to 1.4 μg/mL and LOQs from 1.3 to 3.9 μg/mL. These values were slightly better than those provided by Kostić et al., 2012 [28] (LOD: 4.2 LOQ: 13.3 μg/mL) which proved that the method exhibited satisfactory sensitivity.

Intra-day and inter-day precision were satisfactory both in related to the system and the method, with RSD values lower than 6%, in accordance with the acceptance criterion (<10%) [41]. Analytical recoveries, were ranging from 92% to 105%, which were within the established range (80–120%) (Fig. 3 and Table 3) [41].

Upon validation, the method was applied to five commercial formulations (Table 4). Since the concentration of BAK in the cutaneous formulation is over the linearity limit, a previous dilution 1:10 in mobile phase was necessary. As it can be noted, only two homologues were detected (C12-BAK and C14-BAK) in all samples, C12 being two or three times higher than C14 (Fig. 3). The average total BAK content was within the range established by the European Medicines Agency for each type of formulation (cutaneous: 0.05–1 mg/g; ophthalmic: 0.01–0.1 mg/mL; otic: 0.02–0.2 mg/mL). It is worth noting that most of the published methods were applied only to one or two formulations; the study conducted by Labranche et al. (2007) [25] was the only one that analyzed four ophthalmic formulations.

Finally, since the formulations have different active drugs and excipients, the reliability of the method was evaluated in terms of recovery by spiking the four formulations at intermediate level (0.035 mg). The obtained recovery values ranged between 93% and 104%, with RSD lower than 4.5%, indicating that there were neither matrix effects nor interferences observed for the different formulations. On the other hand, other components of some of the tested formulations (formulations "B" and "F") were eluted before C12-BAK and they were detected by their characteristic spectra. Therefore, the method has potential for the simultaneous separation of BAK and other ingredients, but this is not one of the objectives of the present work.

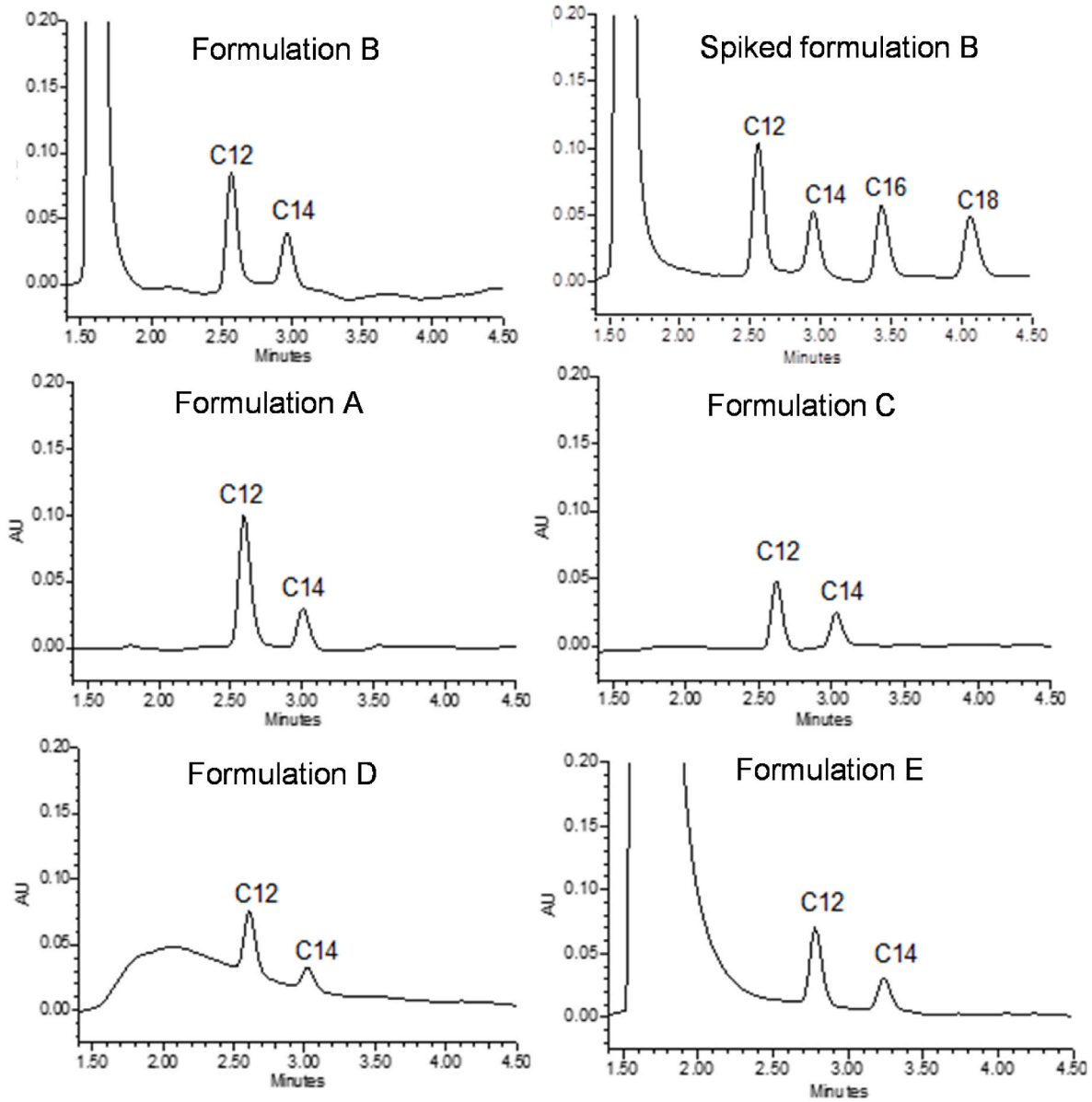

Fig. 3. Chromatograms of the analyzed pharmaceutical formulations with the selected conditions. (Formulation B were spiked with 0.035 mg of each homologue. For more details, see Tables 1 and 3).

Table 4
Concentration (mg/mL) found in several pharmaceutical formulations and recoveries adding 0.035 mg for each homologue.

Formulation	C12-BAK		C14- BAK		C16-BAK		C18-BAK	
	Av.(%RSD)	%R±%RSD	Av.±%RSD	%R±%RSD	Av.±%RSD	%R±%RSD	Av.±%RSD	%R±%RSD
Cutaneous (A)	0.68 ± 0.8	96 ± 0.4	0.22 ± 2	97 ± 1	n.d.	100 ± 1	n.d.	93 ± 2
Ophthalmic (C)	0.031 ± 3	104 ± 2	0.017 ± 2	104 ± 4	n.d.	102 ± 4	n.d.	101 ± 4
Ophthalmic (D)	0.030 ± 2	98 ± 2	0.010 ± 3	99 ± 2	n.d.	102 ± 2	n.d.	101 ± 1
Otic (E)	0.039 ± 2	98 ± 2	0.020 ± 3	102 ± 3	n.d.	104 ± 3	n.d.	103 ± 4

Av. = average.

4. Conclusion

The retention of the four BAK homologues followed a U-shaped behavior using a core-shell column functionalized with biphenyl groups. This typical behavior of functionalized columns in mixed mode has not been referenced neither in the literature for the BAK analysis nor on the used column on this work. The experimental data were adjusted to polynomial equations of different order depending on the aqueous phase used. These fittings can be used to predict the retention times of analytes in each one of the tested modes.

Some variables of the mobile phase were studied. An increase in salt concentration and a decrease in pH led to a decrease on BAK retention in the RP and in HILIC modes indicating an important contribution of the electrostatic interactions. Linear relationships between the retention factors of the four homologues were found when the salt concentration and the pH were changed. The relationship encompasses the entire range of acetonitrile except to 95%, which showed a different behavior.

The type of anion and cation of the salt had influence on the retention. Using formate as the salt anion, it was possible to separate the homologues in a wider range of conditions with shorter retention time. The retention factors of BAK homologues showed a good relationship between two tested anions. Regarding the salt cation, a similar retention of the homologues in the RP mode was obtained with a lower TEA concentration than ammonium concentration. In the fitting of the polynomial equations, different polynomial grades were found depending on the salt anion. For the two tested TEA concentrations, the relationship between the obtained retention factors was established in a wider range of acetonitrile levels than the other mobile phase variables.

Based on the retention study, a method for the analysis of BAK homologues in pharmaceutical formulations was proposed. Minimum handling of the sample, a reduced sample volume and short analysis time are its main characteristics. The method provides good precision, accuracy, LOD and LOQ and a wide linear range for the four homologues. The analytical recoveries in five commercial samples indicated a good specificity and lack of matrix effects. Therefore, the proposed method demonstrated its ability to perform a BAK analysis in a wide range of formulations.

Credit author statement

M. C. Prieto-Blanco: Conceptualization, Supervision, Formal analysis, Writing-original draft, Review & editing, **A. Planas-Franco:** Investigation, Formal analysis, Validation, **S. Muniategui-Lorenzo**: Funding acquisition, Resources, **M.J. González-Castro:** Supervision, Conceptualization, Validation, Review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

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