Ultra-high performance separation of basic compounds on reversed phase columns packed with fully/superficially porous silica and hybrid particles by using UV transparent hydrophobic cationic additives.

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Non-standard abbreviations: UHPLC, ultra-high performance liquid chromatography; **ILs**, ionic liquids; **ER-RPLC**, electrostatic repulsion reversed phase liquid chromatography; **CSH,** charged surface hybrid, **TBAOH**, tetrabutylammonium hydroxide; **$TBAHSO_4$**, tetrabutylammonium hydrogen sulfate; **TBA**, tetrabutylammonium; **SPP**, superficially porous particles; **FPP**, fully porous particles; **$RP^{plusTBA}$**, reversed phase added of TBA.

Keywords: basic analytes, hydrophobic cationic additives, peak tailing, reversed phase columns.

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Abstract

The use of the tetrabutylammonium additive was investigated in the ultra-high performance reversed phase liquid chromatographic elution of basic molecules of pharmaceutical interest. When added to the mobile phase at low pH, the hydrophobic tetrabutylammonium cation interacts with the octadecyl chains and with the residual silanols, thus imparting a positive charge to the stationary phase, modulating retention and improving peak shape of protonated basic solutes. Two sources of additive were tested: a mixture of tetrabutylammonium hydroxide/trifluoroacetic acid and tetrabutylammonium hydrogen sulfate. Retention and peak shape of eleven basic pharmaceutical compounds were evaluated on commercially available ultra-fast columns packed with octadecyl stationary phases (Ascentis Express C18 2.0 µm, Acquity BEH C18 1.7 µm, Titan C18 1.9 µm). All columns benefit from the use of additive, especially tetrabutylammonium hydrogen sulfate, providing very symmetric peaks with reasonable retention times. Focusing on the probe compounds amitriptyline and sertraline, efficiency and asymmetry values were investigated at increasing retention factor. The trend is very different to that obtained in reversed phase conditions and the effect lies in the complex molecular interaction mechanisms based on hydrophobic and ion exchange interactions as well as electrostatic repulsion.

Introduction

Today, more than 70% of small molecules of pharmaceutical interest are bases, about 20% are acids, and only 10% are neutral compounds [1]. However, despite the high number of basic compounds, their analytical control by reversed phase liquid chromatography (RP-LC) poses still particular challenges. Two critical factors should be taken into account in the elution of bases by RP-LC based on silica-based stationary phases: control of retention and minimization of peak broadening and tailing [1-3]. Basic compounds are ionizable and their retention is mainly affected by the ionic behaviors of residual silanols present on the surface of packing materials [4]. Non-negligible amount of underivatized, deprotonated silanols act as cation exchange sites and provide an interaction

mechanism that add to the hydrophobic effect typical of reversed phase stationary phase. In this case, the stationary phase works as a mixed-mode support, the positively charged analytes at the pH condition of the mobile phase are retained longer, and their peak shape is negatively affected (e.g., tailed and broad peaks) [5-7]. Different approaches have been developed to suppress the ion exchange mechanism of silanols. They are gathered in two main groups: i) modification of the mobile phase and ii) use of alternative solid supports. Lowering the pH of mobile phase, addition of tertiary amines at acidic pH, and more recently, the introduction of room temperature ionic liquids (ILs) in mobile phase represent some methods belonging to the first group [8-16]. At pH values between 2.5 and 3.5 silanols are mainly undissociated, the interactions with the ionized basic samples are reduced and peak shape improves [17, 18]. However, pH reduction alone does not seem to be a wide-applicability solution. Tertiary amines are the first choice and the most used mobile phase additives employed to shield silanols [10]. In fact, positively charged amines interact electrostatically with the residual anionic silanols making them no longer available for the interaction with solutes. Moreover, hydrophobic protonated amines can also be applied in combination with chiral mobile phase additives to obtain enantiomeric purity profile of chiral bases [19]. Concerning the ILs, they represents a valid alternative as additives to shield free silanols but the mechanism of action seems to be concentration dependent [20] and not easy to preview. In addition, the main drawback of using ILs as additives in mobile phase lies in their absorption properties in UV region [21].

In the second group, innovative solid supports have been primarily focused on ultra-high performance LC (UHPLC) applications. Among them, some companies produced silica bulk, named high purity silica, with a reduced amount of metals and free silanols; others have commercialized hybrid silica particles, where siliceous skeleton contains organic moieties, typically ethyl groups that provide mechanical resistance and reduction of silanols [22-24]. From the silica derivatization point of view, embedded polar or ionic functional groups were introduced in the long alkyl chain of hydrophobic stationary phase in addition to the standard end-capping procedure [25-27]. Ihara et al. presented an unconventional embedded RP stationary phase [28]. They prepared a long chain

octadecylimidazolium ILs-modified silica stationary where the cationic moiety is located close to the silica network. Compared to the standard C-18 stationary phase, this support can interact with solutes according to a mixed mechanism comprising hydrophobic, anion-exchange, electrostatic, and π-π interactions. In 2010, a new concept of mixed mode stationary phase based on hybrid support was introduced in the market as charged surface hybrid (CSH) materials, which involves positively charged groups chemically bonded to hybrid silica surface [29, 30]. Columns packed with CSH silica provide a wide range of selectivity for ionizable compounds and allows for their rapid equilibration in pH switching protocols. This makes them ideal to be included in the routine method development and to be used in analytical platforms where the pH of mobile phase is frequently changed [28]. Gritti and Guichon have published many papers concerning the use of CSH supports and the theoretical investigation of their interaction mechanism [31-33]. In particular, in a paper dated 2014, the authors introduced the term electrostatic repulsion interactions RP-LC (ER-RPLC) to describe this new version of mixed-mode mechanism [34-38]. The advantages of ER-RPLC lie in the shorter retention times and the reduction of peak tailing when employed at acidic elution conditions.

However, despite all progress in particle technology, peak deformation of basic compounds can be recorded under particular experimental conditions. Taking into account the routine operations in analytical laboratories, low pH in combination with the positively charged additives is often a first favored choice for the RP-LC analysis of basic molecules, like small molecules, peptides, and small proteins, in order to suppress silanol activity effectively. Herein we report on the use of positively charged hydrophobic additives for efficient UHPLC elution of basic molecules of pharmaceutical interest. Commercially available UHPLC columns packed with octadecyl stationary phases, namely Ascentis Express C18 2.0 µm, Acquity BEH C18 1.7 µm and Titan C18 1.9 µm, were evaluated and results compared with the Acquity CSH C18 1.7 µm column.

2. Materials and methods

2.1 Materials

All solvents were LC grade and ~~were~~ purchased from Sigma-Aldrich (St. Louis, MO, USA) as well as trifluoroacetic acid (TFA), tetrabutylammonium hydroxide 30-hydrate (TBAOH) (≥99.0% *w/w*), and tetrabutylammonium hydrogen sulfate ($TBAHSO_4$) (≥99.0% *w/w*). All basic compounds, in active pharmaceutical ingredient form, were available in house from previous studies. Solutions of samples were prepared in mobile phases at a concentration of 1.0 mg/ml.

Four UHPLC columns, of 100 mm L x 3.0 mm I.D., were employed: Acquity BEH C18 1.7 µm and Acquity CSH C18 1.7 µm from Waters Corporation (Milford, MA, USA), Ascentis Express C18 2.0 µm and Titan C18 1.9 µm from Supelco Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.2 Instrumentation

An UltiMate 3000 RSLC LC (Thermo Fisher Dionex, Sunnyvale, California) equipped with dual gradient pumps was employed. The complete configuration of the system includes an in-line split-loop well plate sampler, a thermostated column ventilated compartment (temperature range: 5–110°C), and a diode array detector (UltiMate 3000 RSLC DAD) with a low dispersion 2.5 µl flow cell. The UV detector was set at a time constant of 0.10 s and data collection rate of 100 Hz. UV detection was performed at 220 nm. Data acquisition and processing were performed with Chromeleon 7.2 (ThermoFisher).

2.3 Chromatographic conditions

The mobile phases contained different portions of water (eluent A) and acetonitrile (eluent B), both with added TBAOH ion pair reagent (5 mM) and TFA (0.1% v/v). In addition, the eluent A was buffered at pH 2.5 with TFA. Conversely, when $TBAHSO_4$ (5 mM) was added to water, the pH was at once 2.5. For comparative purpose, the elution conditions of water/acetonitrile 90/10 and 0.1% TFA v/v were used. Mobile phases were filtered through 0.2 µm Omnipore filters (Merck Millipore, Darmstadt, Germany). Injected volumes were 0.5 µL. Hold up volume was estimated by injection of uracil.

For data evaluation, the values of peak asymmetry at 5% of peak height were calculated according to the European Pharmacopeia. The efficiency (N/m) was calculated by the second moment, as reported in Eq. 1 [39].

$$TP = \frac{t_r^2}{\mu_2} \qquad \text{Eq.1}$$

Where *TP* is the number of theoretical plates, t_r the retention time of analyte, and μ_2 the second statistical moment. The μ_2 is defined as reported in Eq. 2:

$$\mu_2 = \frac{\int (t-\mu_1)^2 * f(t)dt}{\mu_0} \qquad \text{Eq. 2}$$

where *t* corresponds to the peak centroid, the μ_1 is the first statistical moment ($\mu_1 = \frac{\int t*f(t)dt}{\mu_0}$) and ~~the~~ μ_0 is the zeroth statistical moment ($\mu_0 = \int f(t)dt$).

3. Results and Discussion

In the first part of the study, a collection of basic compounds of pharmaceutical interest has been eluted on widely used C18 UHPLC columns in reversed phase mode by using only TFA as additive. **Table 1** reports the characteristics of tested columns. Briefly, Ascentis Express and Titan C18 columns are based on superficially and totally porous silica particles, respectively (SPP and FPP). They have very comparable pore (90 Å vs. 80 Å) and particles sizes (2.0 µm vs. 1.9 µm) with different loading of organic portion (8% vs. 13%). Acquity BEH C18 column is packed with hybrid carbon/siliceous 1.7 µm particles (130 Å pore size). The total carbon content is 18% but the portion of octadecyl moiety is not specified. Acquity CSH C18 column has been used as comparative support that was developed to improve both stability at low pH and peak symmetry of basic analytes.

Figure 1 reports structures of investigated compounds grouped for their pharmaceutical activity: samples **1**-**5** are antidepressant drugs, lidocaine (**6**), and ketamine (**7**) are employed as anaesthetics while compounds **8**-**11** belong to the beta-blocker family. In this sample selection, amitriptyline was chosen as a model compound having only an amino group far from the partially aromatic polycyclic portion, so in principle, the effect of cationic additives should be easily evaluated (see later). An

organic/aqueous mobile phase, optimized for each compound, with a constant 0.1% TFA afforded long retention times and broad peaks (**Table S1**). Keeping constant the organic portion in the mobile phase for each compound, the Acquity BEH C18 is the most retentive stationary phase in RP conditions. The Ascentis Express C18 shows capacity factors comparable to those of the Titan C18 although the stationary phase is based on superficially porous particles and has a low carbon content. Besides, focusing on peak distortion and using $As_{5\%}$ as a measure of matrix activity (mainly correlated to silanol activity), significant peak asymmetries were recorded on Ascentis Express C18 column where $As_{5\%}$ values were often > 3.0. On the other hand, lower $As_{5\%}$ values were found on the Acquity BEH C18, while the Titan C18 showed intermediate results. Furthermore, for compounds **1**, **6**, **7**, **9**-**11**, peak symmetries on the Acquity BEH C18 are well comparable or superior to those recorded on Acquity CSH C18 in the same analytical conditions, attesting the better surface inertness of the hybrid organic/siliceous support.

As expected, the last two columns gave higher efficiencies as they are packed with smaller particles. In particular, the plate number of Acquity CSH C18 column, 40-50% higher than those of the Acquity BEH C18, reflects the mixed mode (hydrophobic and charge repulsion) retention mechanism specifically developed for the elution of basic compounds. The fixed positive charge on CSH stationary phase allows electrostatic repulsion with the positively charged analytes reducing capacity factor and increasing the number of plates.

In this contest, taking into account the Acquity CSH C18 column embodies a solution for improving peak shape of basic compounds, we demonstrate how the addition of cationic and hydrophobic additive at low pH, in the mobile phase, represents a valid and flexible additional approach in the elution of the same compounds on conventional UHPLC C18 supports, providing excellent peak shape with asymmetry values close to 1.0. The widely known, not expensive, and UV transparent, tetrabutylammonium (TBA) was chosen as a hydrophobic cationic additive. At acidic pH of the mobile phase, two sources of TBA were evaluated: TBAOH and $TBAHSO_4$. To work at low pH,

TBAOH, as opposed to $TBAHSO_4$, must be added with ~~a~~ TFA. The screening has been done on six samples randomly chosen and data are reported in **Table S2**.

As expected, these additives prevent unwanted interactions with the stationary phase ensuring reasonable elution times and symmetries. In detail, asymmetry values are well comparable in both cases, some slight differences in retention could be ascribed to the nature of counter ion as a kosmotropic effect [40] but this aspect is actually under investigation in our laboratory. When hydrogen sulfate anion is employed, the eluent A reaches a pH of 2.5 directly without any additional pH correction. Therefore, for this facilitating aspect we decided to use only $TBAHSO_4$, namely from now $RP^{plusTBA}$ approach. The elution profiles of amitriptyline **1** on the three investigated columns are given in **Figure 2** together with the chromatographic profile obtained on the Acquity CSH C18. The effect of TBA is clearly shown in the figure. Nice peak shapes with asymmetry values close to 1.00 were recorded in addition to a decrease in retention time.

Comparing the column performances in the absence of additive (blue traces in **Figure 2**), the Ascentis Express C18 seems to be the most sensible to the presence of cationic additive moving the $As_{5\%}$ from 3.48 to 1.24. **Table S3** completes the investigation of all compounds with $RP^{plusTBA}$ elution. In some cases, two different elution conditions were evaluated, aiming to observe the peak distortion at high values of retention (sertraline, paroxetine and citalopram in **Table S3**). Interestingly, the $As_{5\%}$ values remain almost constant. A deeper investigation of retention factor and plate number as a function of organic modifier concentration has been performed for compound **1** on the Ascentis Express C18.

Logarithmic retention of amitriptyline in RP and $RP^{plusTBA}$ modes decreases linearly with % ACN (**Figure 3**). In order to obtain comparable ranges of log k with the two elution modes, lower contents of ACN in the mobile phase were used in the $RP^{plusTBA}$ mode. As an example, the right part of **Figure 3** shows that similar k values (6.17 and 8.51 in $RP^{plusTBA}$ and RP, respectively) are recorded by using 20% and 30% of acetonitrile, with a fourfold reduction of peak asymmetry in $RP^{plusTBA}$ mode.

This chromatographic behaviour indicates a shift in the prevailing retention mechanism from the typical reversed phase to the electrostatic repulsion supported by the TBA additive.

198 Moreover, the higher slope of $RP^{plusTBA}$ straight line provides a reduction of retention time with a tiny
199 increase of acetonitrile. At 35% of organic modifier, as reported at the bottom of **Figure 3**, the
200 retention factor is close to zero, compared to k = 3.53 in RP mode.
201 Different trends were observed for both efficiency and asymmetry as a function of retention factor.
202 **Figure 4 A** shows the variation of plate number per column when retention factor increases with the
203 content of water in the mobile phase. While in acidic RP conditions, we observe a van Deemter like
204 curve, in $RP^{plusTBA}$ conditions experimental data fit with an inverted van Deemter like curve. Peak
205 asymmetry increases rapidly with retention factor in RP, while ~~and~~ it is smaller and remains almost
206 constant in $RP^{plusTBA}$ mode (**Figure 4 B**).
207 Although it is difficult to provide a model detailing the molecular interaction mechanisms operative
208 in our chromatographic systems (due to the coexisting of hydrophobic, electrostatic repulsion, ion
209 exchange interactions), we believe the use of cationic, hydrophobic mobile phase additives is a
210 convenient solution for the elution of basic compounds. Similar results were in fact obtained with
211 sertraline (**Figure S1**) whose structural similarity with amitriptyline is limited to the exocyclic-alkyl-
212 amino portion, thus corroborating the assumption that electrostatic repulsion plays an important role
213 in the elution mechanism. With increased complexity of molecular structure, especially in the
214 presence of additional polar functional groups, the control of chromatographic properties in $RP^{plusTBA}$
215 requires a case-by-case investigation respect to the previous models. However, $RP^{plusTBA}$ elution mode
216 results effective in any case as shown by the radar plots in **Figure 5**. In these plots, investigated
217 compounds were placed at each vertexes of radars while the axes report the asymmetry values or the
218 efficiency. Data points represent the best elution conditions obtained for each compound and the
219 connecting line gives an idea of the total score for each column. Detailed data of single elution
220 conditions are listed in **Tables S1-S3**. The Ascentis Express C18 column is mainly sensitive to the
221 presence of $TBAHSO_4$ and asymmetry values have been shifted from 3-4 to about 1 in all cases
222 (**Figure 5 A**). Comparing the results achieved on the three UHPLC columns, in **Figure 5 B**, the
223 Acquity BEH C18 column shows better $As_{5\%}$ and N/m values in line with the particle proprieties. The

only compound excluded from data analysis was atenolol that in all tested conditions was eluted with the void volume. A selection of chromatographic traces of different probes, with and without the TBA cationic additives, is presented in **Figure 6**. The chromatograms emphasize the quality of peak shape, like a perfect Gaussian profile, in the elution conditions providing medium to high retentions. Moreover, in two examples related to paroxetine and citalopram, the chromatograms prove that highly symmetric peaks are obtained even with high retention factors, like in the amitriptyline case. The best elution conditions for all tested compounds are given in **Table 2**.

A small sub-class of investigated compounds is represented by the beta-blockers. The effect of electrostatic repulsion for these samples is very large, resulting in small retention factors even in the presence of minor amounts of organic modifier (2% ACN, **Figure 7**). Moreover, the beta-blocker nadolol has more than one stereocenters and exist as a pair of diastereomers that can be in principle separated by achiral chromatography. Indeed, all UHPLC columns can separate the two diastereomers (data in **Table S2**) by using $TBAHSO_4$ additive demonstrating a diastereoselectivity and efficiency higher than that recorded by using TBAOH/TFA mixture in the mobile phase or recorded on the Acquity CSH C18 column. As shown in **Figure 7**, two well separated peaks were observed at reasonable retention time with a resolution of 2.34 (α = 1.12), while the charged surface hybrid stationary phase missed this target and showed a chromatographic performance similar to classical C18 stationary phase (no diastereomer separation) except for the better peak symmetry under the same RP conditions.

4. Conclusions

Recent years have seen a great research effort to finding solutions for the UHPLC separation of basic compounds in reversed phase conditions. One advantageous approach exploits mobile phase cationic hydrophobic additives. In this study, the $TBAHSO_4$ additive has been employed to improve the elution of basic molecules of pharmaceutical interest on several UHPLC columns packed with C18

stationary phases. $TBAHSO_4$ resulted convenient for the analysis of basic compounds providing highly symmetric peaks in a wide range of retention factors. All tested UHPLC columns, based on either pure silica or hybrid organic-silica, benefit on the use of this mobile phase additive. In addition, the mixed retention mechanism based on electrostatic repulsion and hydrophobic effect, results in high efficiencies that usually compensate for low selectivities. Finally, the obtained results showed the $TBAHSO_4$/C18 stationary phase combination represents a valid alternative to the use of CSH materials.

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Conflict of interest statement

The authors declare no commercial or associative conflict of interest.

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Figure captions.

Figure 1. Structures of investigated compounds.

Figure 2. Amitriptyline eluted on the four investigated columns. Analytical conditions: in RP H_2O/ACN 65/35 + 0.1% TFA (blue traces); in $RP^{plusTBA}$ H_2O/ACN 75/25 + 5mM $TBAHSO_4$ (orange traces). Flow rate 0.5 mL/min, T = 35°C, Sample: 1µg on column. Geometry columns: 100 mm L x 3.0 mm I.D.

Figure 3. Log k vs %ACN plot in RP and $RP^{plusTBA}$ and chromatograms relating to the two areas highlighted in the graph (right: similar k, bottom: similar %ACN). Sample: amitriptyline. Column Ascentis Express C18, 100 mm L x 3.0 mm I.D. Analytical conditions: in RP H_2O/ACN + 0.1% TFA (blue dots and trace); in $RP^{plusTBA}$ H_2O/ACN + 5mM $TBAHSO_4$ (orange dots and trace). Flow rate 0.5 ml/min. T = 35°C.

Figure 4. **A)** Plates and **B)** $As_{5\%}$ vs k plots in RP and $RP^{plusTBA}$. See details in **Figure 3**.

Figure 5. A) Radar chart corresponds to the Ascentis Express C18 for both RP (blue trace) and $RP^{plusTBA}$(orange trace) conditions. Vertexes are investigated compounds, axes the $As_{5\%}$.. **B)** Radar charts correspond to the three UHPLC columns (Titan C18 (green), Acquity BEH C18 (black) and Ascentis Express C18 (gray)) in only $RP^{plusTBA}$ conditions. Vertexes are investigated compounds, axes are $As_{5\%}$ (left) and N/m (right). Eluents composition is shown in Supporting Information, **tables S1-S2-S3**.

Figure 6. $RP^{plusTBA}$ (orange traces) vs RP (blue traces) approaches. Columns and samples are reported in the graphs. Eluents as reported in **Figure 3**. Detailed elution conditions: Sertraline: A/B 35/65 and A/B 75/25 in RP and $RP^{plusTBA}$ respectively. Citalopram: A/B 65/35 and A/B 80/20. Ketamine: A/B 85/15 and A/B 98/2. Paroxetine: A/B 65/35 and A/B 80/20. Lidocaine: A/B 85/15 and A/B 98/2. Mirtazapine: A/B 90/10 and A/B 90/10.

Figure 7. Elution profiles of beta-blockers. Columns and samples are reported in the graphs. Eluents as reported in **Figure 3**. Detailed elution conditions: Pindolol: A/B 90/10 and A/B 98/2, Sotalol: A/B 95/5 and A/B 98/2, Nadolol: A/B 90/10 and A/B 98/2 in RP (blue traces) and $RP^{plusTBA}$ (orange trace) respectively.