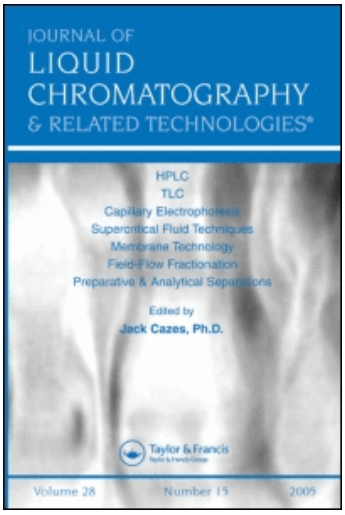

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Enhancing Productivity in the Analytical Laboratory Through the Use of Ultra Fast-HPLC in Preformulation/Formulation Development

Rosario LoBrutto,[1] Alexey Makarov,[1] Anton Jerkovich,[1] Raymond McGill,[1] Yuri Kazakevich,[2] and Richard Vivilecchia[1]

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Abstract: The pharmaceutical industry today is driven to create new, more efficient ways to discover, develop, deliver and monitor drugs. Pharmaceutical companies are being faced with major challenges in reducing drug discovery and development timelines. Automation and the introduction of new analytical technologies that increase speed of analysis are integral in the analytical laboratory. The development of rapid chromatographic methods in preformulation and formulation development is playing an increasing role to support this drive in efficiency and productivity. The introduction of ultra fast-HPLC systems that can operate at pressures of up to 15,000 psi with columns packed with sub-2-μm particles have allowed for high speed and efficient separations. The consequent reduction of time, solvent, and waste disposal, and the analysis of more samples per unit time makes ultra-fast HPLC a very attractive technology. Faster method development and decision making can be achieved during late-phase preformulation/formulation development for the analysis of both singly charged and multiply charged basic compound analysis.

The use of ultra fast-HPLC (UHPLC) for pH scouting experiments and the determination of the analyte's ionogenic nature was shown to be an effective

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tool for rapid and systematic method development. The implementation of this technology was also evaluated for the analysis of drug product formulations and excipient compatibility studies. Increased speed of analysis and significant gains in resolution per unit time were obtained compared to separations performed using conventional HPLC systems (operating pressures of <5,500 psi). Also, the use of liophilic ions as mobile phase additives with operation under high pressures led to enhanced separation selectivity, retention, and peak symmetry of multiply charged basic compounds.

Keywords: Basic compounds, Formulation development, Liophilic, pK_a, Preformulation developments, UHPLC, UPLC

INTRODUCTION

The pharmaceutical industry today is being faced with major challenges in reducing drug discovery and development timelines.[1,2] Analytical Departments can have significant and positive impact on timelines by employing fast and efficient problem solving. Automation and the introduction of new analytical technologies such as ultra fast-HPLC that increase the speed of analysis are integral to the success of our business.[3–5]

The advances in fast LC can be primarily attributed to development in the following three areas: (1) ultrahigh pressure liquid chromatography[6–9] (2) monolithic columns[10–12] and (3) high temperature LC.[13–15] The introduction of columns packed with sub-2 μm particles has allowed for high speed and increased efficiency in chromatographic separations.[16–22] However, in order to overcome the high pressure drop generated by columns containing small particles (<2 μm), ultrahigh or very high pressure pump LC systems have been developed and introduced into the marketplace. Ultra high (UHPLC) or very high pressure LC systems are generally those that can operate at pressure limits of greater than 5,500 psi. The reduction of time, solvent, waste disposal, and resources makes it a very attractive alternative to traditional HPLC. These advantages allow faster method development and decision making during late phase preformulation/formulation development. The types of analyses employed during this phase of development include determination of solubility, excipient compatibility studies, dissolution studies, formulation screening, and stability studies for the API (active pharmaceutical ingredient) as well as drug product formulations. As increasingly larger number of samples are generated, fast turnaround of results is needed so that optimal formulations can be developed in shorter periods of time. Any shortcomings that arise during stability can then be addressed more expeditiously.

Three main areas in which ultra fast-HPLC was applied will be discussed in this article:

1. Transfer of HPLC methods to UHPLC methods;
2. Methods development (pH screening);
3. Analysis with liophilic mobile phase additives for challenging separations of multiply charged species.

Method Transfer of HPLC Method to UHPLC Method

Transfer of an existing HPLC method to a UHPLC method is desirable in the pharmaceutical industry and this has been successfully demonstrated in the literature.[23–25] The packing material and the geometric scaling (scaling factors to address differences in column dimensions) relationships for the flow, gradient and injection volume especially needs to be taken into account for a successful transfer of an HPLC method to a UHPLC method.

Column Considerations

Significant gains in speed and increase of resolution per unit time are strong drivers for transferring methods from traditional HPLC systems to ultra-high pressure instruments. A few key requirements need to be considered for columns that are chosen to be run on a UHPLC. Selection of a column that is packed with sub-2 μm particles that can withstand high pressures (>5,500 psi–15000 psi) is needed. Moreover, the stationary phase packing material must be stable within a wide pH/temperature range and the columns should be robust with respect to different batches of base silica, the bonding process and the packing process. A column with selectivity similar to that of the original column is preferred, however not all manufacturers have sub-2-μm counterparts to 3–5 μm column packing materials, which can make method transfer challenging.

Particle Size Considerations

The practical goal of most separations is not to achieve the greatest resolution possible, but rather to obtain sufficient resolution to separate all components in the shortest amount of time. This approach has been coined as "enhancing the resolution per unit time" for the separation. Therefore, in order to optimize for speed, the minimum resolution requirement for the separation needs to be determined. Generally, a resolution of >1.5 for a critical pair is deemed acceptable.

Resolution is proportional to the square root of the number of theoretical plates in the column (N)

$$Rs \propto \sqrt{N} \tag{1}$$

where N is proportional to the column length (L), and inversely proportional to the efficiency (H) (theoretical plate height).

$$N = \frac{L}{H} \tag{2}$$

From Eq. (2), it is shown that column efficiency scales directly with column length and inversely with the plate height. Reducing the plate height is one way to obtain higher theoretical plates without increasing the column length. Reducing the diameter of the packing material particle size is a powerful approach for reducing the plate height, and consequently allows the number of theoretical plates to be increased. However, a reduction in particle size will lead to an increase of the column backpressure, due to the inverse dependence of pressure drop to the square of the particle diameter.

$$\Delta P = \frac{uL\eta\phi}{d_p^2} \tag{3}$$

where u is the mobile phase velocity, L is the column length, η is the mobile phase viscosity, and ϕ is the empirical flow resistance factor, which is essentially dependent on particle size distribution and packing density.[26] If the average backpressure on the column packed with 5 μ particles is in the range of 500 to 1500 psi, the decrease of the particle size to 1.7 μ causes the backpressure to increase approximately 8 fold, to between 4,000 and 13,000 psi, respectively, which necessitates the use of ultra high pressure liquid chromatographic systems.

Reduction of plate height allows for the use of shorter columns since the same value of N (the plates needed to achieve the desired resolution requirement), can be obtained compared to a separation carried out on a longer column and that is packed with larger particles. The plate height is also dependent on the linear velocity of the mobile phase. The minimal attainable plate height for a column, $H_{\min}$, is the plate height occurring at the optimum linear velocity, u_{opt} and this is dependent upon the particle size. When operating near the optimal linear velocity, u_{opt}, the approximation can be made that H is proportional to dp[27] and therefore N is proportional to L/dp. Using smaller particles allows the use of faster flow rates, since the operation at higher velocities does not cause a significant impact on the u_{opt}. The smaller particles allow faster linear velocities to be used without much sacrifice in plate

height due to the shallow C-term (resistance to mass transfer) of the van Deemter equation. Thus, when optimizing for speed, the two van Deemter parameters that must be considered together are H and u. The goal, then, is not just to reduce H, but to minimize H/u. This will favor both high resolution and short analysis times. Minimizing H/u, then, encompasses the heart of what is desired in a fast HPLC method—achieving the greatest *resolution per unit of time*.

Scaling Considerations

Working with ultra fast HPLC systems allows the chromatographer to work at higher backpressures. When a method is to be transferred from HPLC to UHPLC, it should be scaled to account for differences in column dimensions to maintain equivalency.[27] The flow rate is scaled to maintain the same linear velocity as employed in the original method. This scalar is performed based on the ratio of the column diameters squared:

$$F_{\text{column 2}} = F_{\text{column 1}} \times \frac{d^2_{\text{column 2}}}{d^2_{\text{column 1}}} \tag{4}$$

Next, scale the injection volume needs to be scaled according to the ratio of the two column volumes (V_M):

$$\text{inj. vol.}_{\text{column 2}} = \text{inj. vol.}_{\text{column 1}} \times \frac{V_{M_{\text{column 2}}}}{V_{M_{\text{column 1}}}} \tag{5}$$

Here column volume is defined as the volume of mobile phase in the column and may be estimated as $V_M \approx 0.7\pi r^2 L$, where r and L are the column radius and length, respectively, and 0.7 is the approximate fraction which mobile phase occupies inside a column that is packed with porous particles.

Finally, the gradient times and total run time must be scaled. An equivalent gradient profile will have the same gradient slope—that is, it will deliver the same number of column volumes of mobile phase per gradient step. The number of column volumes per step can be calculated by multiplying the flow rate by the step duration, and dividing by the column volume for the particular column in question. The new gradient time t_g for a given step is therefore:

$$t_{g_{\text{column 2}}} = t_{g_{\text{column 1}}} \times \frac{F_{\text{column 1}}}{V_{M_{\text{column 1}}}} \times \frac{V_{M_{\text{column 2}}}}{F_{\text{column 2}}} \tag{6}$$

If the flow rate has been scaled according to Eq. (4), then linear velocity remains constant, and then Eq. (6) simplifies and gradient time can be scaled in proportion to the column lengths:

$$t_{g\ \text{column 2}} = t_{g\ \text{column 1}} \times \frac{L_{\text{column 2}}}{L_{\text{column 1}}} \tag{7}$$

The other parameters of the separation, such as the mobile phase composition, gradient range (Δ%B), and column temperature should be kept the same. A difference in particle size between columns does not affect the geometric scaling (scaling factors to address differences in column dimensions) relationships; however, the location on the H vs. u curve (i.e., column efficiency) and the column backpressure changes may require an increase or decrease in flow rate or column length. In addition, the decrease of the particle diameter while maintaining the same particle porosity will facilitate the achievement of thermodynamic equilibrium in the system (decrease of the impact of mass transfer on efficiency), which allows the use of even faster gradients and additional decrease of analysis time.

Once these parameters are scaled, the flow rate can then be increased to enhance the speed of the separation. With an increase in flow rate the gradient times must again be adjusted proportionally (e.g., doubling the flow rate requires gradient times to be halved). The optimum flow velocity for the separation must be kept in mind, however. A column with smaller stationary phase particles will have a higher optimum velocity, i.e., a newly scaled method may not be at optimum conditions. The molecular weight of the analyte also plays a role—large molecules such as proteins and large peptides will have a lower optimum velocity compared to small molecules (>1000 MW) due to slower diffusion in the mobile phase.[28] At this point the method may be further optimized using standard method development strategies.

Method Development

There are many factors to consider when developing HPLC methods. The initial steps include collecting information about the analyte in regards to the physicochemical properties (pK_a, $\log P$, $\log D$, solubility, etc.) and determining which mode of detection would be suitable for analysis (i.e., suitable wavelength in the case of UV detection). Sample preparation development includes optimization of such factors as sample concentration and choice of diluent type as well as centrifugation, and/or filtration, and sonication. The choice of diluent/solvent type plays an integral role in method development since this may affect the

chromatography and the recovery of the analytes. Determination of the solution stability in the diluent is also an important factor that must be determined during early development. If the analyte is not stable in solution it will become increasingly more challenging to compare subsequent analyses during method development. Choice of the mobile phase and gradient conditions is dependent on the ionogenic nature and the hydrophobicity of the analytes. This is a crucial step in the method development process since these two factors have a major impact on the variation in selectivity especially for ionizable compounds. Therefore, choosing the optimal pH is a prerequisite to perform further method optimization experiments (variation gradient slope, temperature, flow rate, etc.) and is usually the first step in the development of robust reversed phase HPLC methods.

For the separation of ionogenic (ionizable) solutes, the variations of mobile phase pH can lead to extreme changes in selectivity. The mobile phase (eluent) pH affects the ionization of ionogenic species and consequently their HPLC retention. However, the pH in the aqueous phase is not equivalent to the pH in the aqueous/organic eluent and consequently the variation of the mobile phase composition leads to the variation in pH under both isocratic and gradient conditions.[29–31] Due to the shift of the mobile phase pH upon the addition of the organic modifier during the gradient, it becomes imperative to properly describe the ionogenic nature of the retention process of the analyte during development of the HPLC method of such compounds. This will lead to more robust and rugged methods, since methods could be developed at mobile phase pH values that are not close to the pK_a of the target analyte and/or critical pairs in the sample.

The pK_a is a characteristic constant of the specific analyte, and from Eq. (8), one can conclude that relative amounts of neutral and ionic forms of the analyte could be easily adjusted by varying the mobile phase pH. If the eluent pH is at least one unit away from the component pK_a, more than 90% of the analyte will be in either the ionic or neutral form. Moreover, if the eluent pH is at least two units away from the component pK_a, more than 99% of the analyte will be in either ionic or neutral form and method development/method optimization in these pH regions is generally preferred in order to obtain robust chromatographic methods. The development of HPLC methods where the mobile phase pH is close to the analyte pK_a is not recommended because of potential peak distortion. Methods developed near the analyte pK_a may not be rugged and will not be easily transferable to other laboratories (manufacturing facility/contract laboratory). Any minor variation in the mobile phase pH in this case will lead to the significant variations in the

analyte retention and to the selectivity of the method.

$$pK_a = \text{pH} + \log\left(\frac{[AH]}{[A^-]}\right) \tag{8}$$

Neutral and ionic forms of any analyte have significant differences in their apparent hydrophobicity and thus tend to migrate though the column with different velocity. These retention profiles can be described by the following equation as a function of eluent pH and analyte pK_a[32]

$$k = \frac{k_0 + k_1 \frac{[H^+]}{K_{a(B^+)}}}{1 + \frac{[H^+]}{K_{a(B^+)}}} \tag{9}$$

or

$$k = \frac{k_0 + k_1 10^{(pK_a - \text{pH})}}{1 + 10^{(pK_a - \text{pH})}} \tag{10}$$

where for bases k_1 is the limiting retention factor of the protonated form and k_0 is the limiting retention factor of the neutral form. However, for acids k_0 is the limiting retention factor of the anionic form, and k_1 is the limiting retention factor of the neutral form. For both acids and bases, k is the retention factor at a given pH and K_a is the analyte ionization constant.

Use of Liophilic Mobile Phase Additives

A large majority of pharmaceutical compounds contain basic functional groups. Therefore, reversed-phase HPLC separation of organic bases with different pK_a values is of particular importance in the pharmaceutical industry. It is generally recommended that the chromatographic analysis of basic compounds to be carried at 2 pH units greater or less than the analyte pK_a. Analysis at mobile phase pHs greater the analyte pK_a could be prohibitive since the bonded phase may not be stable at these pH values. An alternative approach is to analyze the compounds in their protonated state. However, under these conditions the elution of protonated basic compounds may be close to the void volume. The addition of mobile phase additives such as liophilic ions has been found to be an effective approach to enhance the retention of protonated basic compounds.[33–38] The advantages of employing liophilic mobile phase additives at a pH where the basic analyte is in its fully protonated form, provides the chromatographer an additional approach to adjust basic analyte retention and chromatographic selectivity without the need of changing

type of column, pH or organic modifier. The retention behavior of basic compounds containing primary, secondary, tertiary and quaternary amines can be adjusted as a function of the concentration of liophilic anion in the mobile phase (ClO_4^-, PF_6^-, BF_4^-, $CF_3CO_2^-$) at low pH. The use of different inorganic counteranions at equimolar concentrations can lead to a concomitant increase in retention, as well as peak symmetry and increased loading capacity. The use of liophilic mobile phase additives provides an attractive approach for the separation of basic pharmaceutical compounds.[39]

EXPERIMENTAL

Apparatus

The chromatographic systems used in the study were:

1. Waters 2695D HPLC system equipped with a 2487 detector and
2. Waters Acquity UPLC™ system equipped with a diode array detector, 80 Hz.

The data sampling rate for the Waters 2695D was 1 point/sec, and was 10 pts/sec for the UPLC runs. Chromatograms were processed using Empower®. Mobile phase pH was measured using a Fischer Scientific Accumet pH meter 15 (Denver Instrument, USA). The pH meter was calibrated with buffer solutions of pH 1.00, pH 2.00 and pH 4.00 or pH 4.00, pH 7.00 and pH 10.00.

Columns

Columns used in this paper include Phenomenex Luna C8(2):3 μm, 150 × 4.6 mm, Waters Acquity BEH C18: 1.7 μm, 2.1 mm × 50 mm, 100 mm, Waters Acquity BEH RP18: 1.7 μm, 2.1 mm × 100 mm, 150 mm, Waters Xbridge C18: 3.5 μm, 150 × 3.0 mm, and YMC ODS-AQ: 3 μm, 3.0 × 150 mm.

Chemicals

Acetonitrile (HPLC grade, 99.93%+), was obtained from Sigma-Aldrich, (St. Louis, MO, USA). Ortho-phosphoric acid (85%), TFA (99.0%+) and Perchloric acid (70%) were obtained from Fluka (GMBH CH-9471 Buchs). Sodium phosphate, dibasic, anhydrous,

(99.5%) was obtained from J. T. Baker (Phillipsburg, NJ, USA). Sodium dihydrogenphosphate, Diammonium monohydrogenphosphate (99.999%) and Potassium hexafluorophosphate, (99.9%+) were obtained from Aldrich (Milwaukee, WI, USA).

$NaClO_4$ (HPLC grade), HCl (37.1%, 12.1N) and NH_4OH (22% as NH_4) were obtained from (Fisher Scientific, Pittsburg, PA).

Lysine (98%+), dilysine (99%+), trilysine (99%+), and tetralysine (99%+) were purchased from Sigma (USA). Diprotoic basic compound (BB), diprotic acidic compound (AA), zwitterionic compound (*E*) and multiply charged basic compounds (*N*, *Q* and *K*) were obtained from Novartis.

Sample Preparation

The concentrations of the dibasic (BB), diacidic (AA) and zwitterionic (*E*) compounds used in the pH screening studies were 0.5 mg/ml and each analyte was dissolved in 90% water : 10% acetonitrile mixture. The concentration of Compound *N* was 1 mg/mL and the diluent used in the last dilution was 80% water : 20% acetonitrile. The final concentration of Compound *Q* was ~0.3 mg/ml and the diluent used was 50 mM NH_4HCO_3 + 0.1% Tween-20 (w/v). The final concentration of Compound K was 1 mg/ml and the diluent used in the last dilution was 50% water : 50%potassium phosphate buffer, pH 7. For Compound *K*, *Q* and *N* solutions, a filter absorption study was done to ensure no adsorption on the filter and to ensure that no leachable components were extracted from the filter housing. The concentration of the lysine and lysine analogs was 0.5 mg/ml and they were dissolved in water. Fresh solutions were prepared prior to each experiment and were analyzed with a cooled autosampler at ~4°C, protected from light.

Mobile Phase Preparations

For the pH screening studies a 10 mM dipottasium hydrogenphosphate buffer (pH 9.3) was prepared. The pH was adjusted to lower values with phosphoric acid. The pH of the aqueous portion of the mobile phase was measured for all studies. All aqueous mobile phases were filtered using a Nylon-66 (Hexamethylenediamine) 0.45 μm membrane filter (Whatman®, Springfield Mill, UK).

For the HPLC to UHPLC method transfer experiments (using Compound *K*) the mobile phase preparation for the HPLC experiments entailed adding 1 ml TFA to 1 L of the aqueous portion of the mobile phase and to 1 L of the organic portion of the mobile phase, and for

the UHPLC experiments, 0.5 ml TFA was added to 1 L of the aqueous portion of the mobile phase and to 1 L of the organic portion of the mobile phase.

For the lysine experiments, to study the effect of hexafluorophosphate counteranion concentration, a 0.5 v/v% phosphoric acid buffer (pH 1.8) was initially prepared. KPF_6 was used to adjust PF_6^- concentration in 0.5 v/v % H_3PO_4 (pH = 1.8) from 10 mM to 35 mM. All aqueous mobile phases were filtered using a Nylon-66 (Hexamethylenediamine) 0.45 μm membrane filter (Whatman®, Springfield Mill, UK).

For the experiments in which different acidic modifiers/salts were used to optimize the separation for Compound *N* various mobile phases were prepared: 1) 0.5 mL of phosphoric acid was added to 900 mL of Mili-Q water +100 ml acetonitrile, 0.05 v/v% phosphoric acid, 2) 0.5 mL of TFA was added to 900 mL of Mili-Q water + 100 mL acetonitrile, 0.05 v/v% TFA, and 3) 0.5 mL of perchloric acid was added to 900 mL of MiliQ water + 100 ml acetonitrile, 0.05 v/v% $HClO_4$ (6.8 mM ClO_4^-). In order to study the effect of perchlorate counteranion concentration, sodium perchlorate was used to adjust the perchlorate concentration in 0.05 v/v% $HClO_4$ (90% Aqueous : 10% acetonitrile), 15 mM and 25 mM were added in subsequent experiments. In order to study the effect of hexafluorophosphate counteranion concentration, KPF_6 was used to adjust hexafluorophosphate concentration in 0.05 v/v% $HClO_4$ (90% Aqueous : 10% acetonitrile) from 5 mM to 15 mM. All aqueous mobile phases were filtered using a Nylon-66 (Hexamethylenediamine) 0.45 μm membrane filter (Whatman®, Springfield Mill, UK). The organic portion of the mobile phase for the phosphoric acid experiments contained 0.05% phosphoric acid, and for the perchlorate and the hexafluorophosphate experiments contained 0.05 v/v% perchloric acid.

RESULTS AND DISCUSSION

HPLC to UHPLC Method Transfer

The implementation of UHPLC was evaluated for the analysis of *Compound Q* drug product formulations. Increased speed of analysis and significant gains in resolution per unit time were obtained compared to original separation carried out on conventional HPLC system. The HPLC method originally carried out on XBridge C18 column (150 × 3 mm, 3.5 μm) (Figure 1a) was directly scaled and run on a BEH C18 column (100 × 2.1 mm, 1.7 μm), Figure 1(b).

The overall run time was reduced from 50 min to 34 min. The elution pattern remained the same in both methods, and an increase in

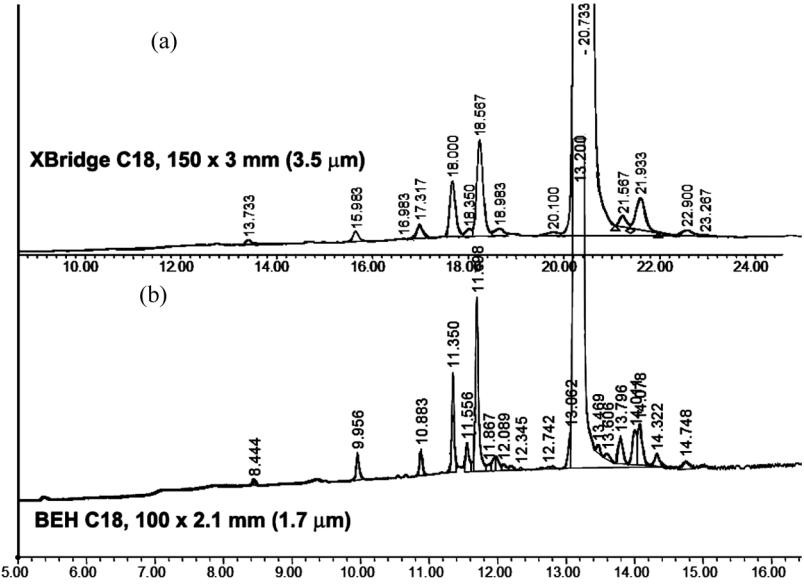


Figure 1. Comparison of separation of *compound Q* on HPLC and UHPLC. (a) Instrument = Alliance 2695D with 2487 detector., Column = Xbridge C18, 3.5 µm, 3.0 × 150 mm, Mobile Phase: a: 15 mM $(NH_4)_2HPO_4$, pH 6.0, b: ACN, Inj. Vol.: 10 µl, Column Temp. = 40°C. Flow: 0.43 ml/min. Initial backpressure ~1,600 psi. Gradient: Initial 20%b to 55%b over 15 minutes, then over 14 minutes to 60%b, then to 95%b over 6 minutes, hold at 95%b for 5 minutes, then re-equilibrate under initial conditions for 10 minutes. Total run time: 50 minutes. (b) Instrument = Acquity with PDA detector., Column = Acquity BEH C18, 1.7 µm, 2.1 × 100 mm, Sample, Inj. Vol.: 2.0 µL full loop, Flow rate: 0.21 ml/min, Initial Back Pressure ~4,200 psi. Gradient: Isocratic hold for 0.46 min at 20%b, then increase to 55%b over the next 10.00 min, then to 60%b over the next 9.33 minutes, then to 95%b over the next 4.0 minutes, then hold at 95% for 3.33 minutes, then re-equilibrate at initial conditions for 6.6 minutes. Mobile phase and column temp same as in (a). Total run time 34 minutes.

resolution of some critical pairs was observed on the UHPLC method. For example in Figure 1(a) (HPLC) the *Rs* between peaks eluting at 21.567 and 21.93 minutes is 1.09 and for the same critical pair in Figure 1(b) (UHPLC) the *Rs* between same components (peaks eluting at 13.796 and 14.011 minutes) it is 1.32. Also, upon transfer to the UHPLC method, one peak (deemed to be single compound in HPLC, 21.933 min retention) was separated in two in UHPLC (Figure 1b) with retention 14.011 and 14.078 minutes and the resolution between these peaks was 0.37. The increase in resolution of these two sets of critical pairs could be partially attributed to the increase in efficiency due to reduction in

particle size of the packing material resulting in a more favorable L/dp ratio. Since N is proportional to L/dp, the UHPLC column should provide about 1/3 more theoretical plates than the HPLC column. This should correspond to an increase of about 0.2 in the resolution of the critical pairs (as per the master resolution equation). The additional increase in resolution of the critical pairs could be attributed to an increase in the selectivity of the separation due to slight differences in the packing material and/or due to changes in the conformational structure of the analyte(s) exhibited when operating at elevated pressures using UHPLC.[40]

The separation run time was further reduced, optimizing the flow rates on the UHPLC system (0.2–0.5 ml/min, refer to Figure 2). The chromatographic conditions were scaled using the equations in the introduction section (method transfer considerations). The overall speed of the separation from the HPLC method (50 min) was decreased to 14 min on the UHPLC (0.5 ml/min) and the separation selectivity and resolution of critical pairs was maintained (refer to Figure 2).

The HPLC method for *Compound K* formulation was used as another example in HPLC-to-UHPLC method transfer. The original separation carried out on a YMC ODS AQ column (polar embedded column) resulted in a total run time of 45 minutes, Figure 3(a). The method was transferred to a UHPLC system (see introduction and method transfer sections for scaling considerations), using a polar

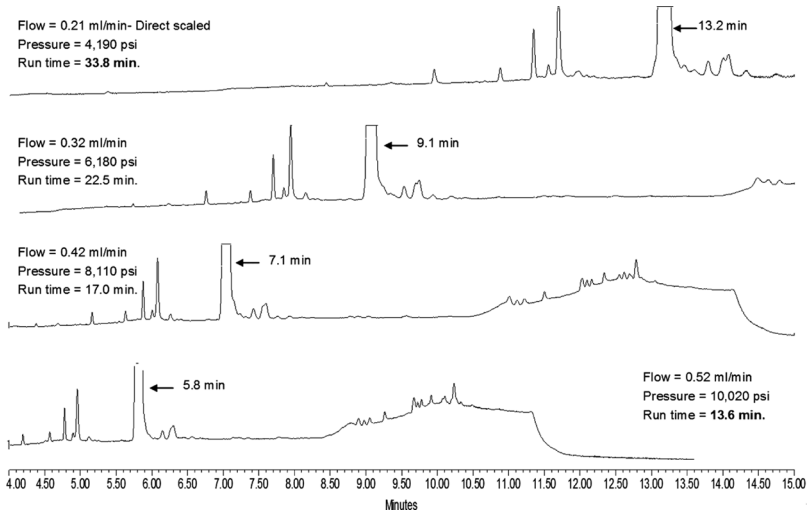


Figure 2. Effect of flow rate on UHPLC separation of *Compound Q* (conditions as in Figure 1, except with variation of flow rate).

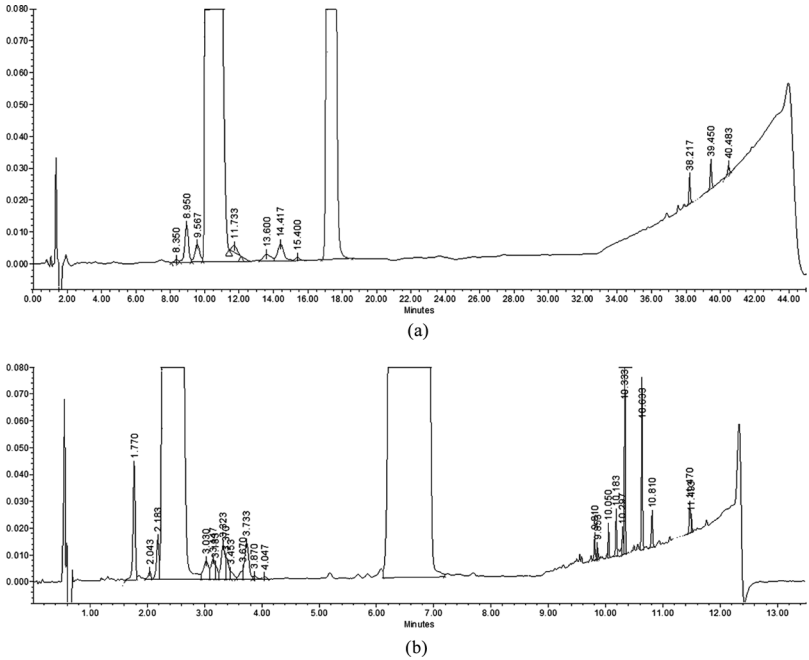


Figure 3. Method transfer from HPLC to UHPLC method for *Compound K*. (a) Instrument = Alliance 2695 with 2487 detector., Column = YMC ODS AQ 3 μm, 120 Å, 3.0 × 150 mm Compound *K* Conc.: ~1 mg/ml, Sample Solvent = ACN/pH 7 Buffer, 50:50, Mobile Phase A) 0.1% TFA in H_2O, Mobile phase B) 0.1% TFA in ACN, Inj. Vol.: 15.4 μl, Column Temp. = 50°C., Flow: 0.5 ml/min, Initial backpressure ~1,500 psi, Gradient: Isocratic hold for 2 min at 34% B, then over 28 min to 39% B, then over 10 minutes to 80% B and hold for 1 minute and then equilibrate at initial conditions for 4 minutes. (b) Instrument = Acquity with PDA detector., Column = Acquity BEH RP18 Shield, 1.7 μm, 2.1 × 100 mm Compound K Conc.: ~1 mg/ml, Sample Solvent = ACN/pH 7 Buffer, 50:50, Mobile Phase: 0.05% TFA in H_2O/ACN, Inj. Vol.: 5.0 μL full loop, Column Temp. = 55°C, Flow rate: 0.4 ml/min, Initial Back Pressure ~5,700 psi. Gradient: Isocratic hold for 0.8 min at 30% B, then over 7.4 min to 35% B, then over 3.1 minutes to 76% B and hold for 0.3 minutes and then equilibrate at initial conditions for 1.2 minutes.

embedded type column, BEH RP18, resulting in a separation with a total run time of 13 min, Figure 3(b). Although the column chemistries are different, the main goal was to develop a faster separation in which the resolution of the components in the mixture was the same or better. Both methods were used to analyze 20 initial excipient compatibility samples in parallel, and the overall summary of the analysis time, solvent consumption, and injection precision are shown in Table 1. The methods

Table 1. HPLC versus UHPLC methods comparison

System	Column	Void volume	Flow rate	# Inj. per run	Run time per inj.	Total run time	Solvent consumed	Inj. precision (peak area, $n = 9$)
Alliance HPLC	3.0 × 150 mm YMC ODS AQ 3 μm, 120 Å	0.7 mL	0.5 mL/min	44	45.0 min	33 hrs	990 mL	0.61 %RSD
Acquity UHPLC	2.1 × 100 mm BEH SHIELD RP18, 1.7 μm	0.22 mL	0.4 mL/min	44	13.5 min	9.9 hrs	238 mL	0.16 %RSD

were shown to be equivalent and all samples exhibited <1% difference in assay values (average = 0.9%) between both methods. The injection precision for 9 standards run throughout the sequence was less than 1% for both methods. The total number of injections in the sample set was 44, which included blank, system suitability samples, standards, and the 20 excipient compatibility samples. The duration of entire work was 9.9 hours on the UHPLC and 33 hours on the HPLC. There was a 3-fold decrease in the overall analysis time for this sample set using the UHPLC. In terms of productivity, the sample set could be set up to run overnight and the data processing could be performed the following day, if a UHPLC system was employed. During stability testing, the number of samples submitted for testing increase as a function of the number of storage conditions in the study. Subsequent stability time points for 20 samples stored under two different storage conditions would require the analysis of 40 samples. By implementation of the UHPLC method the results for these 40 samples could be generated in less than 24 hours, whereas using the HPLC method it would take approximately 3 days to obtain the results. Another added benefit is that less mobile phase is consumed in the UHPLC runs, so mobile phase preparation and subsequent waste generated is minimized.

Another benefit of using UHPLC is the expeditious testing of samples that might have limited solution stability, further warranting a fast analysis method. Also, if there is a problem with the system (such as instrument shutdown), the same sample set would need to be run the following day. Using the UHPLC method, the overall time for running the 1st sample set and the entire repeat of the sample set if needed for an investigation (due to instrument failure) could be completed much faster when compared to the time that it would take to complete only the 1st sample set analysis on a HPLC system. Solvent consumption can also be significantly reduced by using 1–2.1 mm i.d. columns compared to conventional 3–4.6 mm i.d. columns. For example, if a thousand injections were to be run on both methods in this example, the total volume of solvent used would be 22.5 L for HPLC vs. 5.4 L for UHPLC. A 4-fold reduction in solvent would be realized with a corresponding reduction in solvent disposal cost.

Role of UHPLC in Methods Development (pH Screening)

In this section, the use of the UHPLC for pH scouting experiments was demonstrated as an effective tool for rapid and systematic method development. Most pharmaceutical compounds contain ionizable functionalities such as amino, pyridinal or carboxylic groups. Mobile phase pH and composition are among the main parameters used to

control HPLC retention of most pharmaceutical compounds and to optimize separations. The introduction of new sub-2-μm packings that are stable over a wider pH range up to pH 12 (can be used at pHs > 8, for a large number of column volumes if volatile buffers are used) allows for a broader applicability of mobile phase pH as a retention/selectivity adjustment parameter. As stated in the introduction, the pH of the mobile phase has a strong influence on the retention of protolytic solutes and should be controlled in reversed-phase HPLC. An additional factor that affects the overall pH of the mobile phase is the type and concentration of the organic modifier. Further, the organic modifier can also affect the ionization constants of all ionogenic species dissolved in the mobile phase.

In the following example a pH screening study for a dibasic compound BB (with pK_a values (determined by ACD software[41]) of 3.3 for the pyridinal *N* and 5.3 for the morpholine *N*) was conducted on a HPLC (Figure 4) and UHPLC (Figure 5) systems, respectively.

It is clear that the retention dependence is a function of pH (Figures 4 and 5) and that analysis of the compound in the neutral state would provide for a robust method and that further method optimization experiments were warranted using aqueous mobile phase pHs > 5.

The total time to complete this pH screening experiment using a conventional HPLC system equipped with a 150 × 4.6 mm column was 9 hours (Figure 4). At each pH value, three injections of the analyte were performed to ensure proper equilibration of the system. Once the retention of the second and third injections were equivalent the system was deemed equilibrated. However, on a UHPLC system this entire experiment was accomplished in less than two hours. This resulted in

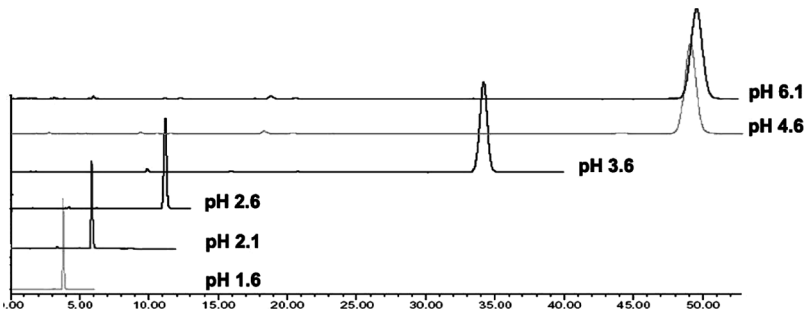


Figure 4. HPLC retention of a diprotic basic compound (BB) as a function of the aqueous: Column: Phenomenex Luna 3 μm C8(2), [150 × 4.6 mm, 3 μm]. MP: 10 mM K_2HPO_4:ACN (71:29, v/v) where pH of the aqueous phase is adj. w/H_3PO_4 Flow rate: 1.0 mL/min. Injection Vol.: 10 μL. Wavelength: 247 nm, Column Temp.: 40°C.

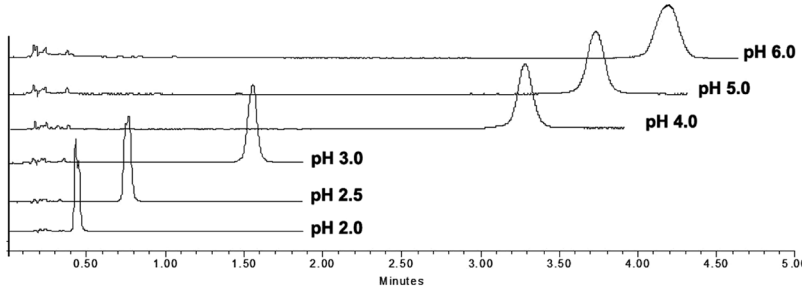


Figure 5. UHPLC retention of a diprotic basic compound (BB) as a function of the aqueous mobile phase pH. Column: Acquity BEH C18 1.7 μm, 2.1 × 50 mm, Flow rate 0.8 ml/min, Temp. 35°C, Inj. 2 μL full loop. Run time 3–5 min. Detection 215 nm. Strong wash: 0.1% NH_4OH 50/50 ACN/H_20. Weak wash: 90/10 H_2O/ACN, Mobile phase A: 15 mM K_2HPO_4 adjusted with HCl (2–6). Mobile phase B: ACN, Starting Pressure: ~9000 psi, Isocratic 30% ACN. Note: pHs indicated in figure indicate the pH of the aqueous phase.

approximately 7 hours of time savings for selection of the optimal pH to perform further method development experiments and to determine the pK_a value of the compound of interest.

If 50 methods per year are developed in a particular department, this corresponds to a time savings of approximately 350 hours. (50 × 7 hours = 350 hours) . This would result in a total time savings of approximately 46 days per year (350 hours/7.5 working hours per day).

Several other pH screening examples are also represented in Figures 6 and 7. The retention as a function of pH is plotted in Figure 6 for an acidic compound, and for a zwitterionic compound at three different organic compositions in Figure 7. For the acidic compound it is prudent to conduct further method optimization experiments at mobile phase pHs where the molecule is in its neutral state (aqueous pH values < 4) at either 30 or 40% organic concentrations. If experiments are conducted at mobile phase pHs where the molecule is ionized, this would result in early elution near the void volume, even at the lowest studied organic composition.

For the zwitterionic compound, there is one region (aqueous mobile phase pH > 6) of interest which the chromatographer could focus on when performing further method optimization experiments. In this pH region, however the chromatographer would be limited to working at the lowest studied organic concentration (30%) to avoid early elution near the void volume.

In conclusion, the pH screening experiments for each compound using three organic compositions took less than 3 hours to perform using the UHPLC system.

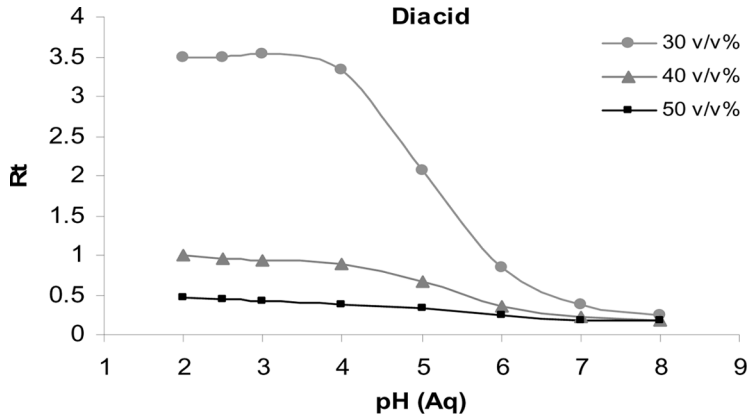


Figure 6. UHPLC retention of an acidic compound (AA) as a function of the aqueous mobile phase pH. Isocratic run 30% ACN – 5 min, 40% ACN – 1.5 min, 50% ACN – 1 min, Flow: 0.8 ml/min, Column: Acquity BEH C18, 2.1 × 50 mm 1.7 μm, Inj. 3 μl, PDA (220 nm), Mobile phase: A: 15 mM K_2HPO_4 adjusted with HCl to target pH (2–8), B: Acetonitrile (10–50%). Sample diluent: 90% H_2O/10% ACN. Total run time for 30% ACN for all exp: ~60 min, for 40% ACN for all exp: ~45 min, for 50% ACN for all exp: ~30 min.

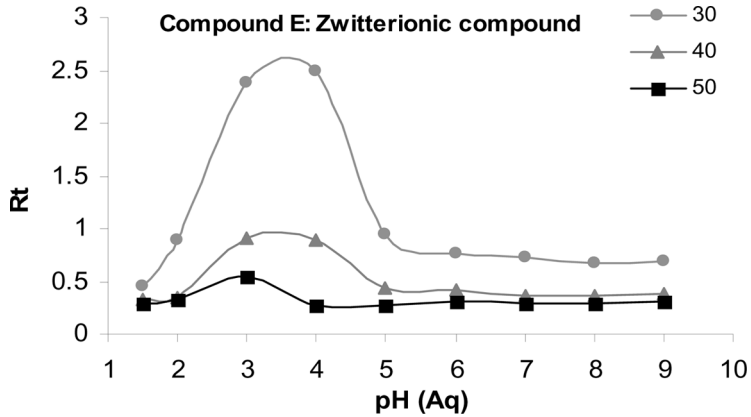


Figure 7. UHPLC retention of a zwitterionic compound (E) as a function of the aqueous mobile phase pH. Isocratic run 30% ACN – 5 min, 40% ACN – 1.5 min, 50% ACN – 1 min, Flow: 0.5 ml/min, Column: Acquity BEH C18, 2.1 × 50 mm 1.7 μm, Inj. 3 μl, PDA (220 nm), Mobile phase: A: 15 mM K_2HPO_4 adjusted with HCl to target pH (2–8), B: Acetonitrile (10–50%), Sample diluent: 90% H_2O/10% ACN. Total run time for 30% ACN for all exp: ~70 min, for 40% ACN for all exp: ~40 min, for 50% ACN for all exp: ~30 min.

Role of UHPLC in pK_a Determination

The dependencies of analyte retention as a function of pH could be used for estimation of the potentiotemetric pK_a ($^{w}{}_{w}pK_a$, the pK_a in water) of the target molecule or an unknown impurity/degradation product. This is a very efficient approach for simultaneously evaluating the pK_a values of the target compound, as well as any associated synthetic byproducts and potential degradation products that are ionizable within the pH region studied. This is performed by plotting the retention factor (under isocratic conditions) of the desired component versus the true mobile phase $^{s}_{s}$pH (taking into account the pH shift of the aqueous mobile phase upon addition of organic). The $^{s}{}_{s}pK_a$ of the analyte (pK_a of the analyte in the hydroorganic solvent in which it was analyzed) can be determined from these experiments using nonlinear regression analysis software.

The $^{w}{}_{w}pK_a$ of the analyte can be calculated when the $^{s}{}_{s}pK_a$ of the analyte and the type and concentration of organic modifier used are known. For acetonitrile/water systems the $^{w}{}_{w}pK_a$ can be calculated by using the following empirical formula for basic and acidic compounds:

$$^{w}{}_{w}pK_a = {}^{s}{}_{s}pK_a + (x\%\text{Organic}) * B \quad \text{(Basic Compounds)} \qquad (11)$$

$$^{w}{}_{w}pK_a = {}^{s}{}_{s}pK_a - (x\%\text{Organic}) * A \quad \text{(Acidic Compounds)} \qquad (12)$$

where $B = 0.02$ (corresponds to basic analyte pK_a shift per 1 v/v% ACN) and $A = 0.03$ (corresponds to acidic analyte pK_a shift per 1 v/v% ACN).[42]

Enhancement of Retention/Selectivity/Resolution of Multiply Charged Basic Compounds with the Use of Liophilic Additives in UHPLC

Fast HPLC methods for the separation of complex mixtures may require the use of columns packed with sub-2-μm particles using high mobile phase velocities on UHPLC systems. The separation of multiply charged basic pharmaceutical compounds may be a challenging task due to their early elution, poor peak shapes, and limited loading capacity. Occasionally ion-pairing reagents are used for HPLC separation of basic compounds and peptides to enhance retention and reduce peak tailing. Recently inorganic anions (liophilic mobile phase additives) have been used for selective variation of the retention of ionic analytes, improvement of loading capacity and peak symmetry.[38,39] These liophilic ions, are usually small inorganic ions and are characterized by significant delocalization of their charge, absence of surfactant properties, and that they are primarily symmetrical, usually spherical in shape and are absent in surfactant properties. They possess an important

ability for electrostatic interactions with the analyte in the mobile phase and in the adsorbed organic layer on top of the bonded phase. It has been found that the retention as well as the peak shape of protonated basic analytes is dependent upon the type and concentration of inorganic salt added to the mobile phase.[39]

Employment of liophilic mobile phase additives using gradient elution at elevated pressures (>9000 psi) for enhancement of multiply-charged basic compound retention and peak symmetry was investigated. The effect of type and concentration of different inorganic mobile phase additives (i.e., KPF_6, $NaClO_4$, TFA) on the chromatographic figures of merit (resolution of critical pairs, retention and tailing factor of API) were studied.

UHPLC was used for the analysis of lysine (amino acid) and multiply-charged hydrophilic peptides to study the effect and utility of liophilic inorganic mobile phase additives. Mono, di, tri, and tetralysine have two, three, four and five positively charged residues respectively. Mono-, di-, tri-; and tetralysine were analyzed with and without the addition of liophilic salts in the mobile phase. Liophilic mobile phase additives were used to enhance the retention, selectivity and resolution of multiply charged hydrophilic species.

Lysine and multiply-charged peptides (Figure 8) are very polar molecules and typically show early elution on traditional C18 reversed phases. Also, lysine has a weak chromophore and UV detection below 215 nm is necessary. Detection of lysine may be challenging when TFA is employed since TFA has a UV cut off of 210 nm. Therefore experiments were performed with hexafluorophosphate, which usually has the greatest impact on the analyte retention of protonated basic molecules and which does not absorb in this UV region. Mobile phases

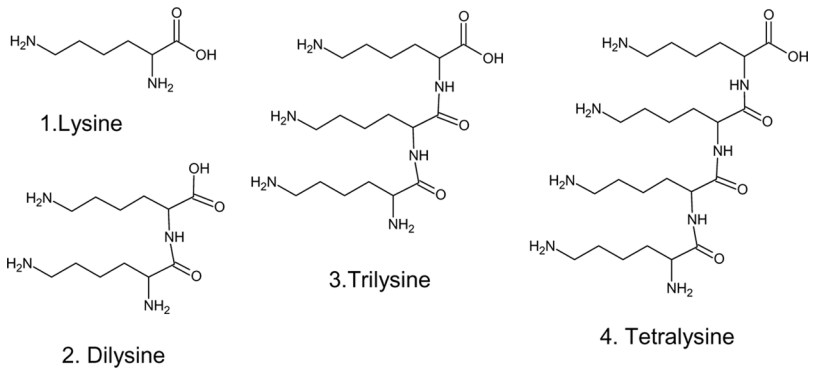


Figure 8. Structures of lysine and multiply charged peptides.

were prepared by keeping the pH constant at 1.8 using 0.5 v/v% H_3PO_4 and the concentration of PF_6 was increased by the addition of KPF_6.

With a mobile phase containing solely dihydrogen phosphate up to 20 mM (pH 1.8), co-elution of lysine and multiply-charged peptide species was observed (Figure 9). In subsequent experiments, the pH was kept constant at pH 1.8 with phosphoric acid, and the concentration of hexafluorophosphate was increased from 0–35 mM. With the increase of the hexafluorophosphate concentration, significant increase in the retention of all the multiply-charged species was observed. (Figures 9 and 10). Moreover, at 35 mM concentration of PF_6, all the multiply-charged species were resolved from the nonbasic impurity eluting at

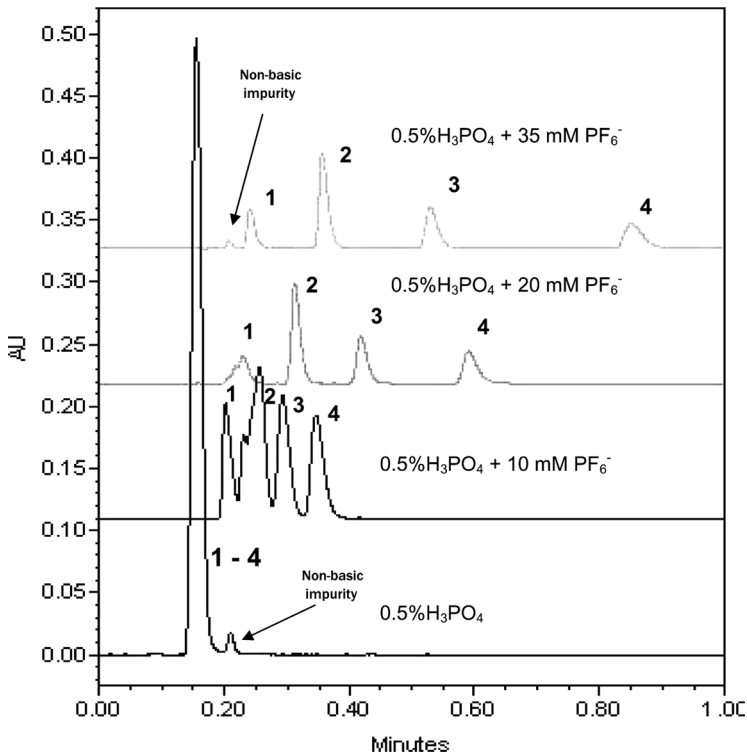


Figure 9. Enhancement of separation selectivity with addition of liophilic additive (KPF_6) Column: Acquity BEH C18 1.7 μm, 2.1 × 50 mm, Flow rate 0.7 ml/min, Temp. 40°C, Inj. 1 μL Conc. 0.5 mg/ml. Run time 1 min. Detection 210 nm. Strong wash: 50/50 ACN/ H_2O. Weak: 90/10 H_2O/ACN Mobile phase A: 0.5% H_3PO_4 pH 1.8 + 0, 10, 20, 35 mM KPF_6. Mobile phase B: ACN. Starting Pressure: ~6800 psi. Isocratic: 85% A. 15% B. 1-lysine, 2-di-lysine, 3-tri-lysine, 4-tetra-lysine.

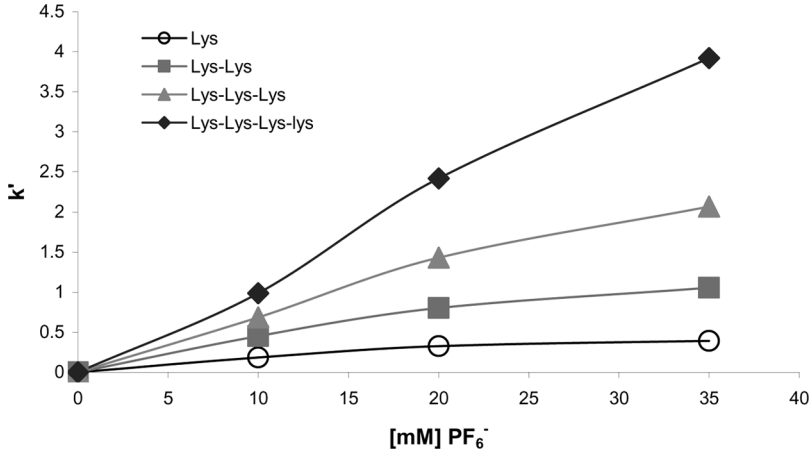


Figure 10. Effect of concentration of hexafluorophosphate on retention factor of lysine analogs.

the solvent front and from each other. The variation of type and concentration of liophilic additives for very polar compounds can be used as an effective approach to enhance the retention and selectivity of the separation.

The effect of the addition of three different acidic modifiers, TFA, perchloric acid and phosphoric acid on a 2.1×100 mm column was explored for the analysis of *compound N* formulation (Figure 11). *Compound N* is a multiply-charged basic compound and is fully charged at pHs below 4. The use of TFA or perchloric acid modifiers led to increased retention, decreased tailing factor and better resolution of the critical pairs in the separation (Table 2). TFA due to its absorbance in the low UV region can lead to reduced sensitivity of low level impurities in the drug product formulation. Perchloric acid is UV transparent in the low UV region (>205 nm), therefore the concentration of the perchlorate was further optimized (Figure 12). The pH was kept constant with the addition of 0.05 v/v% perchloric acid, which is equivalent to 6.8 mM perchlorate The concentration of the perchlorate anion was further increased by the addition of 15 mM and 25 mM sodium perchlorate. The gradient was modified with the 25 mM sodium perchlorate mobile phase (Figure 12c) in order to keep the gradient slope the same as in Figures 12(a) and (b) in the critical separation region. Upon increase in the perchlorate concentration the separation was significantly enhanced. An increase in resolution of the critical pairs (AP1, Imp. 1, Imp. 2, and Imp. 3) was obtained with subsequent increases in retention and significant reduction in the tailing factor of the API (Table 3). Moreover,

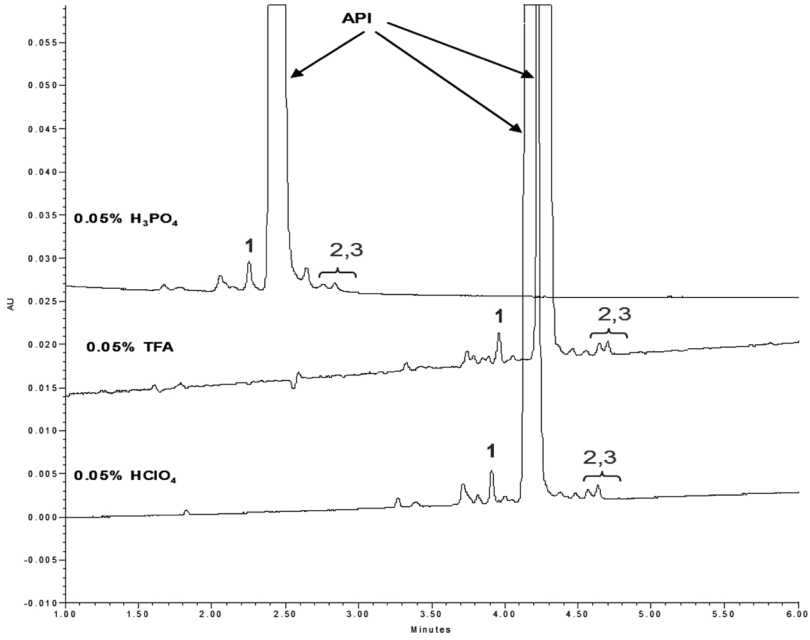


Figure 11. Effect of type of acidic modifier on separation of Compound *N*. Column: BEH RP18 Shield 1.7 μm, 2.1 × 100 mm, S/N of column 01051516410K (12 and 16), Flow rate 0.6 ml/min, Temp. 50°C, Inj. 2 μL full loop, Strong wash: 0.1% NH_4OH 50/50 ACN/H_2O, Weak wash: 90/10 H_2O/ACN, Initial back Pressure: ~7000 psi. Mobile Phases: Top: Mobile phase A: 90% H_2O/10% ACN + 0.05 v/v% H_3PO_4, Mobile phase B: ACN + 0.05 v/v% H_3PO_4, Middle: Mobile phase A: 90% H_2O/10% ACN + 0.05 v/v%TFA, Mobile phase B: ACN + 0.05 v/v% TFA, Bottom: Mobile phase A: 90% H_2O/10% ACN + 0.05 v/v% $HClO_4$, Mobile phase B: ACN + 0.05 v/v% $HClO_4$, Gradient: Isocratic hold for 0.5 min at 20% B, then over 5.5 min to 37% B, then over 1 minute to 80% B and equilibrate at initial conditions for 0.9 minutes, Total Run time – 8 min.

at increased concentrations of the perchlorate anion a new impurity (marked with #) was resolved from the API. This impurity eluted prior to the API with the addition of 15 mM and 25 mM sodium perchlorate to the aqueous portion of the mobile phase. The addition of another liophilic mobile phase additive, hexafluorophosphate, was also used to study the effect of different modifiers on the separation (Figure 13, Table 4). With the addition of 15 mM hexafluorophosphate, the impurity (marked with #) was also resolved from the API. At all prior conditions with 0.05% TFA, 0.05% phosphoric acid, and 0.05% perchloric acid this impurity (marked with #) was unresolved

Table 2. Influence of different acidic modifiers: Chromatographic figures of merit

	0.05 v/v%		
	H_3PO_4 (4.6 mM)	TFA (6.4 mM)	$HClO_4$ (6.8 mM)
Rs (1, API)	1.9	4.5	3.6
Rs (2, 3)	1.1	1.3	1.3
Rt API	2.4	4.3	4.2
Rt Imp. 1	2.3	4.0	3.9
Rt Imp. 2	2.6	4.6	4.57
Rt Imp. 3	2.8	4.7	4.64
T_f (API)	2.5	1.6	2.1

Rs = Resolution, *Rt* = Retention time (min.), T_f = Tailing factor at 5% peak height.

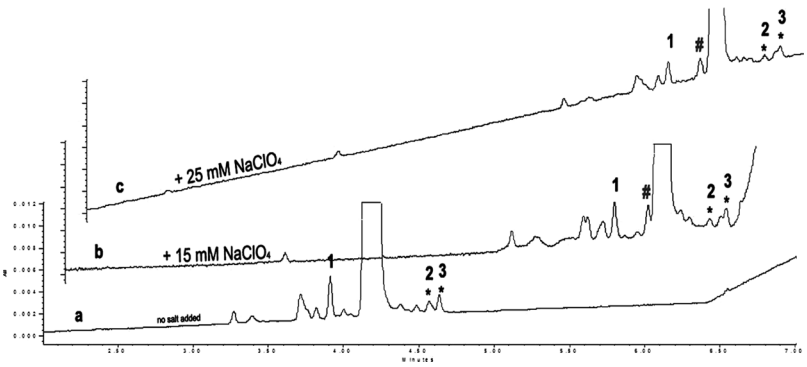


Figure 12. Effect of perchlorate concentration on the separation of Compound *N*. Column: Acquity BEH RP18 Shield 1.7 μm, 2.1 × 100 mm, S/N of column 01051516410K, Flow rate 0.6 ml/min, Temp. 50°C, Inj. 2 μL full loop, Run time – 8 min, Strong wash: 0.1% NH_4OH 50/50 ACN/H_2O, Weak wash: 90/10 H_2O/ACN, Mobile phase A: 90% H_2O/10% ACN + 0.05 v/v% $HClO_4$ + 0, 15, 25 mM $NaClO_4$ Mobile phase B: ACN + 0.05 v/v% $HClO_4$. Initial back Pressure: ~7000 psi, Gradient a/b: Isocratic hold for 0.5 min at 20% B, then over 6 min to 37% B, then over 1 minute to 80% B and equilibrate at initial conditions for 0.9 minutes. Gradient c: Isocratic hold for 0.5 min at 20% B, then over 8.1 min to 45% B, then over 1 minute to 80% B and equilibrate at initial conditions for 0.9 minutes.

Table 3. Influence of perchlorate modifier concentration: chromatographic figures of merit

	$HClO_4$	$HClO_4$ + 15 mM $NaClO_4$	$HClO_4$ + 25 mM $NaClO_4$
Rs (1, API)	3.60	5.88	6.46
Rs (2, 3)	1.33	1.57	1.50
RtAPI	4.15	5.97	6.21
Rt Imp. 1	3.91	5.66	5.89
Rt Imp. 2	4.57	6.29	6.53
Rt Imp. 3	4.64	6.41	6.64
T_f (API)	2.15	1.22	1.23

Rs = Resolution, *Rt* = Retention time (min.), T_f = Tailing factor at 5% peak height.

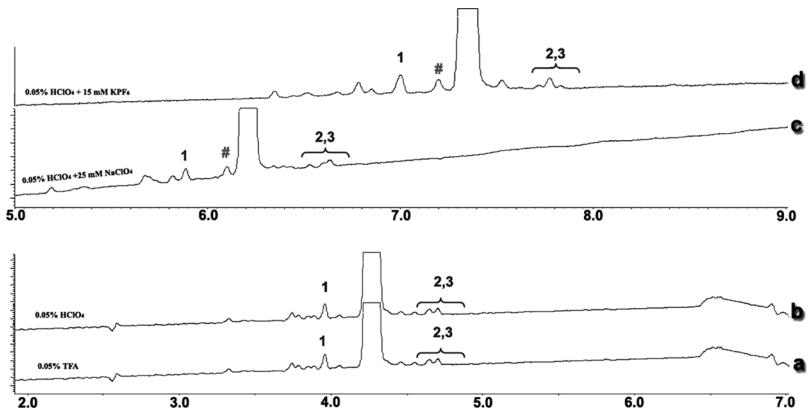


Figure 13. Effect of different type of modifiers/salts on separation of compound *N*. Column: Acquity BEH RP18 Shield 1.7 μm, 2.1 × 100 mm, S/N of column 01051516410K, Flow rate 0.6 ml/min, Temp. 50°C, Inj. 2 μL full loop, Run time – 8 min, Strong wash: 0.1% NH_4OH 50/50 ACN/H_2O, Weak wash: 90/10 H_2O/ACN. Mobile phases a) Mobile phase A: 90% H_2O/10% ACN + 0.05 v/v% $HClO_4$ + 15 mMKPF_6: Mobile phase B: ACN + 0.05 v/v% $HClO_4$; b) Mobile phase A: 90% H_2O/10% ACN + 0.05 v/v% $HClO_4$ + 25 mM $NaClO_4$: Mobile phase B: ACN + 0.05 v/v% $HClO_4$; c) Mobile phase A: 90% H_2O/10% ACN + 0.05v/v% $HClO_4$: Mobile phase B: ACN + 0.05 v/v% $HClO_4$; d) Mobile phase A: 90% H_2O/10% ACN + 0.05 v/v%TFA: Mobile phase B: ACN + 0.05 v/v% TFA. Initial back Pressure: ~7000 psi, Gradient a/b: Isocratic hold for 0.5 min at 20% B, then over 6 min to 37% B, then over 1 minute to 80% B and equilibrate at initial conditions for 0.9 minutes. Gradient c/d: Isocratic hold for 0.5 min at 20% B, then over 8.1 min to 45% B, then over 1 minute to 80% B and equilibrate at initial conditions for 0.9 minutes.

Table 4. Influence of type of liophilic mobile phase additive: chromatographic figures of merit

	TFA	$HClO_4$	$HClO_4$ + 25 mM $NaClO_4$	$HClO_4$ + 15 mM KPF_6
Rs (1, API)	4.5	3.6	6.5	5.2
Rs (2, 3)	1.3	1.3	1.5	?
Rt API	4.3	4.2	6.2	7.4
Rt Imp. 1	4.0	3.9	5.9	7.1
Rt Imp. 2	4.65	4.57	6.5	7.7
Rt Imp. 3	4.71	4.64	6.6	7.8
T_f (API)	1.6	2.1	1.2	1.2

Rs =Resolution, *Rt* = Retention time (min.), T_f = Tailing factor at 5% peak height.

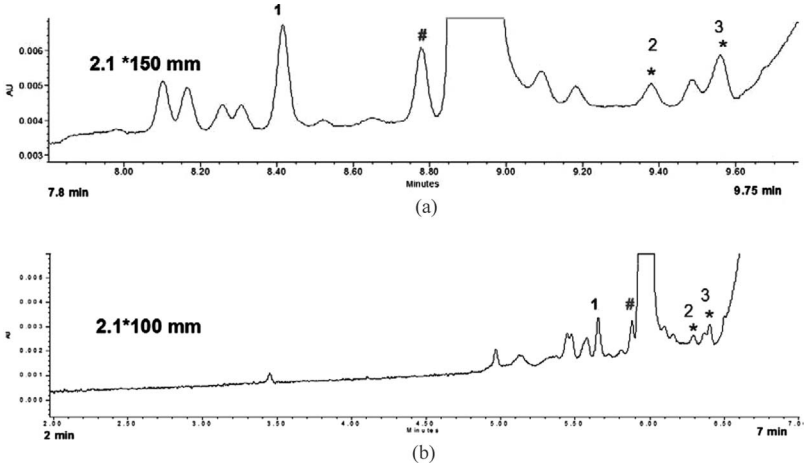


Figure 14. Effect of column length on Resolution: Perchlorate anion (UHPLC). Column: Acquity BEH RP18 Shield 1.7 μm, 2.1 × 100 mm, S/N of column 01051516410K or Acquity BEH RP18 Shield 1.7 μm, 2.1 × 150 mm, S/N: 01065520710B04, Flow rate 0.6 ml/min, Temp. 50°C, Inj. 2 μL full loop, Run time – 8 min, Strong wash: 0.1% NH_4OH 50/50 ACN/H_2O, Weak wash: 90/10 H_2O/ACN, Mobile phase A: 90% H_2O/10% ACN + 0.05 v/v% $HClO_4$ + 15 mM $NaClO_4$, Mobile phase B: ACN + 0.05 v/v% $HClO_4$, Initial back Pressure: ~7000 psi, Gradient a (2.1 × 150 mm): Isocratic hold for 0.75 min at 20% B, then over 8.25 min to 37% B, then over 1.5 minute to 80% B and equilibrate at initial conditions for 1.6 minutes. Gradient b (2.1 × 100 mm): Isocratic hold for 0.5 min at 20% B, then over 5.5 min to 37% B, then over 1 minute to 80% B and equilibrate at initial conditions for 0.9 minutes.

Table 5. Influence of column length with TFA mobile phase: chromatographic figures of merit

	0.05 v/v%	
	TFA (2.1 × 100 mm)	TFA (2.1 × 150 mm)
Rs (1, API)	4.5	6.0
Rs (2, 3)	1.3	1.6
Rt API	4.2	6.6
Rt Imp. 1	4.0	6.2
Rt Imp. 2	4.6	7.2
Rt Imp. 3	4.7	7.3
T_f (API)	1.6	2.3

Rs = Resolution, *Rt* = Retention time (min.), T_f = Tailing factor at 5% peak height.

from the API. Therefore the addition of perchlorate (>22 mM) and hexafluorophosphate (15 mM) induced positive changes in the observed selectivity.

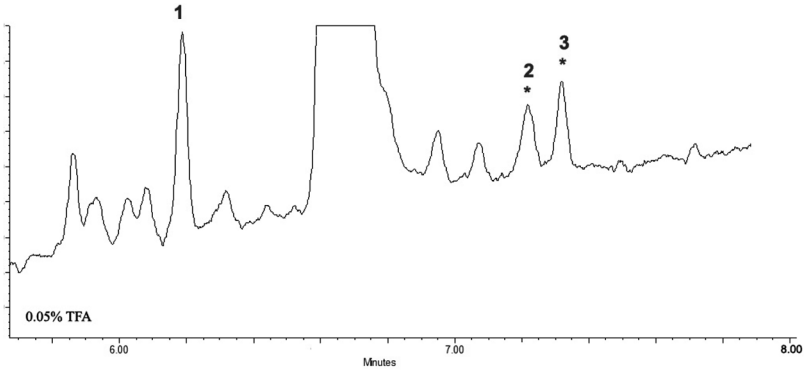
The best separation obtained thus far was with 15 mM sodium perchlorate + 0.05% perchloric acid (total perchlorate concentration: ~22 mM) in which most critical pairs and other impurities within the formulation were better resolved. In order to further improve the resolution of the critical pairs, a longer column (15 cm) was used and the gradient was scaled accordingly using both the 15 mM sodium perchlorate + 0.05 v/v% perchloric acid mobile phase (Figure 14, Table 6) and the 0.05 v/v% TFA mobile phase (Table 5). The initial backpressure was ~13,000 psi but this was not a limitation using the

Table 6. Influence of column length with perchlorate mobile phase: chromatographic figures of merit

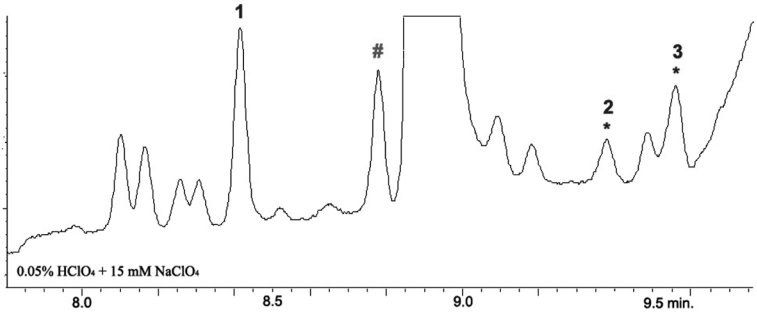
	0.05 v/v% $HClO_4$ + 15 mM $NaClO_4$ (2.1 × 100 mm)	0.05 v/v% $HClO_4$ + 15 mM $NaClO_4$ (2.1 × 150 mm)
Rs (1, API)	5.9	6.5
Rs (2, 3)	1.6	2.8
Rt API	6.0	8.9
Rt Imp. 1	5.7	8.4
Rt Imp. 2	6.3	9.4
Rt Imp. 3	6.4	9.6
T_f (API)	1.2	1.9

Rs = Resolution, *Rt* = Retention time (min.), T_f = Tailing factor at 5% peak height.

UHPLC system, since pressures up to 15000 psi are acceptable. As expected, there was an increase in resolution of the critical pairs (API, Imp 1, Imp 2 and Imp 3) when a longer column was employed. However, with the TFA in the mobile phase even though a longer column was used the impurity that coeluted with the API was still not resolved, further indicating that the addition of the liophilic mobile phase additive (i.e., perchlorate anion) induced a change in selectivity.



(a)



(b)

Figure 15. Enhancement of separation selectivity by use of perchlorate mobile phase additive. Column: Acquity BEH RP18 Shield 1.7 μm, 2.1 × 150 mm, S/N: 01065520710B04, Flow rate 0.6 ml/min, Temp. 50°C, Inj. 2 μL full loop, Mobile phases: a) Mobile phase A: 90% H_2O/10% ACN + 0.05% TFA, Mobile phase B: ACN + 0.05% TFA, Starting Pressure: ~13,000 psi; b) Mobile phase A: 90% H_2O/10% ACN + 0.05% $HClO_4$ + 15 mM $NaClO_4$, Mobile phase B: ACN + 0.05% $HClO_4$, Starting Pressure: ~13,000 psi. Gradient time table for both a and b:. Isocratic hold for 0.75 min at 20% B, then over 8.25 min to 37% B, then over 1.5 minute to 80% B and equilibrate at initial conditions for 1.6 minutes.

Table 7. Optimized separation: chromatographic figures of merit

	0.05 v/v% TFA	0.05v/v% $HClO_4$ + 15 mM $NaClO_4$
Rs (1, API)	6.0	6.5
Rs (2, 3)	1.6	2.8
Rt API	6.6	8.9
Rt Imp. 1	6.2	8.4
Rt Imp. 2	7.2	9.4
Rt Imp. 3	7.3	9.6
T_f (API)	2.3	1.9

Rs = Resolution, *Rt* = Retention time (min.), T_f = Tailing factor at 5% peak height.

Figure 15 shows the direct comparison of the separation with 15 mM sodium perchlorate + 0.05 v/v% perchloric acid versus 0.05 v/v% TFA on a 150 × 2.1 mm column. It is evident that the separation with perchlorate gave excellent resolution of the critical pairs, and led to the resolution of a new impurity (#) from the API (Table 7) compared to TFA mobile phase. The addition of liophilic reagents has been shown in the literature to reduce secondary interactions with the stationary phase and increase mass transfer[39] for basic compounds. In this particular example, the addition of perchlorate anion at concentrations of greater than/equal to 22 mM led to a decrease in the tailing factor and also led to increased resolution of impurities eluting on the tail of the API peak. The use of liophilic mobile phase additives is an effective approach for method development of multiply charged basic compound separations.

CONCLUSIONS

The use of commercially available ultra fast HPLC systems has been shown to be beneficial in pharmaceutical development. Faster analysis can be achieved with ultra high pressure HPLC systems compared to conventional HPLC, without sacrificing loss in resolution of critical pairs when L/dp is kept constant and when the same type of bonded phase is used. Ultra high pressure HPLC systems can lead to expeditious generation of results and faster decision making. Also, the use of liophilic mobile phase additives coupled with the speed of ultra high pressure HPLC systems can be used for selectivity enhancement of multiply-charged basic compound separations in the pharmaceutical industry.

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