Correlation of Analyte Retention in Organic and Inorganic Mobile Phases to aid Liquid Chromatography Method Development

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Many liquid chromatography methods are developed using organic mobile phase additives which allow compatibility with mass-spectrometric (MS) detection. However, these types of additives often give high UV absorbance which can lead to low level impurity quantitation issues. Additionally, these additives often have little or no buffering capacity at the pH they are typically used, which in turn can lead to variability in analyte retention time. A rational approach for the selection of phosphate buffers from organic based mobile phase additives of the same pH (acidic or neutral) in liquid chromatography stability indicating method (SIM) development may provide a solution to this problem. Excellent correlation was observed for analyte retention (33 test analytes) in switching from an organic based mobile phase additive to an appropriate potassium phosphate buffer at low and mid pH. This approach provides a basis for developing SIM methods under mass-spectrometer friendly conditions and converting them directly to phosphate methods (or *vice-versa*) which typically provide higher UV sensitivity and retention robustness while maintaining the elution order and chromatographic resolution observed with the organic mobile phase additives.

Keywords: UHPLC, phosphate buffers, retention correlation, ion-pairing

Introduction

In the pharmaceutical industry, early clinical phase analytical method development requires a balance between spending sufficient time and effort in developing a fit-for-purpose method against the very real possibility that the compound may be halted before the next project milestone is reached (e.g. due to toxicology or compound absorbance issues). Accordingly, the starting point for chromatographic method development typically involves screening relevant samples through a set of generic methods and choosing the conditions offering best retention, analyte resolution and peak shape [1-4]. The method providing best global resolution is generally selected and further optimisation undertaken. This process is often supplemented using other key information (both measured and predicted *in-silico e.g.* physical-chemical parameters such as pK_a and/or Log D) and chromatographic predictive software such as Drylab™ (Molnar Instiut, Berlin, Germany) [5,6], ChromSword™ (Software Entwicklung, Muehltal, Germany) [7] or LC Simulator™ (ACDLabs, Toronto, Canada) [8]. This approach often greatly reduces both the amount of resource and time required to develop a suitable method.

The generic screening systems may be based on HPLC or UHPLC instrumentation, but are typically hyphenated to both UV and mass spectrometric (MS) detectors. The use of MS detection, particularly in early-phase pharmaceutical development, is essential for early characterisation of new and unknown impurities, while UV detection (often diode-array detection (DAD), also known as photo diode array (PDA)) is typically used for quantitation of impurities by area normalisation. The use of MS detection requires LC mobile phases which are both volatile and promote ionisation of the sample

Effective pH range or commonly used pH	Additive	pK_a
1.9	0.1% trifluoroacetic acid (TFA)	<1.0
2.8 – 4.8	formic acid	3.8 ($HCOO^-$)
3.8 – 5.8	acetic acid	4.8 (CH_3COO^-)
6.8 – 11.3	ammonium bicarbonate	7.8 ($H_2CO_3^{2-}$) 9.2 (NH_3^+) 10.3 (HCO_3^-)
3.8 – 5.8 8.2 – 10.2	ammonium (acetate)	4.8 (CH_3COO^-) 9.2 (NH_3^+)
2.8 – 4.8 8.2 – 10.2	ammonium (formate)	3.8 ($HCOO^-$) 9.2 (NH_3^+)
9.7 – 11.7	Triethylamine (TEA) (acetate)	4.8 (CH_3COO^-) 10.7 (TEA^+)
10.6	0.1% ammonium hydroxide (NH_4OH)	9.2 (NH_3^+)
10.3 - 12.3	pyrrolidine	11.3 [9,10]

Table 1. Some common mobile phase additives employed in LC/MS work. Note – higher pH mobile phases require suitably stable columns such as Agilent Zorbax Extend, Phenomenex Gemini or Waters BEH/Acquity phases.

analytes. One benefit of these mobile phases is they are typically quick and easy to prepare (for example they are not often pH adjusted and are used 'as is'). However, as these additives are organic molecules (in comparison to inorganic buffers such as phosphate or borate), they often have appreciable background absorbance and (in our experience) often contain impurities which can negatively impact chromatographic gradient profile, peak identification and limits of detection. Additionally, these additives are used to simply adjust the pH of the mobile phase and typically offer little buffering capacity. For example, 10 mM ammonium acetate has an aqueous pH of 6.8 which has no buffering capacity. If these mobile phases are used for project progression activities such as SIMs, this may lead to issues with irregular and non-reproducible analyte retention times, poor peak-shapes and ultimately poor method robustness. A list of some organic additives typically used in LC/MS analyses are listed in Table 1.

During method development if lower limits of detection or increased robustness are required, then inorganic additives should be used. Most often, the additive chosen is a phosphate based salt. Phosphate has a wide buffering capacity (pK_a values of = 2.15, 7.20 and 12.30) and excellent UV opacity down to wavelengths as low as 200 nm [11]. This often makes phosphate the buffer of choice for method development, particularly in late-stage pharmaceutical development where method transferability is very important and many, if not all, process impurities and degradents are known [e.g. 12,13]. The downside of utilising phosphate buffers are obviously incompatibility with MS detection, they are more aggressive on silica based columns [14] and also have lower solubility in organic solvents meaning that gradients to high organic fractions are not possible (typically <80% (v/v) solvent depending on organic solvent and salt type, salt concentration and pH).

The primary focus of this investigation was to identify if retention correlations existed between organic buffers and their potassium phosphate alternatives at the same pH, solvent and column types. Analysis at the same pH was important in this work as pH is the dominant contributor to analyte retention for ionisable compounds under reversed-phase conditions [15] and could lead to significant deviations in retention and selectivity if not held constant. Correlations were assessed by comparing retention times of the analytes under both sets of conditions and assessing any changes in chromatographic selectivity.

Method	Column	Mobile Phase ('A')	Organic Solvent ('B')	Gradient
1	Waters Acquity Shield RP18	0.1% formic acid (pH 2.6)	acetonitrile	0-1.7 min 5% B 1.7-8.7 min linear to 95% B 8.7-10.4 min 95% B
2	Waters Acquity C18	10 mM ammonium acetate (pH 6.8)	methanol	0-1.7 min 5% B 1.7-8.7 min linear to 95% B 8.7-10.4 min 95% B
3	Waters Acquity Phenyl	0.1% ammonium hydroxide (pH 10.6)	methanol	0-1.7 min 5% B 1.7-8.7 min linear to 95% B 8.7-10.4 min 95% B

Table 2. Organic buffers, solvents and columns investigated in this study.

If correlations were identified, then this approach could be used as part of a method development strategy whereby a method developed with MS compatible mobile phases for impurity identification could be transferred directly to an appropriate phosphate method for increased UV sensitivity and method robustness. This could be incorporated into a Quality-by-Design (QbD [16]) framework for SIMs or used alone e.g. for transferring phosphate based methods to MS-friendly buffers for new impurity identification.

Additionally, high pH buffers were also investigated for SIM development. A comparison of analyte retention in alternative buffers to a commonly utilised generic method employing ammonium hydroxide was examined. Baseline characteristics (such as prevalence of system peaks and effect on peak sensitivity) of the alternative buffers were noted.

Experimental

Samples & Reagents

33 compounds were utilised in this study which were families of proprietary Pfizer molecules including the active pharmaceutical ingredients (API), synthetic precursors and process related impurities (PRIs) covering a range of acid, basic, neutral and zwitterionic character. These were typically combined in groups of related compounds at 0.1 mg/mL each in methanol - water (50:50, v/v).

Acetonitrile (Sigma-Aldrich Chromasolv) and methanol (Sigma-Aldrich Chromasolv) were used in this study. Formic acid (HCOOH – Fluka Analytical *puriss* LC-MS grade ampoules) and ammonium acetate (NH_4OOCCH_3 – Fluka Analytical *puriss* HPLC grade) were investigated in this study. Additionally, alternatives to ammonium hydroxide (NH_4OH – Fluka Analytical >25% in water ampoules LC-MS grade) for high pH separations were investigated in an effort to identify alternative mobile phase additives offering higher UV sensitivity and reduced baseline artefacts.

Dihydrogen potassium phosphate and dipotassium hydrogen phosphate (both anhydrous) were purchased from Fluka Analytical (HPLC grade). Phosphoric acid (Fluka Analytical puriss – 85% in water, HPLC grade) was used to pH adjust the mobile phases to the identical pH of the organic buffers they were being compared with.

Purified water was obtained from a Millipore MilliQ Gradient A10 system producing water of 18.2 MΩ.cm and < 3 ppb total organic carbon quality.

The following organic additives and buffers were utilised for the high pH investigation; ammonium formate, acetate and bicarbonate, pyrrolidine, 1-methylpiperidine, triethylamine acetate and the Goods buffers (BioXtra grade) - CAPS, CAPSO and glycine which were all obtained from either Fluka Analytical (*puriss* LC-MS grade) or Sigma (SigmaUltra grade) except pyrrolidine (Alfa-Aeser). These additives were compared to a mobile phase of 0.1% ammonium hydroxide (unadjusted pH = 10.6). The additives for comparison were prepared at 10 mM (solids) or 0.1% (liquids) concentration and adjusted to a pH of 10.6 with ammonium hydroxide (Fisher) or glacial acetic acid (Sigma Aldrich) as appropriate.

Instruments

UPLC experiments were performed on a Waters Acquity UPLC (with PDA detection). The system was controlled through Waters' Empower 2 software. Both organic mobile phase additive and phosphate experiments were performed on the same system with the same column, thus mitigating any effects from different system dwell volumes or column differences and aging in the correlations. This

allows direct comparison of retention times in this work (rather than a more formal use of retention factors, *k*, to be used).

Methods

The generic methods listed in Table 2 were used in this investigation. Columns were 100 x 2.1 mm i.d. (1.7 µm) and utilised a flow rate of 0.4 mL/min. Columns were thermostatted at 30°C. 2 µL of test mixes (typically 0.1 mg/mL of each analyte) were injected, as well as individual stock solutions for peak tracking. While PDA detection was used, data was reprocessed and compared at 210 nm as a 'worst-case' scenario for observing system peaks and other baseline artefacts.

Potassium phosphate buffers have lower solubility in organic solvent-water mixtures than organic buffers. 10 mM dipotassium hydrogen phosphate (K_2HPO_4 – pH 6.8) was found to be compatible to a maximum methanol volume of 80% (v/v), while 10 mM potassium dihydrogen phosphate (KH_2PO_4 – pH 2.6) was soluble up to 85% (v/v) acetonitrile (both at room temperature). The same gradient was mimicked in both sets of experiments i.e. the same gradient slope was used in the phosphate experiments as the organic buffer experiments. However, when the maximum organic solvent level was reached in the phosphate gradients, the mobile phase was held isocratically for the remainder of the analysis.

Results and Discussion

UV spectra

A comparison of the UV spectra for potassium dihydrogen phosphate and formic acid (pH 2.6), and dipotassium hydrogen phosphate and ammonium acetate (pH 6.8) are shown in Figure 1. Quite clearly the spectra for equivalent pH phosphate buffer exhibits much lower absorbance than the respective organic buffers. Significant absorbance is observed for formic acid from 240 nm and below, while ammonium acetate shows high levels of absorbance from 225 nm and below. The obvious result of this is significantly better signal to noise and baseline characteristics when using phosphate allowing lower levels of analyte quantitation. For example, up to 25-30% increase in analyte response in the dipotassium phosphate buffer was observed compared to the ammonium acetate mobile phase. This exemplifies one of the reasons why phosphate is often the preferred buffer for chromatographic method development. Comparisons of separations using the different buffers are shown in Figures 2 and 3.

Retention

The correlation coefficients for the test analytes retention and applying a linear relationship between the organic and

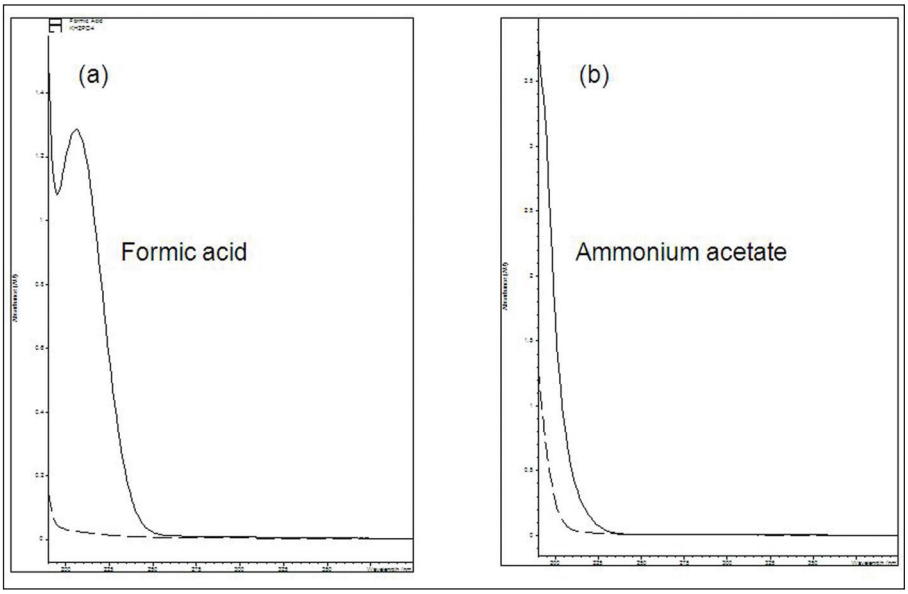

Figure 1. A comparison of UV spectra for (a) 10 mM potassium dihydrogen phosphate and 0.1% formic acid (pH 2.6), and (b) 10 mM dipotassium hydrogen phosphate and 10 mM ammonium acetate (pH 6.8). In both cases, the phosphate spectra (dashed line) exhibits much lower absorbance from wavelengths above 225 – 250 nm.

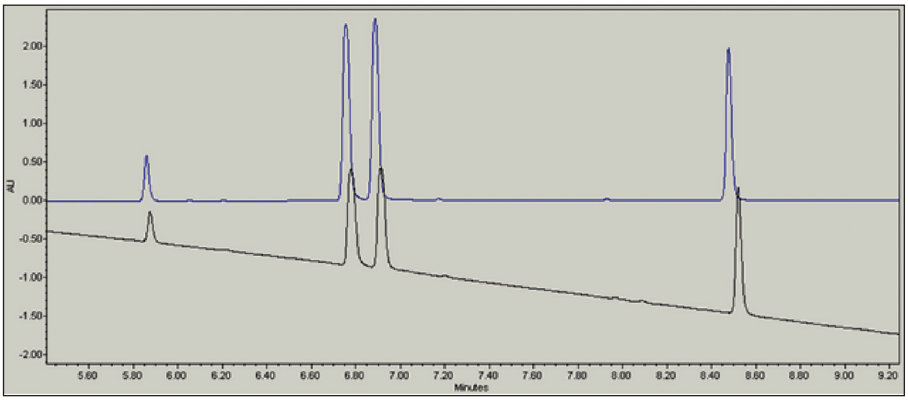

Figure 2. Overlay of one set of test analytes using method 1 with 0.1% formic acid (pH 2.6 – black line) and 10 mM potassium phosphate (pH 2.6 – blue line). The sloping nature of the formic acid baseline can make quantitation difficult and the increase in analyte response with the phosphate buffer is obvious. The signals were both collected at 210 nm and the same sample injected with both mobile phases.

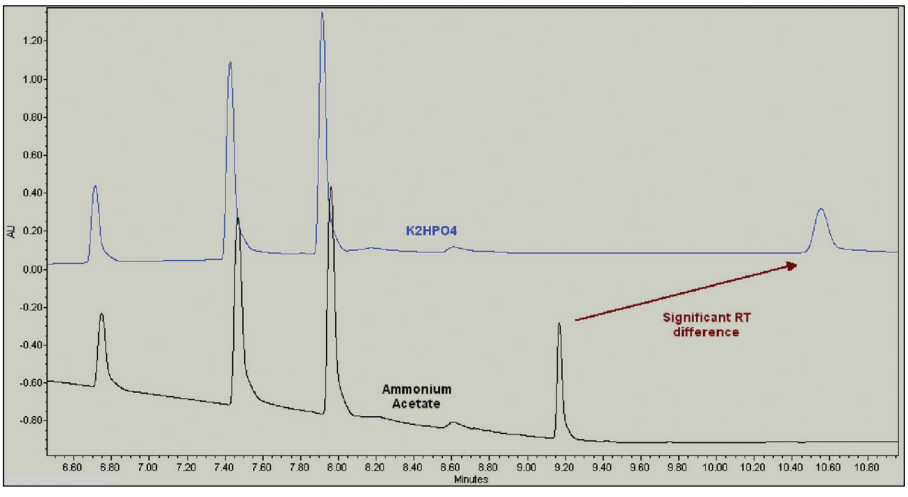

Figure 3. Overlay of one set of test analytes using method 2 with 10 mM ammonium acetate (pH 6.8 – black line) and 10 mM potassium phosphate (pH 6.8 – blue line). This is an extreme case where retention (for one analyte) is significantly different between the two methods (corresponding to extreme top-right data point in Figure 5).

phosphate buffers is shown in Table 3. Correlation coefficients for the low and mid-pH data demonstrate excellent correlation (see Figures 4 and 5 for the low and mid-pH plots respectively). However, the absolute retention times between the two methods can vary. In the largest variation observed (1.18 and 1.20 min under method 1 and 2 conditions respectively), this equates to approximately an 11% deviation in retention over the length of the whole analysis.

The lower pH formic acid buffer demonstrates a larger maximum retention range variation than the pH 6.8 systems. This is most often observed with early eluting polar or charged analytes. This is due to significant ion-pairing of basic analytes with the phosphate counter-ion leading to longer retention. For acidic and neutral analytes (18 out of 33 compounds) at pH 2.6 where no ion-pairing with phosphate would be anticipated to occur, near perfect correlation of retention was observed (r^2 = 0.9997) supporting this conclusion.

In the low pH mobile phase comparison, only two analytes (from all 33 analytes analysed) were found to change elution order when switching from formic acid to potassium dihydrogen phosphate. At pH 6.8, no changes in elution order were observed and the observed retention correlation is superior to that at low pH. As may be expected, ion pairing effects for the basic analytes were much less pronounced at this higher pH, possibly due to less ionisation of the bases under these conditions. Again, this reinforces the premise that retention order and peak selectivity is largely maintained when switching mobile phases.

Peak shape

A qualitative assessment of peak shape was undertaken as part of this study. Generally peak shapes were consistent when switching between organic and phosphate buffers. On occasion where different peak shapes were noted, the organic additives were generally found to give worse peak shapes than their phosphate equivalent. At low pH, this might be slightly surprising as the potassium diphosphate buffer has lower ionic strength than the formic acid mobile phase (Table 4) and higher ionic strength mobile phases is one factor that generally provides better peak shapes [17,18]. As noted above, a likely factor for the improved peak shape with the phosphate buffer is phosphate ion-pairing with the basic analytes. This prevents secondary interactions with silanol groups on the stationary phase and leads to improvements in peak shape.

At pH 6.8, peak shape for the dipotassium

Methods	Organic additive	Phosphate buffer	Correlation coefficient (r^2)	Maximum retention time variation/min
1	0.1% Formic acid (pH 2.6)	10 mM KH_2PO_4 (pH 2.6)	0.9492	-1.09 to +1.18
2	10 mM Ammonium acetate (pH 6.8)	10 mM K_2HPO_4 (pH 6.8)	0.9826	-1.20 to +0.08
3	10 mM Ammonium hydroxide (pH 10.6)	N/A (see text)	0.8290 (0.9837 – see text)	N/A (see text)

Table 3. Correlation coefficients for test analyte retention of MS compatible and phosphate methods.

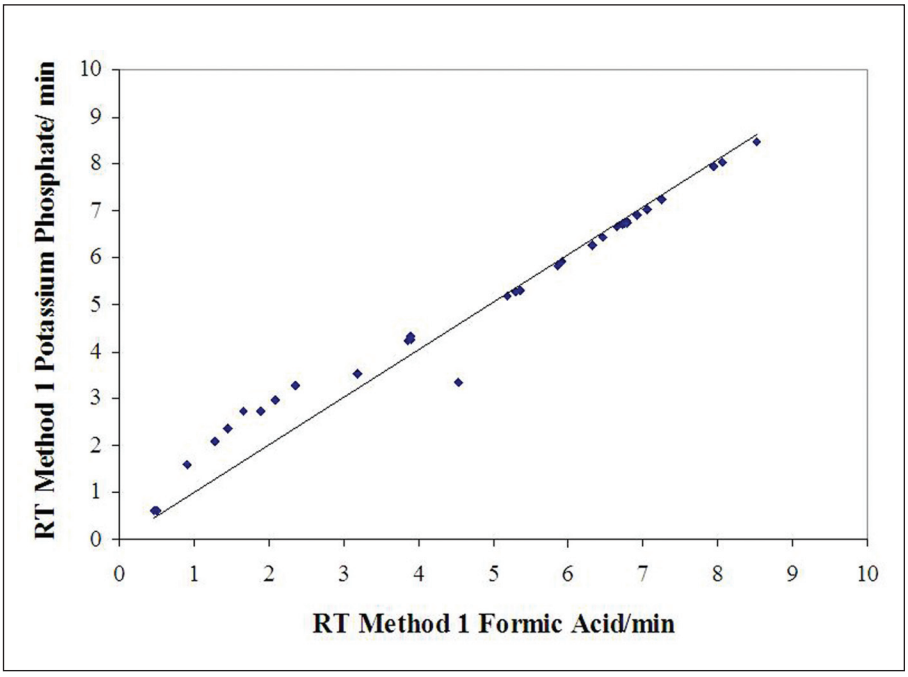

Figure 4. Comparison of analyte retention times for method 1 with 0.1% formic acid (pH 2.6) and 10 mM potassium phosphate (pH 2.6). Note the higher retention with phosphate buffer for early eluting analytes which is believed to be due to ion-pairing effects. Full method details are provided in the Experimental section. The correlation coefficient was 0.9492.

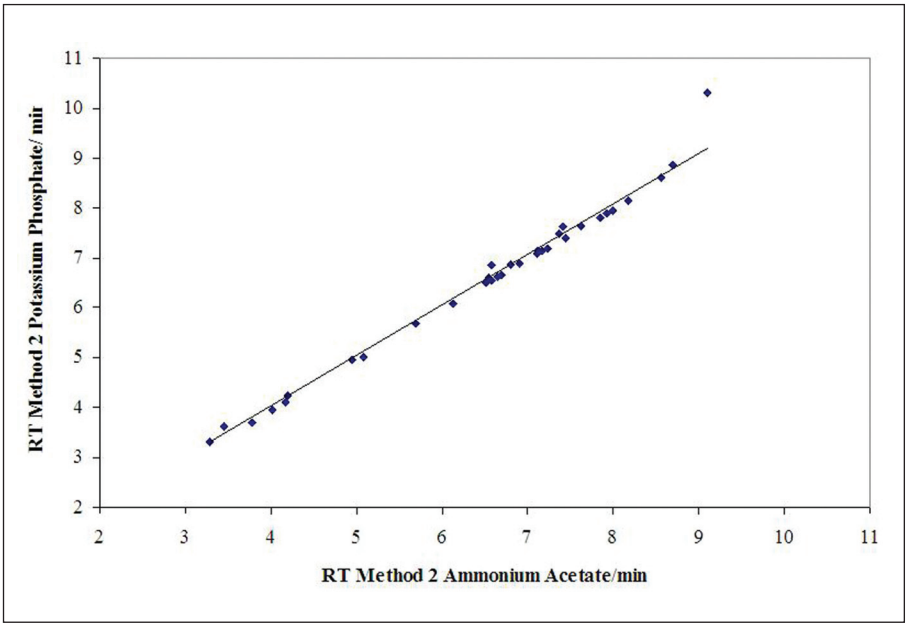

Figure 5. Comparison of analyte retention times for method 2 with 10 mM ammonium acetate (pH 6.8) and 10 mM potassium phosphate (pH 6.8). Full method details are provided in the Experimental section. The correlation coefficient was 0.9826.

phosphate buffer was nearly always better than the ammonium acetate (up to a 20% decrease in peak tailing was typically observed). As reported in Table 3, the analyte retention correlation at this pH is very good and indicates a smaller phosphate ion-pairing effect than at lower pH. The peak shape in this case can be rationalised through the significantly higher ionic strength of the mobile phase compared to the ammonium acetate mobile phase. In this instance it is believed that residual analyte-silanol interactions are decreased by mobile phase cation (K^+) competition for these sites.

Baseline noise & artefacts

In this investigation, it was found that shifting to phosphate buffers not only increased analyte signal-to-noise, but also led to a decrease in baseline artefacts when comparing organic and phosphate buffer mobile phases at both low and mid-pH. An example of the improved baseline with phosphate compared to ammonium acetate at 210 nm is shown in Figure 6. It should be reiterated that phosphate buffer solubility is significantly lower in organic solvents than alternative organic additives. If the maximum organic solvent levels in the gradient are breached, this can lead to the phosphate salt 'crashing-out' in the instrument or column which in turn may lead to increased baseline noise and instrument back-pressure issues.

Additives at high pH

The use of phosphate buffers at high pH is deleterious to silica based column stability which at high buffering pH's (>11.3) leads to rapid silica dissolution and collapse of the phase [14,19]. For this reason, the majority of mobile phases used in high pH chromatography utilise organic based buffers which are less aggressive on the column. A 'buffer' commonly used for high pH work is ammonium hydroxide as it is both easy to prepare and MS compatible. However, in our experience ammonium hydroxide tends to degrade in aqueous solution quickly and this can be observed as baseline artefacts in the chromatogram. Additionally, at 0.1% (v/v) concentration, ammonium hydroxide offers very limited buffering capacity which can lead to separation reproducibility issues (the pH of 0.1% ammonium hydroxide is around 10.6. The pK_a of the ammonium ion is 9.2).

One other point of note is that at high pH, on-column degradation of analyte molecules may be observed. This is usually confirmed by comparing a high pH separation with analysis of the analyte at low pH. In the higher pH separation, additional peaks may be observed which are not present in the low pH separation (e.g. identified by mass tracking using MS detection) and care must be taken not to misidentify these high pH impurities as originating from the sample under analysis.

A number of high pH buffers were therefore investigated as alternatives to ammonium hydroxide (see Experimental section for full details). The ammonium hydroxide mobile phase was chosen as a 'baseline' generic method having been previously found to provide a high degree of orthogonality [20] to other generic methods employed in our department. Note that not all of the buffers investigated are MS compatible, but were chosen as potential alternative mobile phase additives that might exhibit improved sensitivity characteristics. The main criteria assessed was the ability to provide similar analyte retention to ammonium hydroxide

Mobile phase	Ionic Strength / mol dm-3
0.1% HCOOH (unadjusted pH 2.6)	0.025
10 mM NH_4OOCCH_3 (unadjusted pH 6.8)	0.010
10 mM KH_2PO_4 (adjusted to pH 2.6 with H_3PO_4)	0.013
10 mM K_2HPO_4 (adjusted to pH 6.8 with H_3PO_4)	0.026

Table 4. Ionic strengths of aqueous portion of low and mid-pH mobile phases used in this study.

High pH additive	Concentration	Correlation coefficient (r^2)
Ammonium hydroxide	0.1% (v/v)	N/A
Triethylamine acetate	0.1% (v/v)	0.8290 (0.9837)
Pyrrolidine	0.1% (v/v)	0.5639
1-Methyl piperidine	0.1% (v/v)	0.7786
Ammonium acetate	10 mM	0.6272
Ammonium bicarbonate	10 mM	0.6860
Ammonium formate	10 mM	0.6685
CAPS	10 mM	0.8992
CAPSO	10 mM	0.8541
Glycine	10 mM	0.7466

Table 5. Correlation coefficients for test analytes comparing retention in the specified mobile phase with the ammonium hydroxide mobile phase. All solutions were titrated to pH 10.6 with ammonium hydroxide or acetic acid as required except ammonium hydroxide which was naturally pH 10.6 in aqueous solution at this concentration.

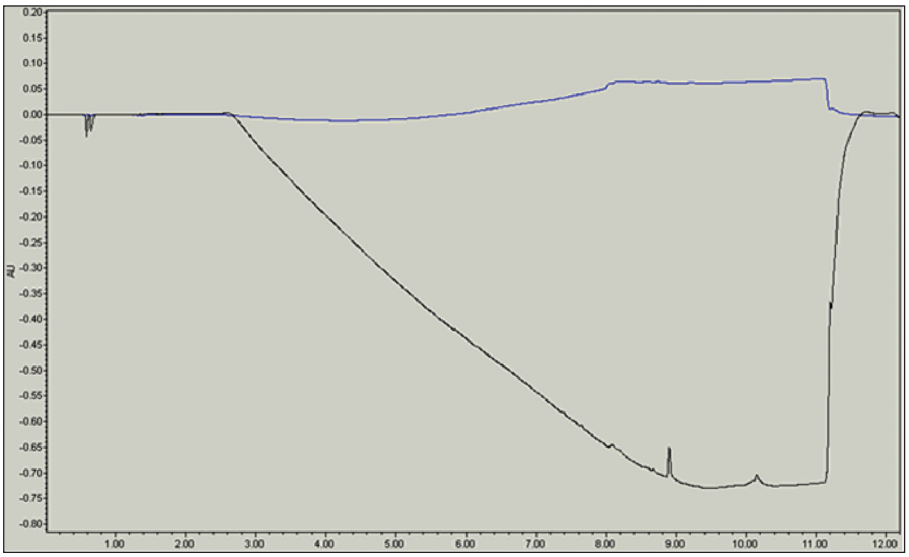

Figure 6. UV baselines of blank injections with the dipotassium phosphate buffer and ammonium acetate mobile phases for method 2. Clearly the ammonium acetate baseline (black trace) exhibits a greater number of baseline artefacts than the phosphate buffer (blue trace) which can lead to peak quantitation issues when low level analyte related impurities are present.

while providing reduced baseline noise (and hopefully additional buffering capacity to increase separation robustness). The reason for requiring a cleaner baseline is simply that many of these impurities can co-elute with real compound related impurities (which are often present at similar levels) making quantitation difficult.

High pH additive – UV performance

A UV spectral overlay of two of the comparator mobile phase additives (0.1% (v/v) 1-methyl piperidine and 0.1% (v/v) TEA) with 0.1% (v/v) ammonium hydroxide is shown in Figure 7. The figure clearly shows that ammonium hydroxide has a much reduced absorbance at the lower end of the UV spectrum compared to the other two solutions. This was also observed for the other high pH mobile phase additives and illustrates that from a signal-to-noise and sensitivity perspective, ammonium hydroxide is a good choice as a mobile phase additive. Additionally, with these solutions and all the other mobile phase additives investigated, none gave superior sensitivity and at best only a similar baseline artefact count to ammonium hydroxide.

High pH additive – retention correlation

Correlation coefficients for the test analyte retention in the ammonium hydroxide and the other high pH mobile phases were calculated and are listed in Table 5. Of the alternative mobile phases, only triethylamine (as acetate salt) gave similar retention to the baseline ammonium hydroxide method. Two values for this particular correlation are listed in Table 5. The first value (0.8290) is for all 33 test analytes. The second value is the correlation from 32 analytes as one data point exhibited much reduced retention (over 3 minutes less retention) in the triethylamine acetate mobile phase which significantly skewed the correlation. No reason can be attributed for this observation at this time. Significant deviations from linearity were observed for all other mobile phases. An example comparison of one of the test analyte sets with 10 mM triethylamine as acetate salt and 0.1% ammonium hydroxide is shown in Figure 8.

After triethylamine acetate, CAPS provided the closest retention correlation to ammonium hydroxide, but exhibited poorer analyte sensitivity and peak shape. While the baseline artefact count for CAPS was equivalent to ammonium hydroxide, CAPS is not MS compatible and is therefore a poorer choice for SIM development. This was a similar finding for all other high pH mobile phases tested.

The findings of this study suggest that from a

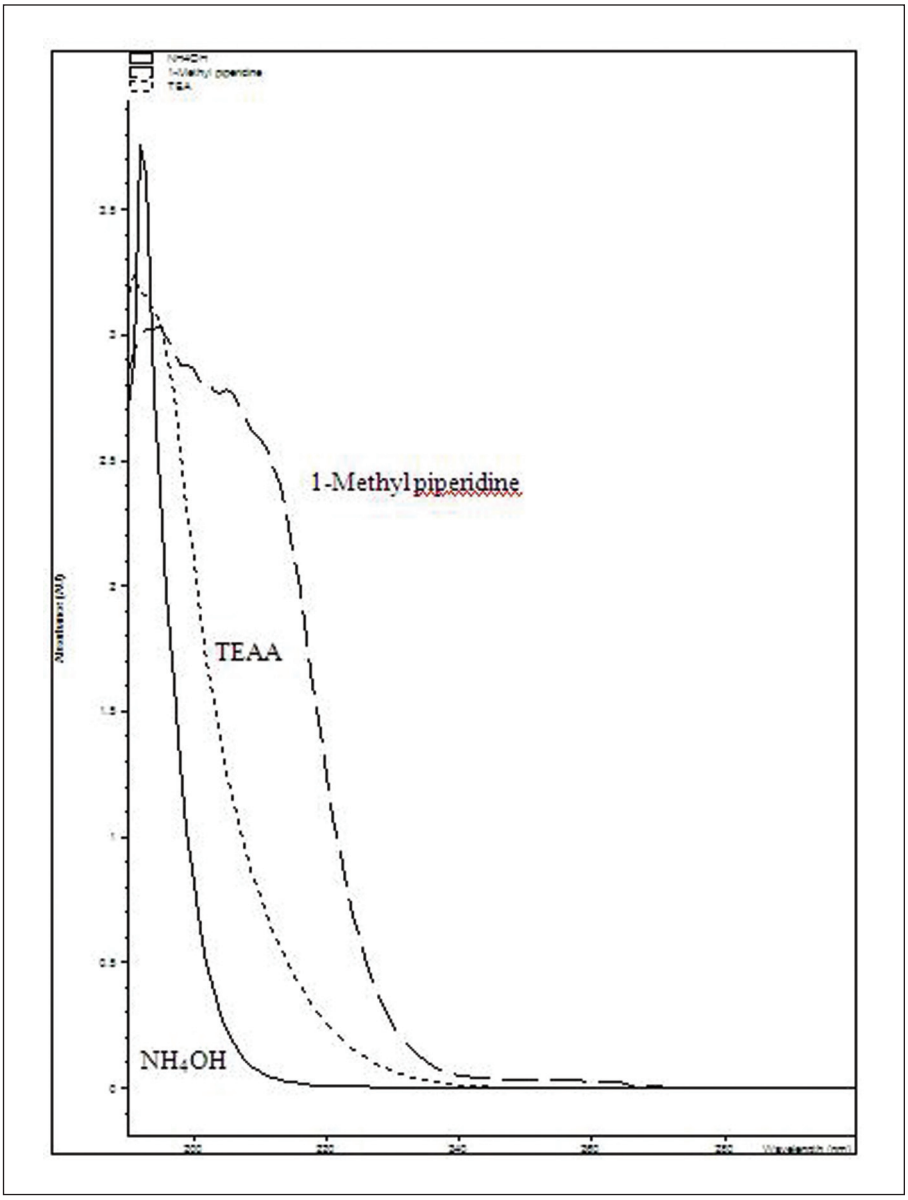

Figure 7. Comparison of some UV spectra for high pH buffers investigated in this study. Ammonium hydroxide is the additive used as the reference mobile phase to which all other high pH systems were compared.

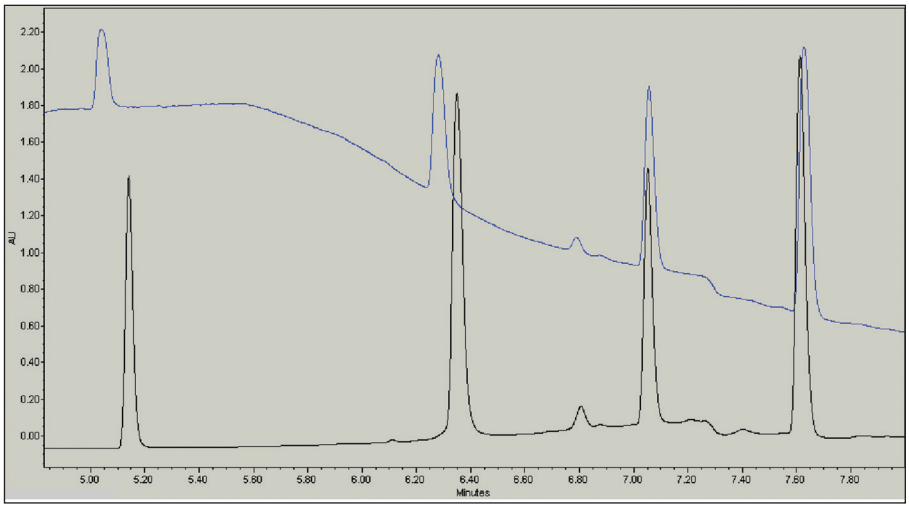

Figure 8. Comparison of analyte response in 0.1% ammonium hydroxide (black line) with 0.1% triethylamine acetate (pH 10.6 – blue line). While triethylamine acetate provided the most similar orthogonality to ammonium hydroxide and a slight improvement in baseline artefacts, this mobile phase gave a much lower analyte response and generally poorer peak shape for the test analytes.

sensitivity, peak shape, baseline artefact and retention perspective, none of the alternative high pH mobile phases tested offered better properties to ammonium hydroxide for MS friendly SIM development work.

Conclusions

Excellent correlation of analyte retention when transferring from organic mobile phase additives to phosphate buffers at low and mid-pH was observed. In most cases absolute elution order and resolution was maintained while significantly increased UV sensitivity and decreased baseline slope were observed with phosphate buffers.

Data from this comparison suggests that many SIMs (or other methods) could be developed from an MS compatible method and transferred directly to an appropriate phosphate method for increased UV sensitivity and method robustness. Alternatively, a phosphate method could be directly transferred to an MS friendly alternative for identification of unknowns with a high degree of confidence.

In the few cases where a change in selectivity is observed and resolution decreases dramatically, it is suggested that experimental modelling is investigated (*e.g.* utilising Drylab or ACDLabs LC Simulator) to re-optimise the separation. This may involve varying gradient slope or temperature or a combination of both to achieve the separation.

In the high pH mobile phase investigation, no alternative additive provided similar properties to the ammonium hydroxide generic method. Low retention correlation, increased baseline artefacts, poorer peak shapes and lower analyte sensitivity were typically observed with the majority of the alternative mobile phases. Alternative approaches to clean ammonium hydroxide mobile phases in order to decrease the artefact level are therefore required e.g. through use of appropriate mobile phase filters.

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