Dr.Krishnasarma Pathy

HOW TO START ANALYTICAL METHOD DEVELOPMENT

Introduction

Prior to the initiation of method development, all the known information about the analyte such as its structure, physical and chemical properties, toxicity, purity, hygroscopicity, solubility, and stability should be determined. These data may be available from preformulation reports, early drug discovery sample screening reports, from the literature on similar compounds, or from past experience with similar compounds. However, many times this information is not available, so preventive measures must be taken in order to ensure that the analyte does not degrade or change during the method development scouting experiments. Implementation of a tray cooler, preparation of fresh samples, storage of samples in a refrigerator, and protection of the solids and sample solutions from light represent some common preventive measures. Depending upon the structure of the compound, potential degradation products can be predicated and forced degradation samples can be generated; these samples can also be used during method development. The goals or requirements of the HPLC method that needs to be developed should be known as well as the analytical figures of merit, which include the required detection limits, selectivity, linearity, range, and accuracy and precision. The potential use of this method needs to be considered: if any regulatory requirements are to be met, if the method is used to analyze multiple samples, or if the method will be eventually transferred to the production site. Some other additional requirements may include sample throughput, analysis time, and instrument limitations. Also, mass balance should always be a consideration during method development. Generally, for a drug substance method it should be established if the area percent method (peak area normalization) and the weight percent method (on a dry basis) are giving similar results, whereas for a drug product method it should be established if peak area normalization and the assay method (based on label claim) are giving similar results. If a bias is obtained between HPLC area percent (peak area normalization) and HPLC weight percent (assay), this may indicate the presence of co-eluting impurities, impurities with different response factors, and/or inadequate elution of all impurities present in the sample.

1. Method development considerations:

There are many factors to consider when developing methods. The initial steps include collect as much information about the analyte in regard to the physicochemical properties (p*Ka*, log solubility) and determining which mode of detection would be suitable for analysis (i.e., suita wavelength in case of UV detection). Sample preparation, which includes centrifugati filtration, and/or sonication and type of diluent, plays an integral role in method developm because this may affect the chromatography and the recovery of the analytes. Determination the solution stability in the diluent is also important during early method development. If solution is not stable, it will become increasingly more challenging to compare subsequ method development analysis. Choice of the mobile-phase and gradient conditions is depend on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixt respectively. This is a crucial step in the method development process because these two fact will probably have the most impact on the change in the analyte selectivity, especially ionizable compounds. Also, the type of stationary phase is very important mainly in regard bonded phase stability at the operational mobile-phase pH. Different stationary phases can a do provide differences in selectivity; however, the change in selectivity is much less predicta compared to varying the pH of the mobile phase to obtain the desired selectivity.

1.1 Sample Properties

1.1.1 Analyte Structure and pKa. In this preliminary step, the ionogenic nature of the compou of interest should be determined. If the target analyte is neutral, the eluent pH will not affect retention. However, the structure of this neutral molecule must be assessed, to postulate i potential ionogenic degradation product may be formed during stress testing and stability testi If this is the case, the HPLC method must be capable of adequately retaining and separating t "potential ionogenic" species from the active and other degradation products or impurit Therefore, in an eluent that has a high pH, the potential acidic impuritiy may be in its ioni form which may result in the elution of the potential degradation product with or even before void volume. If the target analyte is ionizable, the p*Ka* of the analyte should be determined obtained. Software packages such as ACD (Advanced Chemistry Development) may be used get an estimated p*Ka* value for the ionizable functionalities on the molecule. Table 1 shows so common ionizable functionalities present in pharmaceutical compounds. The optimal pH commence method development is at a pH that is at least 1–2 units from the analyte p*Ka* in particular hydro-organic mixture that is employed.

Table 1. p*Ka* of Some Common Functional Groups

Group on aromatic	Pka	Acid/Base
Linear alcohol	>12	Acid
Carboxylic acid	4-5	Acid
Thiol	6-7	Acid
Phenol	10-12	Acid
Alkyl amine	>9	Base
Aromatic amine	4-6	Base
Pyridinal	5-7	Base
Morpholine	8-9	Base
Piperidine	10-11	Base
Imidazole	6-8	Base

For isocratic experiments, this is easily determined by varying the pH of the aqueous phase a monitoring the retention versus the pH, which generally results in a sigmodial type dependence assuming that only one type of ionization center is present. In the event that there a two ionization sites that are acidic and basic, there are competing effects on the retention becau multiple ionization equilibria exist and the overall effect on the retention is dependent on t relative hydrophobicities of the species present at a particular pH. Knowledge of the log *P* for t drug of interest and potential degradation products, metabolites, and synthetic impurities usually helpful to give insight into the types of stationary phases and organic content needed elute and/or retain all the components in the mixture.

1.1.2 Solubility of Components and Diluent Effects (Matrix Effects): Solubility of a particu durg compound is a prerequisite for any salt selection program. Salt formation during a s selection program provides a means of altering the physicochemical and resultant biologi characteristics of a drug substance without modifying its chemical structure, and m compounds with a suitable acidic or basic functionality can potentially be transformed into salt form. The free acid/free base and their corresponding salts will all have different solubilit in the diluent. Generally, salt formation is associated with an increase in the compoun solubility. For example, the free base and phosphate-salt hydrate of codeine have aquec solubilities of 8.3 and 435mg/mL, respectively [6]. The analyte must be soluble in the dilu and must not react with any of the diluent components. It must be determined if the impurities the drug substance observed are actual impurities from the synthesis or if they are formed *in s* in the diluent. The diluent should match to the starting eluent composition of the assay to ensu that no peak distortion will occur, especially for early eluting components. If the analyte is m soluble in the diluent than the starting eluent composition, the compound will tend to reside the "solvent plug" being injected onto the column and a peak fronting or skewing may oc (Figure 1). In Figure peak skewing is occurring with the increase of the concentration methanol in the diluent. However, the solvation of the analyte by the diluent and mobile pha components may also play a role, and peak distortion may occur. In Figure 2a [7], benzoic a (diluent: 50% methanol :50% water) analyzed in 50% methanol: 50% water eluent sho significant peak distortion. In this eluent, this acidic analyte is ionized. The analyte in its ioniz form is expected to show early elution on a C_{18} column; however, solvation of the ioniz analyte with methanol in the mobile phase forms a partially hydrophobic shell that could retained on the reversed-phase adsorbent.

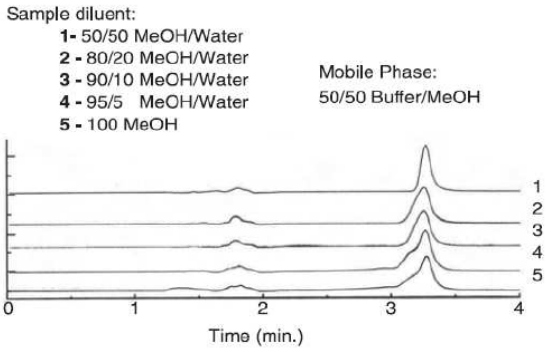

Figure 1. Effect of organic concentration of diluent on peak distortion

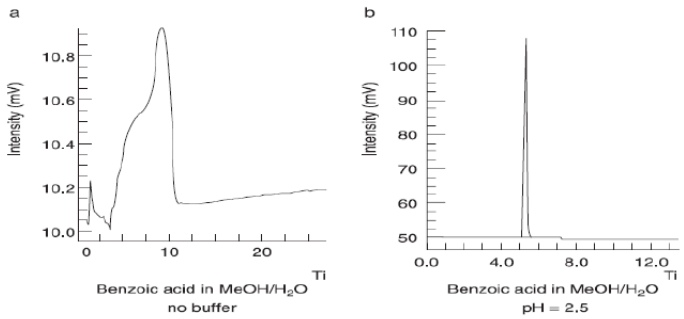

Figure 2. Effect of pH and buffer on analyte peak shape in methanol diluent (150- X4.6-mm C18 column, 1 mL/min, void volume 1.3 mL)

Generally, acetonitrile and methanol elute close to the void volume; however, THF and isopropanol elute later. Co-solvent mixtures with THF/water or isopropanol/water may be an effective way to prevent peak distortion of early eluting peaks such as acids (maleic, tartaric, lactic, fumaric, citric) [8].

Scenario 1. If a compound has a greater solubility in acetonitrile and the diluent is 100% acetonitrile, with the starting eluent composition being 95% aqueous, pH 2: 5% acetonitrile, the early eluting compound may show a peak skewing. Diode array spectra should be obtained to elucidate that this is not an on-column degradation product and/or a coeluting species. If the diode array spectra is the same across this distorted peak, it can be deemed as spectrally homogenous and may be possibly due to a diluent effect. However, if the diluent was changed to 95% aqueous–pH 2/5% acetonitrile, and the peak did not show skewing then the proper diluent has been determined.

Scenario 2. If the diluent was changed to 95% aqueous, pH 2/5% acetonitrile, the sample may not be soluble in this diluent. Therefore serial dilutions may be made such that the compound is first diluted in a diluent in which it is most soluble in and then further diluted to target concentration with a diluent that would be as similar as possible to the staring mobile-phase composition.

Scenario 3. If the diluent was 95% aqueous, pH 2/5% acetonitrile and the sample does show a good peak shape but reinjection of the same solution over time shows an additional impurity that is increasing, this may suggest reaction with the diluent. Multiple steps may be taken here; use an autosampler with a tray cooler to decrease the rate of reaction, adjust the pH of the diluent if pH is catalyzing the reaction in the protic solvent, and/or increase the amount of organic in the diluent. If increasing the organic concentration in the diluent does help in suppressing the formation of the additional impurities but as a consequence peak splitting is observed, then it would be recommended to try another column in order to enhance the retention of the early eluting component such that the compound/impurity would be more retained at a higher initial organic composition. Also, precolumn derivatization may be required to ensure that the desired product is not reacting with diluent prior to analysis. If derivatization is to be employed, then the type, concentration, and derivatization time all need to be explored. This would be considered as a last resort. Other approaches may include the use of aprotic solvents as a diluent and the use of

normal-phase chromatography.Generally, reaction with diluent and mobile phase is sometimes observed for compounds that contain keto functionalities (gem diol, oxycontin [9], active aldehyde [10], active esters such as mesyl sulfonates [11, 12], and enolate intermediates [13]), so protic solvents such as aqueous/methanol should be avoided or derivatizatoin may be required either precolumn or *in situ*.

Scenario 4. Buffered eluents must be used when analyzing ionizable species. Ionizable species are prone to solvation by the mobile-phase components and the solvation equilbira may lead to poor peak shapes. In Figures 3A and 3B, two acidic compounds, benzoic acid (p*Ka* 4.2) and sorbic acid (p*Ka* 4.8), are analyzed at pH 3.5 (a pH lower than the analyte p*Ka*) and at pH 7.0 (a pH greater than the analyte p*Ka*). Acceptable peak shapes are obtained at both these pH values with buffered eluents on this BioBasic C_{18}column[14]. However, when these compounds are analyzed at pH 7.0 without a buffer (only water and methanol), the peak shapes are distorted (Figure 3C).This could be related to the solvation of ionizable species by both the methanol and water which, due to the different secondary equilibria processes, leads to peak distortion.

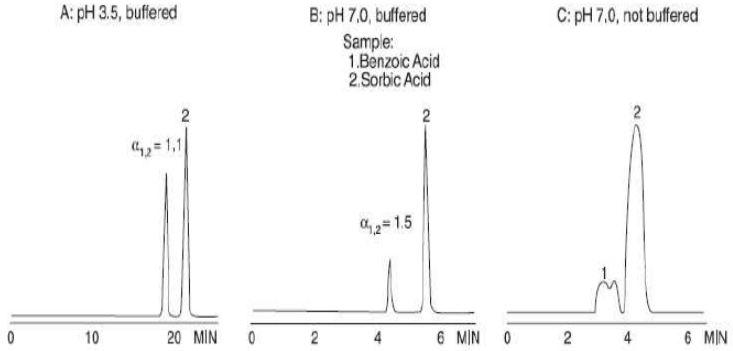

Figure 3. Effect of pH and buffer on the peak shapes of ionizable analytes

1.2 Detector considerations: Choice of the proper detection scheme is dependent on the properties of the analyte. Different types of detectors are available such as ultraviolet (UV), fluorescence, electrochemical, light scattering, refractive index (RI), flame ionization detection (FID), evaporative light scattering detection (ELSD), corona aerosol detection (CAD), mass spectrometric (MS), NMR, and others. However, the majority of reversed-phase and normal-phase HPLC method development in the pharmaceutical industry is carried out with UV detection. In this section the practical use of UV detection will be discussed. A wavelength for UV detection must be chosen so that an accurate mass balance may be determined. Therefore, if area% normalization is to be used, then all the impurities and the active pharmaceutical ingredient must have similar relative response factors (area response/weight). This is sometimes difficult because the impurities may have different electron-donating or electron-withdrawing functional groups, attached to the aromatic ring and/or the impurities may have more complex conjugated systems and the absorption spectra have been shifted to longer or shorter wavelengths compared to the parent compound. Therefore the UV spectra of target analyte and impurities must be taken and overlaid with each other, and the spectra should be normalized due to different amounts present in the mixture. A wavelength must be chosen such that adequate response is

obtained for the active and that at least a 0.05 v/v% solution of the active at target concentration could be quantified (S/N greater than 10). The wavelength chosen should not be on a distinct slope of the spectrum, and the relative difference in the absorbance at a certain wavelength is not significantly different from the impurities/degradation products present. Figure 4 shows the diode array overlay for an API and its related impurities. The optimal wavelength for detection is 280nm because the impurities and API have similar absorbance at this particular wavelength. Although at 250 nm all the compounds have similar absorbance and even higher absorbance compared to 280 nm for some of the components. However, at the 250 nm wavelength greater variability in the response factors may be obtained if an analysis is run on different systems with different detectors (Figure 4). Most detectors are calibrated at ±2nm. If an analysis were to be carried out at 250 nm the spectral bandwidth becomes very important. The spectral bandwidth is dependent on the slit width. The linearity of the detector is inversely proportional to the spectral bandwidth (as the spectral bandwidth gets narrower, the linearity gets better).

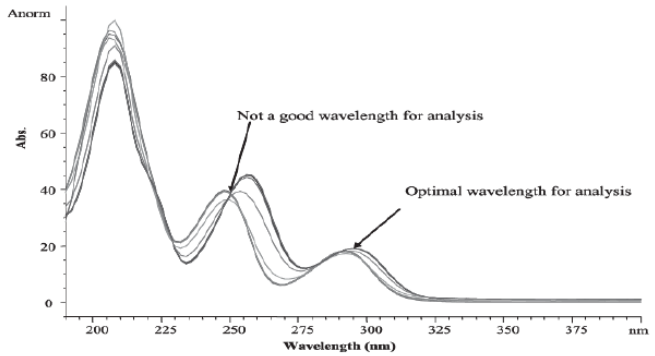

Figure 4. Optimal wavelength selection for API and related impurities

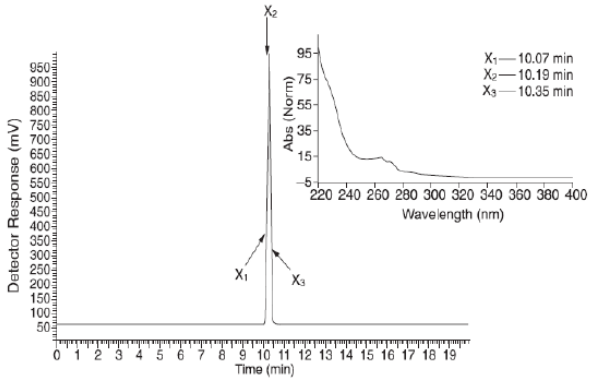

Figure 5. Determination of peak homogeneity: Diode array detection

Diode array spectra at least three points across the peak should be taken to ensure the peak is spectrally homogenous see Figure 5. If the peak is not spectrally homogenous, the overlay of the spectra will show distinct differences. However, even if the diode array spectra do overlay, this does not absolutely ensure that the peak does not contain any coeluting impurities, because the impurities could have similar diode array spectra and/or if there is a low level of a coeluting species with a different diode array spectrum, it may not be determined by this approach.

1.3 Solution stability and sample preparation: It should be determined if the drug substance being analyzed is stable in solution (diluent). During initial method development an autosampler tray cooler and preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. Also, since it is not known if dimeric species or more hydrophobic compounds are present in the sample solutions during the initial method development or are formed in stability studies, gradient elution should always be performed with a hold at higher organic conditions or up to the buffer stability limit. The reduction of downtime of the instrument (i.e., operations of pump components, injectors, and detectors) can be controlled to some degree if sample solutions are filtered and/or centrifuged; the use of a 0.2- or 0.45-µm pore-size filter is generally recommend for removal of particulates [15]. Filtration is a preventive maintenance tool for HPLC analyses [16–18].

Sample preparation is a critical step of method development that the analyst must investigate. For example, the analyst should investigate if centrifugation (determining the optimal rpm and time) shaking and/or filtration of the sample is needed, especially if there are insoluble components in the sample. The objective is to demonstrate that the sample filtration does not affect the analytical result due to adsorption and/or extraction of leachables. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts (i.e., extractables) into the filtrate. Extractables are often the result of inappropriate material construction and improper handling of the device during the manufacturing process. Particular attention should be paid to potential extractables from the membrane and housing material. The sample preparation procedure should be adequately described in the respective analytical method that is applied to a real in-process sample or a dosage form for subsequent HPLC analysis. The analytical procedure must specify the manu facturer, type of filter, and pore size of the filter media.

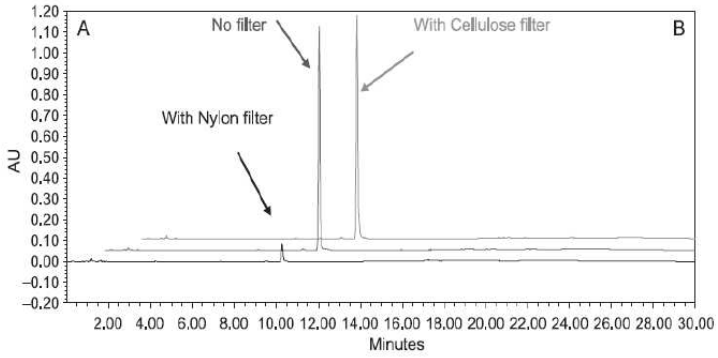

Figure 6. Comparison of filtered (nylon filter versus cellulose filter) versus no filter (centrifuged). Column: Luna C18 (2). Mobile phase: (A) 10 mM ammonium bicarbonate, pH 7.5; (B) MeCN, linear gradient from 0 to 15 minutes, 20—70% of B. Sample concentration: 1 mg/mL

If any additional peaks are observed in the filtered samples, then the diluent must be filtered to determine if a leachable component is coming from the syringe filter housing/filter. In Figure 6 a solid oral dosage form was prepared at 1 mg/mL concentration. The initial stock solution was centrifuged (no filter) and two additional samples from the centrifuged solution were filtered

with a nylon filter and a cellulose filter. Further optimization of the sample preparation would include removing the centrifugation step and just filtering the supernatant (solution above the undissolved excipients) with the cellulose acetate filter.

1.4 Choice of stationary phase: Ideally for a reversed-phase separations, the retention factors (*k*) for all components in a sample should lie between 1 and 10 to achieve separation in a reasonable time. For a given stationary phase the *k* of a particular component can be controlled by changing the solvent composition of the mobile phase. However, the impact of eluent composition will depend on the type of stationary phase and the nature of the components in the mixture. In reversed phase HPLC the most common solvent mixtures are: water and acetonitrile, water and methanol, and water and THF. The elution strength increases as the organic portion of the modifier increases. Thus, to optimize a chromatographic separation, the concentration of the organic modifier is adjusted so that the *k* of the components in the sample are in the range of 1 to 10. However, sometimes due to the hydrophobic nature of the compound, even high concentrations of organic modifier will not allow elution of all components in a single run and the chromatographer can try one or a combination of the following approaches:

- Use a stronger modifier
- Apply a steeper gradient
- Use a less hydrophobic stationary phase.

The type of column chosen for a particular separation depends on the compound and the aim of analysis. Pharmaceutical companies may have a preferred list of columns that have good demonstrated performance in regard to pH/temperature stability. These columns that have been selected by a specific laboratory are known to be stable within predefined pH and temperature regions in which method development/column screening are employed. Screening columns from each of the following various column classes should provide for the desired chromatographic selectivity, even for the most challenging separations: (1–3) C_8 or C_{18} stable at pH≤2, pH 2–8, and pH ≥8–11; (4) phenyl; (5) pentafluorphenyl; (6) polar embedded and stationary phases that could be run in 100% aqueous. A certain number of columns in each of the six column classes and subclasses could be chosen as standard columns that the chromatographers choose as a first choice for performing method development. These standard columns could be chosen based on some set of internal criteria (ie., chromatographic selectivity for a set of compounds, bonded phase stability, and lot-to-lot reproducibility). The criteria for selection may include that the column is stable for a certain number of column volumes (efficiency, tailing factor, retention time criteria for predefined probe analytes) at the recommended max and min pH at a particular maximum temperature. By tracking the column usage (number of column volumes run at a particular pH/temperature), this will reduce the number of system suitability failures and decrease the cost of the consumables for a particular laboratory. It is generally recommended not to use the same column for multiple projects, especially when performing release and stability testing. For more hydrophobic compounds, a stationary phase that has a lower surface area should be used. For very polar compounds that cannot be retained on traditional C_{18} phases, less hydrophobic columns such as C_4 and polar embedded stationary phases could be used. However, all this is also dependent on the pH of the analysis since some columns are not stable at low pH (<2) and higher pH (>7) for extended periods of time. This should be taken into careful consideration when defining a column(s) during the development of a method.

1.5 Mobile-phase considerations

1.5.1 Choice of pH. If analytes are ionizable, the proper mobile-phase pH must be chosen based on the analyte p*Ka* so the target analyte is in one predominate ionization state ionized or neutral. If possible, method development at both of these defined mobile-phase pH values is encouraged to maximize the potential gains that may be obtained in regard to selectivity (for the neutral and ionized forms of the target analyte and related substances). Alteration of the mobile-phase pH is one of the greatest tools in the "chromatographers toolbox" allowing simultaneous change in retention and selectivity between critical pair of components. Analytes may be analyzed in their ionic form or neutral form. This may be dependent on the type of analysis that is required. If fast analysis is required, then analysis of the component in its ionized form may be acceptable if the desired resolution from the matrix components is achieved. However, if adequate resolution of the active from its process related impurities/degradation products/excipients are not obtained, then mobile-phase additives may be added to the mobile phase or the mobile phase pH may be adjusted so the analyte may be analyzed in its neutral form in order to potentially enhance the electivity/resolution between critical pairs of components. Increasing flow rate, increasing temperature (up to column stability limit at a particular pH), increasing the concentration of the organic eluent, and using shorter columns with narrower dimensions may be used to obtain more desirable run times. However, speed does not come without a price, and the influence of the aforementioned parameters on the resolution of the critical pairs in a mixture/sample needs to be evaluated.

1.5.2 Buffers. In order to develop rugged HPLC methods, knowledge of choosing the right buffer is very important. Buffers that are selected should have a good buffering capacity at the specified mobile-phase pH. Also, the concentration of the buffer should be at least 10 mM to provide the needed ionic strength to suppress any undesired analyte solvation effects that may lead to poor peak shapes. Methods that specify a phosphate buffer in the pH range of 4 to 6, or an acetate buffer in the range of 6 to 7, are, unfortunately, not good buffers. These buffers are not just useless in these pH ranges, they complicate the preparation of mobile phase unnecessarily and give the analyst a false sense of controlling the reproducibility of the separation.

1.5.3 General considerations for buffers. A judicious choice of type and concentration of buffer must be made to ensure mobile-phase compatibility.

- Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
- Some salt buffers are hygroscopic. If an analyst makes a 20mM buffer and the original buffer salt contains 20 w/w% water, then the buffer concentration would be 16mM. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).
- Ammonium salts are generally more soluble in organic/water mobile phases than potassium salts, and potassium salts are more soluble than sodium salts.
- TFA can degrade with time, is volatile, absorbs at low UV wavelengths, and is not a buffer at pH ≥1.5.
- Citrate buffers can attack stainless steel.When using these buffers, be sure to flush them out of the system as soon as the analysis is completed, but this is a recommendation for any buffer system.

- Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and can damage chromatographic performance.
- At pH greater than 7, phosphate buffers accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
- Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 to 48 hours. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.
- After buffers are prepared, they should be filtered through a 0.2-µm filter.
- A "test tube test" should be conducted to determine if the buffer at the concentration it is prepared will precipitate in the column/system when it is exposed to the highest organic concentration in the gradient. The temperature should also be considered as well. Buffers generally will have a higher solubility at higher temperatures. The test tube test can be performed by preparing the mobile phase in a 10-mL test tube and then putting the test tube in the refrigerator and/or water bath (to mimic higher temperatures) to determine if any precipitation occurs.
- Mobile phases should be degassed if an on-line degasser is not available on the HPLC system.

1.5.4 Concentration of buffers. A buffer concentration in the range of 10 to 50 mM is adequate for most reversed-phase applications. However, some times the concentration of the buffer does lead to improvement of peak shape, presumably because the cation of the buffer suppresses silanophilic interactions of the protonated base with accessible ionized residual silanols. This concentration should also be low enough to avoid problems with precipitation when significant amounts of organic modifiers are used in the mobile phase and, in the case of phosphate buffers, low enough to minimize the abrasive effect on pump seals. It is seldom advisable to use a buffer concentration more than 100 mM and less than 10mM. Increasing the concentration of the mobile phase buffer, and thereby increasing the ionic strength of the mobile phase, will sometimes suppress this ion-exchange interaction and reduce this "secondary retention" effect.

1.5.5 Practical aspects of preparing a buffered mobile phase. The following steps are suggested for preparing a buffered mobile phase:

- Define the appropriate wwpH for the separation and then select an appropriate buffer. Refer to Table 3 to determine the appropriate buffer for your application.
- Prepare an aqueous buffer solution of the desired concentration and wwpH (pH of aqueous buffer solution).
- Measure the pH of the solution and adjust, if necessary, to the desired wwpH with dilute acid or dilute basic solution. When adjusting the wwpH of a buffer solution, make sure to wait until the solution reaches equilibrium after adding additional acid or base before measuring the pH.

1.5.6 Choice of organic modifier. Selection of the organic modifier type could be viewed as relatively simple: The usual choice is between acetonitrile and methanol (rarely THF). Acetonitrile as an organic modifier may offer these variations due to the introduction of a dual

retention mechanism. The viscosity of water/organic mixtures should be considered as an additional parameter in the selection of organic modifier. Acetonitrile/water mixtures show roughly 2.5 times lower viscosity than equivalent methanol/water eluents; this means that one can use 2.5 faster flow rates with acetonitrile as organic modifier and develop faster separation methods. Acetonitrile is not ionogenic and is not a hydrogen bonding agent, but its four electrons offer strong dispersive interactions that should also be taken into account in the solvent selection. Changing the type of organic eluent may have an effect on the resulting selectivity of the two species in a mixture.There is no definite way to predict if changing the type and concentration of the organic eluent will impart a difference in selectivity of the closely eluting species. The types of solvent that are recommended are pure acetonitrile, pure methanol, and a mixture of acetonitrile/methanol. Sometimes, a small addition of THF (up to 5 v/v%) or isopropanol to either acetonitrile or methanol may lead to changes in the selectivity. The adsorption of the organic eluent component on the stationary phase and the interactions of the eluent with the analyte molecules play a significant role in determining the resultant selectivity of the separation.

1.6 Gradient separations

1.6.1 Isocratic versus gradient separations. Traditionally, isocratic separations are deemed as more reproducible than gradient separations. Indeed, constant eluent composition means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte-eluent and analyte-stationary-phase interactions are also constant throughout the whole run. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak. Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). The condition where the tail of a chromatographic zone is always under the influence of a stronger eluent composition leads to the decrease of the peak width. Peak width varies depending on the rate of the eluent composition variation (gradient slope).

1.6.2 Changing Gradient Slope. Gradient elution is usually employed with complex multicomponent samples since it may not be possible to get all components eluted between *k(*retention factor*)* 1 and 10 using a single solvent strength under isocratic conditions. This leads to the general elution problem where no one set of conditions is effective in eluting all components from a column in a reasonable time period while still attaining resolution of each component. This necessitates the implementation of a gradient. Employing gradients shallow or steep allows for obtaining differences in the chromatographic selectivity. This would be attributed to the different slopes of the retention versus organic composition for each analyte in the mixture. Therefore, changing the gradient slope or steepness of the gradient is an important variable that should be considered in reversed phase method development for controlling the retention of components and adjusting the selectivity for components. Take, for example, the two gradient runs in Figure 7, where a shallow and steep gradient were run at both 35^0C and 50^0C. Increase of separation selectivity is observed for the impurities eluting between peak H and peak A using the shallow gradient for both temperatures (35^0C and 50^0C). Using the steep gradient at both temperatures, a coelution of two of the impurities eluting between H and A was obtained.

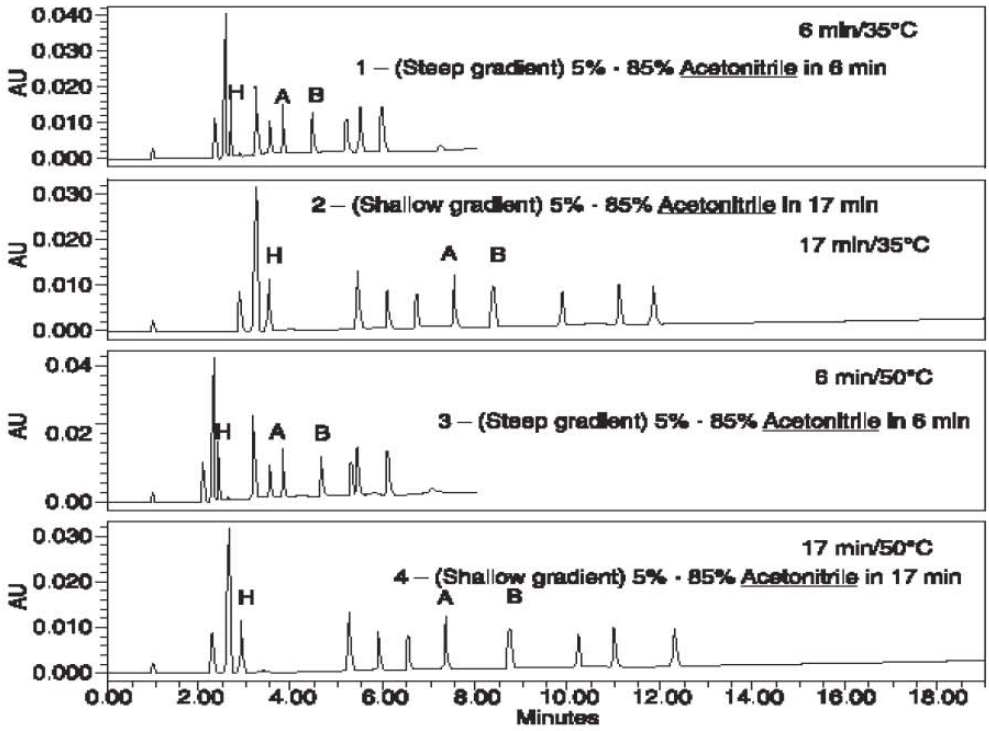

Figure 7. Effect of gradient slope on the chromatographic separation. Symmetry C_{18}, 4.6 ×100 mm, 3.5 µm

1.6.3 Linear versus multi step gradients. If the gradient separation mode is selected, it is generally recommended to use a simple linear gradient because of better reproducibility, less column stress, and better resolution of critical pairs. If any isocratic step is included in the run, the peak width of all analytes is broadened during this step and the whole method loses its main advantage, namely, high apparent efficiency (which results in higher resolution).

1.6.4 Equilibration. When a gradient method is used, the column must be allowed to equilibrate at the starting mobile-phase conditions prior to the next sample injection and the start of the next gradient run. Generally, if a gradient is used from 5% organic to 95% organic on bonded phases that are not polar embedded or that should not be run at 100% aqueous conditions, at least three to five column volumes should pass through the column before starting the next injection. The gradient method can be programmed to immediately change (after completion of the separation) from 95% organic to 5% organic in <0.1 min (this depends on instrument limitations). This sharp change of the eluent composition will actually help to clean the column. How is a column volume (V_{column}) calculated? A good rule of thumb is that the volume of the column can be approximated from the volume of a cylinder. Seventy percent of the column volume is approximately the volume inside the porous space and around the particles:

$$V_{column}=0.7\pi R^2 L$$

The time needed to equilibrate the column is determined by the flow rate. The number of column volumes (three to flve column volumes) can be divided by the flow rate (mL/min) to determine the time (in minutes) needed. Therefore, the lower the flow rate, the longer the equilibration time.

2 Method development approaches

2.1 ***If analyte structure is known*****:** Determine if analytes are acidic, basic, or neutral. This will allow the chromatographer to choose a pH such that the analyte is being analyzed predominately in one ionization state. Use the rules for pH shift and p*Ka* shift to ensure that the analyte is one predominant ionization state and choose the appropriate mobile-phase pH. Some general guidelines are as follows: If target analyte is an acidic analyte (p*Ka* ≥3), use a 0.2 v/v% phosphoric acid mobile phase. If target analyte is a basic analyte (p*Ka* ≥7-9), use an ammonium acetate buffer (pH 5.8) to analyze in its ionized form or use a 25mM ammonium hydroxide buffer (pH 10.5) or 25mM *N*-methyl pyrrolidine buffer (pH 10.5) to analyze in its neutral form.

If changing the organic modifier does not work, consider changing the mobile-phase pH (analyze the molecule in a different ionization state). For example, if a basic compound was originally analyzed under basic conditions (pH <≤p*Ka*), try to use acidic conditions (pH >≥p*Ka*) with the acetonitrile in the initial gradient. If that still does not work, then consider using a different stationary phase (phenyl or polar embedded) employing the initial gradient, with initial aqueous mobile phase and acetonitrile organic modifier, and repeat the process that was performed on the original column used for initial method development. The final method optimization may include varying the gradient slope, column temperature, and flow rate. Note that multiple pH values and columns can be screened in gradient mode at the same time as well. This will increase the efficiency/probability of obtaining the best column/conditions and the best demonstrated chromatographic selectivity. Note that the aqueous phase pH values that would be chosen for these pH/column screening studies should be based on knowledge of the physicochemical properties of the molecule, taking into consideration the mobile-phase pH and analyte p*Ka* shifts in the hydro-organic media.

2.2 ***If method is being developed for separation of active and unknown component*****:** Define the criteria for the method such as the LOQ, maximum run time, wavelength detection, and so on. Look at the structure of the target analyte (estimate p*Ka*) or use ACD (advanced chemistry development) and determine the best pH to run the method. Try to use shorter columns for gradient scouting experiments (5cm×4.6mm) packed with 3-µm columns or use a high pressure system (max pressure 15,000 psi) with 10-cm×2.1-mm, 1.7-µm particles. Use 35-45^{0}C as starting temperature. If pH scouting studies are needed, run a probe linear gradient using 0.2 v/v% phosphoric acid on a short column (5-cm×4.6-cm column) to determine the isocratic conditions for the pH studies. Run pH studies isocratically to determine the desired pH region to understand the behavior of the impurities in the analyte mixture. The desired pH region of the aqueous phase is the pH region where the retention of the components in the mixture do not significantly change their retention as a function of the pH of the aqueous phase. Track impurities using diode array if possible. Run a linear gradient at a pH within the desired pH region and hold at high organic concentration on 5-cm×4.6-mm column. If obtain sufficient resolution, then it is finished. If need more resolution, then use a 15-cm×3-mm i.d. column. If resolution is obtained, then you are finished. If desired resolution/selectivity is not obtained, then screen different organic

modifiers/different stationary phase types. Note that the separation of the critical pair may be obtained on an alternate stationary phase that offers additional selectivity. In addition to the weak dispersive types of interaction that are available on a C_8 or C_{18} phase, phenyl phases may provide additional interactions such as π-π type interactions and may assist in providing additional selectivity. If the impurities/active are very polar, the use of polar embedded phases may provide additional selectivity by introduction of a secondary type of interaction such as hydrogen bonding close to the surface in the organic-enriched layer. Note that Drylab is not suitable for the following types of compounds:

- Chiral compounds
- Achiral isomers or diastereomers
- Inorganic ions
- Carbohydrates
- Proteins and peptides

2.3 *Defining system suitability*: System suitability parameters with their respective acceptance criteria should be a requirement for any method. This will provide an added level of confidence that the correct mobile phase, temperature, flow rate, and column were used and will ensure the system performance (pump and detector). This usually includes (at a minimum) a requirement for injection precision, sensitivity, standard accuracy (if for an assay method), and retention time of the target analyte. Sometimes, a resolution requirement is added for a critical pair, along with criteria for efficiency and tailing factor (especially if a known impurity elutes on the tail of the target analyte).

System Suitability Parameters

- Tailing factor (5% peak height) for peak B≦1.5
- Tailing factor (5% peak height) for peak A≤1.5
- Rt for peak A must be 12.0 ±1.3min
- Rt for peak B must be 21 ±1.0min
- The S/N of the LOQ solution (0.05%) for both actives A and B must be ≥10:1

2.4. *Influence of pH, temperature, and type and concentration of solvent on the retention and selectivity of acidic (phenolic) compounds*: The HPLC retention behavior of a para bromo-monosubstituted phenol inter mediate and its ortho isomer were investigated. The ortho isomer is a common impurity generated during the synthesis of the desired para isomer intermediate. It was critical to control this impurity because it would react at the hydroxyl functionality in the downstream chemistry to produce unwanted synthetic impurities in the API (active pharmaceutical ingredient). Implementing a recrystallization procedure to remove these downstream synthetic impurities, although efficient, would reduce the overall yield of the API. Therefore, control of the starting material, para-bromo phenol, was deemed necessary. The retention of these two isomeric species was found to be highly dependent on the eluent pH, type of organic modifier, and temperature. It was determined that a reversal of elution order could be obtained where the minor isomer elutes prior to the major isomer by optimization of the eluent pH, temperature, and change of type of organic modi.er from acetonitrile to methanol.

2.4.1 Effect of pH on the retention/selectivity of the isomers. The first step in method development is to understand the effect of pH on the separation characteristics of the method.

The p*Ka* values of the ortho and the para isomers was estimated by ACD to be 9.0 and 9.5 respectively. Obviously the best pH to carry out the separation would be at pH that is less than 2 units lower than the analyte that has the lowest p*Ka*. This would be at wwpH values less than 7.0. However, to illustrate the effect of pH on the separation selectivity of the isomers, a controlled pH study at isocratic conditions was conducted. Figure 8 (*k* versus wwpH) and Figure 9 (selectivity versus wwpH) show the effect of pH on the retention of the para and ortho isomers at a constant mobile-phase composition of 50 : 50 15mM dihydrogen potassium phosphate acetonitrile, at 25^0C over the aqueous wwpH range 2.0-10.7 analyzed on a Luna $C_{18}(2)$ column. Both of these isomeric compounds are acidic, and it is expected that an increase in the mobile phase pH will cause a decrease in the analyte retention because these compounds are becoming progressively moreionized. At 25^0C for these isomers analyzed at wwpH $\leq$8 the undesired isomer, ortho isomer, is eluting after the para isomer and at wwpH $\geq$9 the ortho isomer elutes before the para isomer (desired elution order).

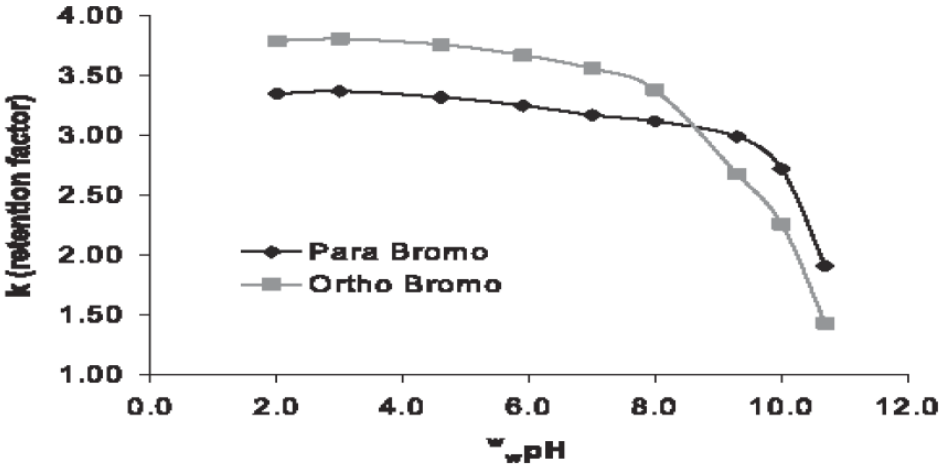

Figure 8. Effect of pH on retention of ortho and para isomers

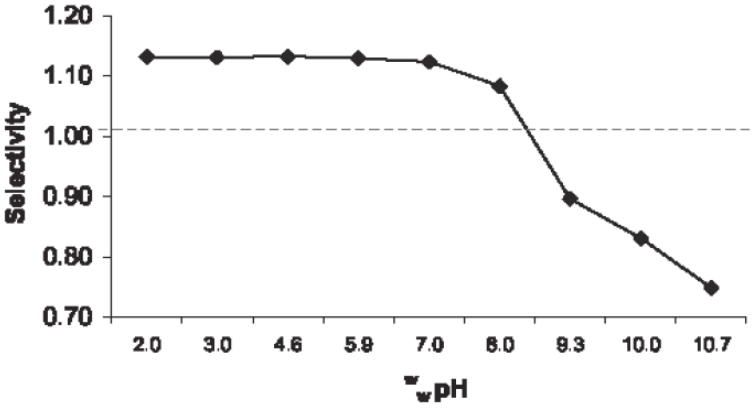

Figure 9. Effect of pH on selectivity

2.4.2 Effect of organic concentration on the retention/selectivity of the isomers. The effect of organic concentration on the selectivity and retention (Figure 10) of para and ortho isomers at three different pH values (wwpH 2, wwpH 8, and wwpH 8.6) were determined. The optimal pH for the separation is at wwpH 2. However, studies were performed at wwpH 8, and wwpH 8.6 to illustrate why working at these higher pH values would not be ideal for the separation from a robustness point of view. Although favorable changes in selectivity may occur at a pH near the p*Ka* values of the components in the mixture, the method may not be robust due to minor changes in pH and organic concentration.

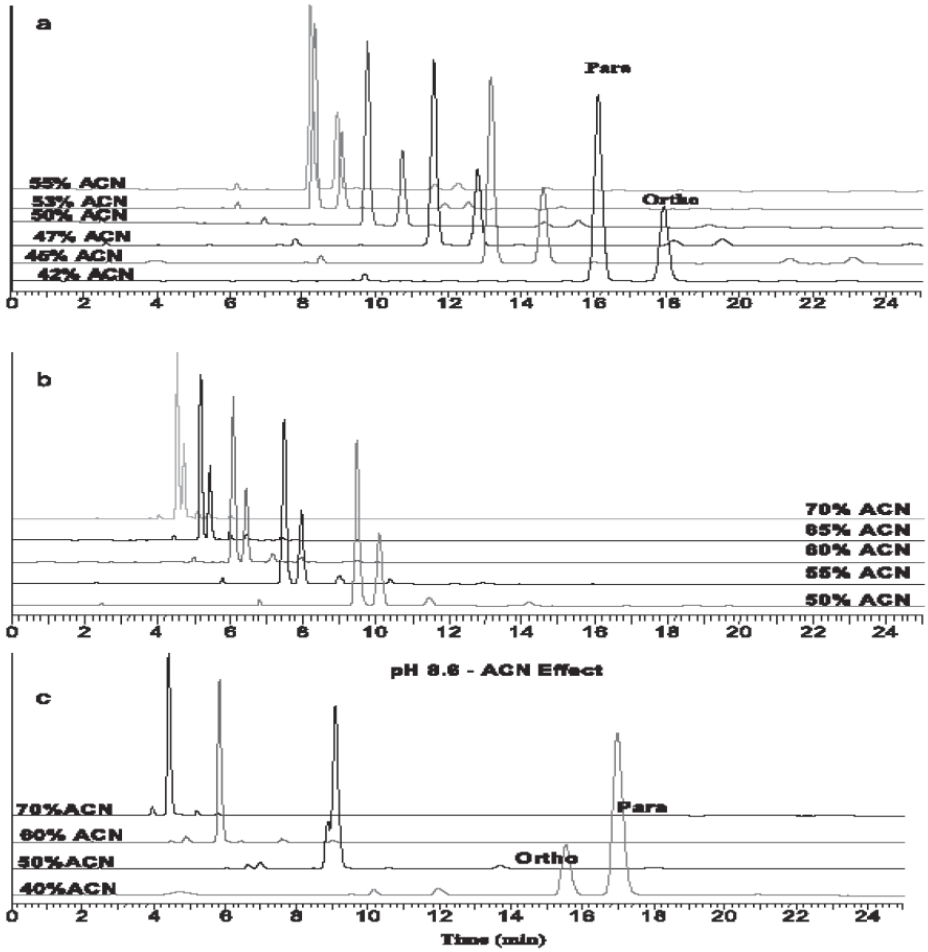

Figure 10. Effect of organic content on the analtye retention. (A) wwpH 2, (B) wwpH 8.0, (C) wwpH 8.6

% ACN Study w wpH 2.0. The amount of acetonitrile in the mobile phase was varied over the following range: 42-55% ethanenitrile. Plots of ln (*k*) versus % organic for both isomers were linear in this v/v% acetonitrile region (R^2 =0.999). Increasing the v/v% acetonitrile led to a decrease in the retention of both isomers (Figure 19A), and a decrease in resolution between both

isomers however the selectivity and efficiency for each isomer remained constant. Ideally, the eluent composition should not affect the selectivity between two species if their ionization state is not changing with an increase in the organic composition. Also, the efficiency did not change as a function of the organic composition because the capacity factor of the analytes was such that extra-column band broadening (column and extra-column effects) leading to peak dispersion were avoided. The selectivity also was constant within this studied organic composition range at pH 2. The decrease in resolution from 4.1 to 3.2 was obtained upon increasing the organic composition from 42% to 55% acetonitrile.

% ACN Study ww*pH 8.0.* A similar study was carried out at wwpH 8 (Figure 10B).The range of v/v% acetonitrile studied was from 50% to 70%. Increasing the v/v% acetonitrile over the range studied caused a decrease in resolution to the point where baseline resolution between the isomers was lost. Also at this pH and these acetonitrile compositions studied, the selectivity between the isomers was independent of % organic. Plots of ln *k* versus % organic for both isomers were linear in this v/v% acetonitrile region (R^2 =0.997).

% ACN Study pH 8.6. A similar study was carried out at wwpH 8.6 (Figure 10C), where the pH of the mobile phase is approaching the analyte p*Ka* values. The p*Ka* of the para isomer is greater than p*Ka* of the ortho isomer as observed by the injections points in Figure 17. At wwpH 8.6 the ortho isomer elutes prior to the para isomer. The range of % acetonitrile studied was 40-70 v/v%. Plots of ln (*k*) versus % organic for both isomers showed slight curvature at high organic concentrations, and a change in the selectivity between the two isomers was observed. The addition of organic component to an aqueous mobile phase shifts the pH of the acidic aqueous eluent (phosphate buffer) upward (0.2 pH units per 10 v/v% acetonitrile) and shifts the acidic analyte ionization state of the isomers to higher p*Ka* values (~0.3p*Ka* units per 10 v/v% acetonitrile, determined in an independent study).

2.4.3 Effect of temperature on the retention/selectivity of the isomers: The next variable investigated was the effect of temperature on the analyte retention. The effect of temperature on the retention and selectivity of the para and ortho isomers at wwpHs 2, 8, and 8.6 was studied (Figures 11, 12, and 13). The effect of temperature could be used to optimize the run time and the apparent efficiency of the separation. At a buffer pH of ww2.0, the effect of temperature on retention/resolution was studied over the temperature range 5^0C-70^0C with 50/50 acetonitrile/buffer (Figure 11).The retention and resolution of both isomers decreased with increasing temperature, with the major isomer (para isomer) eluting first at all temperatures studied. This study was also conducted at a buffer wwpH of 8.0 over the range 5-70^0C at 50/50 acetonitrile/buffer (Figure 12). The retention and resolution of both isomers again decreased with increasing temperature; however, a reversal in elution order was observed when the temperature was increased above 50^0C, where the ortho isomer eluted prior to the para isomer. The temperature study was also performed at w wpH 8.6. At 5^0C, the para isomer eluted first, at 20^0C they coeluted, and at temperatures greater than or equal to 30^0C a reversal of elution order was obtained where the ortho isomer eluted prior to the para isomer (Figure 13).

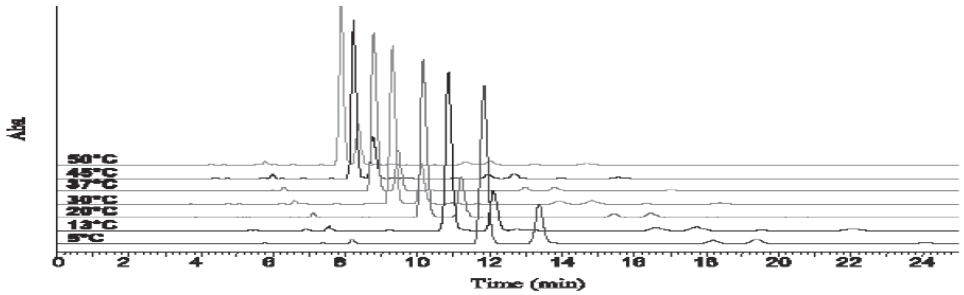

Figure 11. Effect of temperature on the analyte retention, wwpH 2

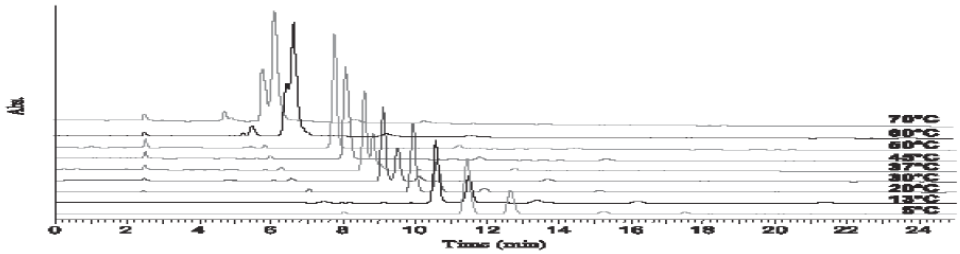

Figure 12. Effect of temperature on the analyte retention, wwpH 8

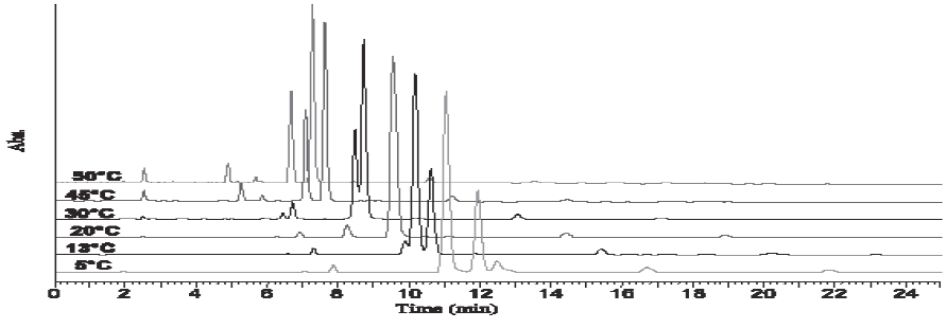

Figure 13. Effect of temperature on the analyte retention, wwpH 8.6

2.5 Effect of pH on UV absorbance: The extent to which an organic molecule absorbs electromagnetic radiation in the ultraviolet region (UV light) depends on the structure of the molecule. Generally, molecules that contain one single double bond absorb weakly in the UV region. However, if multiple double bonds are present in a molecule and they are conjugated, the molecule absorbs more strongly and the absorbance is shifted to longer wavelengths. The pH of the mobile-phase effects the ionization of ionogenic solutes and also the analyte UV response. The change in pH can change the electronic structure of the molecule and result in changes in the molar absorptivity and the absorption maximum of the molecule [25]. Ionization of aromatic compounds containing a pyridinal nitrogen, amino, carboxylic acid, and phenolic group can lead to significant changes of their UV response. Understanding the effects of charge delocalization and conjugation on the UV response and detection wavelength will allow the chromatographer to choose the proper pH and wavelength of detection to obtain a method with higher sensitivity. *Silverstein et al.* [26] and *Shenk* [27] provide a good overview for predicting how the structure of

a molecule and its environment will affect its molar absorptivity and the wavelength of the absorption maximum. Most applications of absorption spectroscopy to organic compounds are based upon transitions for *n* or π electrons to the π* excited state. Energies required for these processes bring absorption peaks into the spectral region (200-700nm) π-electrons are further delocalized by conjugation. The effect of this delocalization is to lower the energy level of the π* orbital and give it less antibonding character and as a result absorption maxima are shifted to longer wavelengths [28]. UV spectra of aromatic hydrocarbons are characterized by three sets of bands (E1, E2, and B bands) that originate from * transitions. Generally the E2 and B bands are of most interest to chromatographers, since the solvent cutoff for most mobile phases is <200nm For example, benzene has strong absorption peaks at

- E1: 184nm, ε_{max} ~ 60,000
- E2: 204nm, ε_{max} =7,900
- B: 256nm, ε_{max} =200

Value shows E2 and B bands for some organic molecules. Auxochromes are a functional group that does not itself absorb in the UV region but have the effect of shifting chromophore peaks to longer wavelengths and increaseing their intensity. The -OH and -NH_2 groups have an auxochromic effect on benzene chromophore. These substituents have at least one pair of *n* electrons capable of interacting with electrons of the ring. This stabilizes the π* state and lowers its energy. The phenolate anion auxochromic effect is more pronounced than for phenol because the anion has an additional pair of unshared electrons. Aniline has a pair of π electrons capable of interacting with the electrons of ring. This stabilizes the π*state by the relationship shown in Equation, thereby lowering its energy [28].With a decrease in protonation, the absorption maxima would be shifted to longer wavelengths and increasing intensities and a red shift occurs However, upon protonation the nonbonding electrons are lost by formation of the anilinium cation, and the auxochromic effect disappears as a consequence. The change in the mobile-phase sspH at a constant organic composition may have an effect on an ionizable analyte's UV response (Figure 14A). Also, at constant wwpH as the organic concentration is increased, this may also lead to a change in the analytes absorbance at a particular wavelength. Increasing concentration of the organic shifts the pH of the mobile phase upward (for an acidic modifier) and changes in UV absorbance may be observed (Figure 14B).

At 232nm there is a decrease in aniline's absorbance as this analyte becomes progressively more ionized. A plot of the UV absorbance at a particular wavelength versus the wwpH of the aqueous phase will lead to a sigmoidal dependence (Figure 15). The injection point corresponds to the analyte p*Ka* (not corrected for pH shift of the mobile phase).When performing method development experiments a judicious choice for the wavelength of the detection should be carefully considered because this can lead to desired/undesired effects (change in sensitivity at particular wavelength as a function of pH) on the resulting chromatography. Figure 16 demonstrates that a greater response for aniline is observed at wwpH where the analyte is in its neutral state. As the analyte ionization state varies with pH so does the conjugation.

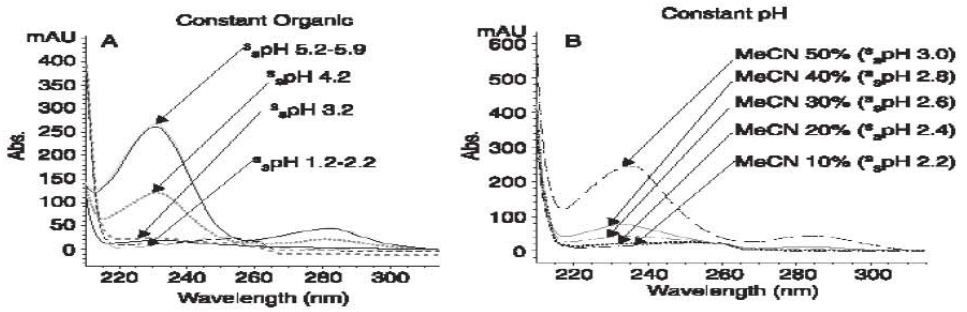

Figure 14. Effect of pH on UV absorbance for aniline (obtained from diode array). (A) 10 v/v% acetonitrile and pH of 15mM dipotassium hydrogen phosphate adjusted to wwpH 1-9 with phosphoric acid. (B) wwpH 2.0 and acetonitrile concentration changed from 10 to 50 v/v%

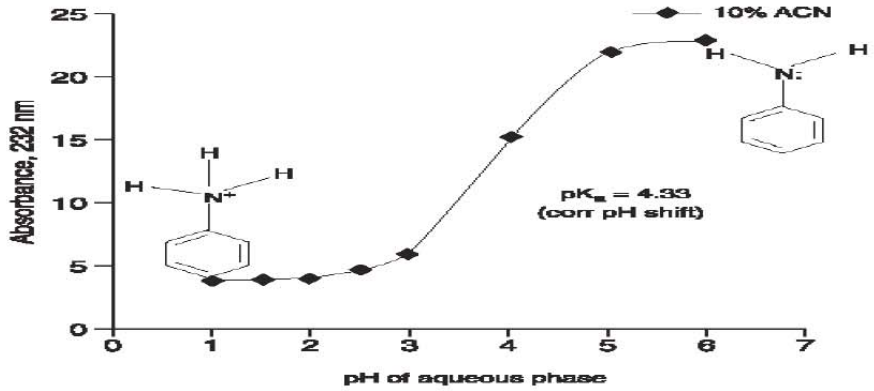

Figure 15. Absorbance at 232 nm versus the wwpH of the aqueous phase. Mobile phase contains 10 v/v% acetonitrile

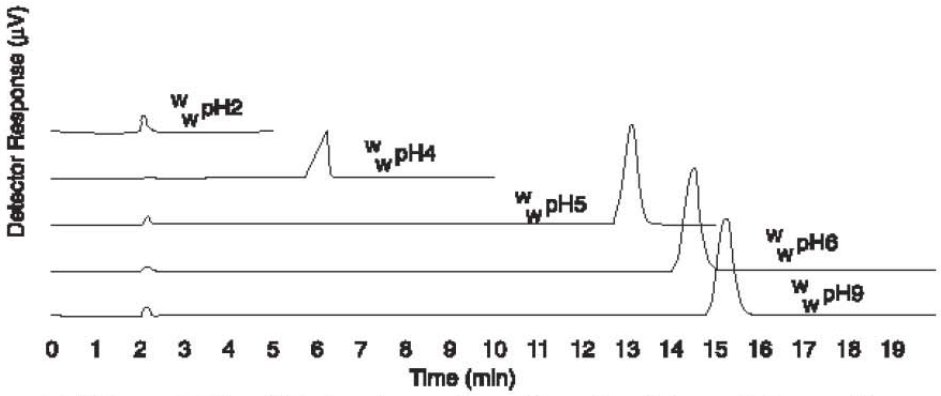

Figure 16. Effect of pH on UV absorbance for aniline. Conditions: Column: 15-cm ×0.46-cm Luna C_{18}(2). Eluent: 90% aqueous:10% ethanenitrile. Aqueous: 15Mm dipotassium hydrogen phosphate adjusted to w wpH 1.5-9 with phosphoric acid, Flow rate, 1 mL/min; temperature, 25^0C; detection, PDA

2.6 Reversed-phase versus normal-phase separations: Reversed-phase HPLC is the dominant method used for the majority of pharmaceutical applications (>95%). Normal-phase chromatography may be required for separations that are not compatible with reversed-phase mode. Solutes that are labile (i.e., reacts with protic solvents) or exhibit poor solubility in aqueous media are prime candidates for normal-phase chromatography. Normal phase is well-suited for the separation of isomers and diastereomers, as well as for separating compounds with saturated and unsaturated side chains. Generally, the greater is the amount of unsaturation the greater the retention due to increased polarizability of double bond. Diol phases are a good starting point for normal-phase application. Silica, amino, and cyano are alternative phases. Silica tends to strongly retain solutes that can interact with its highly active sites. Hexane or heptane modified with a polar organic solvent is generally utilized as the mobile phase. The polar organic solvent can be chosen based on it physicochemical properties (dipole, hydrogen bond acceptor/donor).

2.7 Instrument/system considerations: The four common causes for HPLC column failure include column clogging at the inlet frit (from samples/mobile phase), voids generated in the column, strongly adsorbed impurities from solvent/sample, and chemical attack of the stationary phase from the mobile phase or analytes..

2.7.1 ***Column/system backpressure*****:** Column backpressure gives a good indication of how the column and/or system are operating. The initial backpressure of the column should be checked prior to running a method. The backpressure with column attached at initial run conditions should be stated in the method. If high column back-pressure is observed, the column should be taken off and a ZDV (zero dead volume) should be installed and system backpressure recorded and compared to know system backpressure for that particular system under a certain set of mobile-phase conditions. Note that the system backpressure will be different from instrument to instrument due to the different types of tubings that are employed. This depends on the tubing diameters and total tubing length. The system backpressure is also dependent on the mobile-phase composition, the type of organic modifier, the temperature, and the flow rate. If the system back- pressure value is known on that particular system with a certain set of eluent and column conditions and the backpressure value is higher than normal, then the cause of this increased backpressure needs to be investigated. A recommendation is to start removing tubing, starting at the outlet and working your way back to the injector and then the pump, until you see a drop of pressure; then replace that piece of tubing that is leading to the problem with higher backpressure. Also, it is possible that an in-line filter may be clogged. The in line filter is usually made of a 0.5 μm porosity frit and is located either between the pump and injector or between the injector and the column. It is recommended to change this in-line filter on a monthly basis (given continuous flow through the system during that month), especially if buffered eluents are used. These filters should be readily available, and an analyst should know how to replace them to avoid waiting for vendor engineer to change them. Also, a proper maintenance log for each HPLC must be maintained in a regulated environment, and any type of maintenance should be properly recorded according to the pharmaceutical departments standard operating procedures.

2.7.2 ***Column inlet and outlet frits*****:** One of the most common symptoms of column failure is high column back-pressure. Plugging is the most frequently encountered problem by analytical chemists or analysts. Injection of samples containing particulates, along with wear of pump

piston seals and injector valve rotor seals, will eventually block the column inlet, causing high column backpressure, and shorten the normal lifetime of the column. Backpressure also generally increases as particulate matter accumulates on the inlet frit of the column and may lead to band distortion of the peaks in the chromatogram. Columns packed with 5-µm particles typically use 2-µm porosity inlet frits to contain the packing; for the 3-µm particles, 0.5- or 2-µm porosity inlet frits are used, depending on the manufacturer. To a certain extent, particulate matter buildup on the frit is unavoidable, but there are some several simple practices that can slow down this process such as filtering the mobile phase and the sample prior to injection. Also, centrifuging the sample is recommended for at least 2 min at 10,000rpm. If it is determined that a column inlet frit is blocked, then back flushing the column might help. For back flushing, remove the column from the system and connect the outlet end (normal direction flow) to the pump and put the inlet end into a beaker and pump at low flow rate 0.5mL/min using 80%ethanenitrile: 20%water for at least 20 column volumes, (column volume can be estimated by the volume of the cylinder, $\pi R^2 L$ for a 150-˜3.0-mm column the column volume is ~1.1 mL) to displace any particles from the frit.

2.7.3 ***Seals*****:** Maintenance and care of the pump piston seals is recommended. Buffers and other types of salt additives (i.e., ion pairing reagents, chaotropic additives) that are not soluble in organic solvents should not be allowed to reside in the LC system when there is no flow through the HPLC system. Also, the buffer solubility limit in a particular solvent system should be known to prevent precipitation of the buffer salts in the HPLC system. A test tube precipitation test can be used to determine if the concentration of the organic will trigger precipitation. If the buffer solution is left in a dry LC system, the buffer salts can evaporate on the piston surface behind the pump seal, thereby creating an abrasive coating of salt crystals that will damage the seal over time. Therefore it is recommended to wash the HPLC system with acetonitrile/water (20 : 80) for at least 30 min before the system is shut down to remove any potential buffer residues. A shut-down method with this wash method is recommended at the end of the sequence. Pump piston seals usually last for at least 6 months (if system is continually used throughout that duration) and are usually replaced during the preventive maintenance on the HPLC system. Injector rotor valve seals can last for greater than 10,000-20,000 cycles, and these should changed on an annual basis during the yearly preventive maintenance/calibration of the instrument.

2.7.4 ***Mobile-phase preparation*****:** The operations of pump components, injectors, and detectors can be expected to be less troublesome when mobile phases are filtered. For HPLC applications, the 0.2-µm-pore-size filter is typically selected for removal of particulates that may arise from physical contaminants such as fibers. Generally, it is recommended to filter both the aqueous and organic portions of the mobile phase independently. Note that premixed mobile phases with organic and aqueous should not be filtered (using vacuum filtration) since this may change the final composition of the organic in the filtered mobile phase. Also the mobile phases must be covered to avoid evaporation of the buffer components (TFA, acetate, bicarbonate, ammonium hydroxide) and the solvent (especially if premixed mobile phases are used). Also this prevents dust and other particulates from contaminating the mobile phase. The mobile phase should be covered with suitable caps, and the use of aluminum foil is also encouraged. One of the most common mistakes that analysts make is the use of polymeric products to cover the mobile phase (parafilm), since this is not compatible with organic solvents and may lead to potential

contamination of the mobile phase with polymeric components. It is also recommended to add at least 5-10 v/v% of organic solvent to the aqueous phase to prevent microbial growth.

2.7.5 ***Guard columns*****:** A guard column is a small column that contains packing material similar to that in the analytical column. The pore size and particle size of the material can be the same as the packing material in the analytical column. The guard column has a frit at each end, and the frits trap particulate matter. According to the column vendor claims, these guard columns are supposed to help to prevent contamination of the analytical column, but they also may lead to decrease in the efficiency of the peaks of interest due to the addition of extra column volume. It may prevent contamination in the short term, especially for biological samples; however, on a long-term basis, since contaminants are also molecules and they are moving along the column, usually moving slower than analytes, they may elute from the guard column into the main column. The guard column is an excellent mixing chamber and can lead to band dispersion. However, guard columns are often used and recommended for analysis of proteins, lipids, and other high-molecular-weight species, if the sample is biological in origin (urine, plasma, etc.), to prevent contamination of the column with matrix components. The need for replacing the guard column depends on the matrix, the number of injections, and whether decreased performance is observed (change in efficiency for isocratic separations by more than 10% and gradient method change in apparent efficiency by more than 20%).

2.7.6 ***Instrument/system considerations (concluding remarks)*****:** There are quite a few techniques that can be used to extend the useful life of an HPLC column; these include employing "column shock" method after several hundred injections, running at lower operating temperatures, not running at pH extremes, and using moderate concentrations of buffers, to name a few. However, the column should be considered as a disposable item. However, the cost of the column should be viewed in terms of overall analysis costs. Assume that each analysis an analyst performs is on the order of $5 per sample. If the column lasts for 500 injections, this means that the column contributes to 20% of the cost. Many analysts may get 1000-2000 samples analyzed before a column dies, so the cost per sample would be even less: 10% of the cost and 5% of the cost, respectively.

2.8 Column testing (stability and selectivity): There are numerous tests in the literature in regard to the quest for defining universal tests to probe the silanol activity and to define a set of mixture of compounds for selectivity assessment and comparison of reversed phase stationary phases. The tests include different probe molecules run under different conditions (pH and organic composition, isocratic and gradient test procedures). Even the different tests on the same column may lead to different results (in terms of selectivity and silanol activity ranking) [35-39].

2.8.1 ***Column selectivity testing*****:** An area of intense investigation is choosing the column with the best selectivity. The selectivity is dependent on the bonded phase (i.e., bonded ligand, silanol activity), the probe analytes, the pH of the mobile phase, the type and concentration of the buffer, and the type and concentration of the organic modifier, especially if the probe analytes are ionizable. For a given set of probes the selectivity may be high on a specific column; however, for another set of analytes the selectivity may be poor. There is no universal selectivity test that can ensure that a particular column will give the desired selectivity for a set of compounds. However, if enough knowledge is gained on a set of columns in regard to selectivity with certain

probe compounds and these have been good predictors of columns demonstrating adequate selectivity for pharmaceutical compounds in a particular company, then these may be used to screen new columns that come on the market that have demonstrated good bonded-phase stability within a particular pH range.

2.8.2 ***Column stability testing*****:** Due to extended usage of the column under certain pH and temperature conditions, the bonded phase could become compromised, leading the poor chromatographic peak shapes, loss in efficiency, and loss and even sometimes increase in analyte retention. Sometimes, peak shapes can become dramatically distorted upon increased usage of the column without any change in the retention time, and this may also be dependent on the type of analyte in the mixture (base versus neutral). However, if there is a loss of bonded phase, then losses in retention may be observed for all components (neutral and basic). However, if loss of bonded phase and end-capping reagent and/or change to stationary-phase surface occurs and greater exposure of the residual silanols is prevalent, then the increase in retention and peak tailing for protonated basic components may be observed while the retention for neutral compounds may decrease. Columns for a particular laboratory can be chosen based on some set of internal criteria. One of the criteria to select a column should be such that the column is stable for a certain number of column volumes (efficiency, tailing factor, and retention time criteria for predefined probe analytes) at the recommended maximum and minimum pH at a particular maximum temperature. This would allow the chromatographer to employ such phases with a significant degree of confidence and ensure the robustness of the stationary phase during method development and for release and stability testing.

The pH/temp stability limits of the columns usually provided by the vendors are general recommendations. However, if determination of the representative column stability is required, the analyst should use column stability testing conditions similar to those used in their laboratories for testing their samples. The overall column stability depends on many factors such as whether the study is performed in either isocratic or gradient modes, whether an organic or inorganic buffer is used, type and concentration of organic modifier, temperature, pH of the mobile phase, type of counter anion and counter cation of buffer, the flow rate, and backpressure (mechanical stability). Also, the results of the analysis could potentially be confounded if the aqueous component of the mobile phase does not contain any organic due to increased probability of microbial growth, which may lead to the clogging of the inlet frit and cause peak distortion for all peaks in the chromatogram. Therefore, if one is going to use only aqueous solely in line A and organic solely in line B, the aqueous portion A should be replaced every 48 hours.

2.8.3 ***Choice of buffer related to bonded-phase stability:*** The type of pH modifier to make a desired mobile phase pH also has an effect on the column stability, and this is indirectly related to the peak efficiency and the retention of the analyte. As an increasing number of column volumes of the mobile phase are traversed through the column, the stability of the packing material could be comprised. Rearrangement of the packing bead leads to the loss of efficiency, dissolution of silica leads to loss in efficiency and retention, and hydrolytic decomposition of the bonded phase could impact the peak shape and retention. Different compounds, such as neutral compounds, acidic compounds, and basic compounds, could show different behaviors. Different types of buffers at the same ionic strength and w wpH can have a significant impact on the

dissolution of silica. The dissolution of silica is usually measured by the silicomolybdate colorimetric method [41].When determining the bonded-phase stability using different run buffers (effect of buffer counteranion or countercation), the same sspH must be used. The sspH values (pH of the mobile phase: aqueous organic) may be different from the aqueous portion of the mobile phase and may obscure if the dissolution of the silica is directly related to the type of anion/cation and/or the pH.

Conclusion

A well-defined method development plan with clear aim of analysis is critical to the success for fast and effective method development. The general approach for the method development for the separation of pharmaceutical compounds was discussed, emphasizing that modifications in the mobile phase (organic and pH) play a dramatic role on the separation selectivity. The knowledge of the p*Ka* of the primary compound is of utmost importance prior to the commencement of HPLC method development. Moreover, pH screening experiments can help to discern the ionizable nature of the other impurities (i.e., synthetic byproducts, metabolites, degradation products, etc.) in the mixture. The separation of a complex mixture of different ionizible and nonionizible organic components can be challenging, and the development of a rugged separation method can be an adventure. A multitude of approaches can be applied; however, the one that seems to work the best is to screen a limited set of columns at a certain predefined pH range (1-2 pH units below or above the target analyte p*Ka* in a particular hydro-organic mixture) and determine the best permutation and combination of column/mobile phase in order to obtain the specific selectivity that is desired for critical pair of components in the mixture. There are thousands of different columns on the market, and the selection of one that will allow a simple separation of your mixture is more a black magic than a science, but usually the most common columns to carry out initial method development include those comprised of C_{18}, polar embedded/end-capped and/or phenyl bonded ligands. Final optimization can be performed by changing the temperature, gradient slope, and flow rate as well as the type and concentration of mobile-phase modifiers.