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A simple and efficient approach to reversed-phase HPLC method screening

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ABSTRACT

The development and utility of an efficient HPLC method screening strategy using only four columns for the separation of pharmaceutical compounds and related impurities is presented. The strategy established a two-column approach to enable rapid early method development, along with a four-column approach for commercial method development of the analytical methods utilized to verify the quality of drug substance or drug product. Mobile phases consisted of acetonitrile or methanol with aqueous trifluoroacetic acid for low pH screening, and ammonium hydroxide for high pH screening. Examples are provided to demonstrate the practicality and orthogonality of the method screening process. A unique system suitability check, using commercially available compounds, was incorporated as a tool for troubleshooting and for ensuring adequate system performance prior to screening. Initial testing of the strategy revealed that the columns chosen were successful in leading to assay and impurity methods for 40 pharmaceutical compounds.

1. Introduction

Liquid chromatography is the most widely used analytical tool in the pharmaceutical industry and reversed-phase is the most frequently used mode. During the drug development process, stability-indicating liquid chromatographic methods are used to determine the quality of the drug substance (active pharmaceutical ingredient) and drug product. Impurity methods are developed to ensure the patient is receiving a safe product.

Within our laboratory, the method development goal may be quite different depending on the stage of development for the project. For an early stage development project (enabling initial human clinical studies), the goal of method development is typically a broad gradient method which is MS-friendly and can be employed for both assay and impurity control (and possibly for identity, dissolution, and content uniformity). At this stage, the primary factors of an HPLC method (column, organic modifier, and pH modifier) are evaluated and defined in order to narrow the chromatographic design space. As the project approaches later stages of development and product launch, the goal becomes the generation of efficient, robust control methods that are typically supported by quality control laboratories. Throughout development, knowledge of orthogonal HPLC conditions can be useful in the investigation of impurities.

Reducing expenses and improving efficiency have been a focus for many pharmaceutical companies. A simple, harmonized approach to HPLC method screening can reduce cycle time for method development. Several examples in the literature discuss the use of streamlined method development or screening processes. A recent publication by Xiao et al. used the ChromSword® method development software in conjunction with automated column switching for challenging separations (e.g., alpha and beta methylepoxide) while utilizing columns from major vendors [1]. Other examples illustrate automation for peak tracking as well as column and mobile phase screening in addition to the use of software tools for optimization (ChromSword®) [2,3].

Using hyphenated UV-MS detection and comprehensive orthogonal method evaluation technology (COMET), Xue et al. evaluated 32 HPLC columns and 18 mobile phase combinations using 18 proprietary compounds [2]. From this study, five stationary phases were deemed to provide optimum selectivity differences, although the selection criteria were not disclosed. The COMET software provided processing and peak tracking capabilities which greatly improved efficiency. However, since the proprietary structures used in this study were not disclosed, the results may not translate into universal column selectivity for other compounds from other organizations.

The strategy developed by Hewitt et al. utilized 42 parameter combinations with five columns, four aqueous buffers, and two organic modifiers to evaluate an RP screening strategy for lamotrigine and related isomers [3]. Selection of the starting conditions was subjective; however, the ChromSword® software was used to fully optimize the separation. This strategy by Hewitt demonstrated that better selectivity can be achieved in less time as compared to a manual approach. The column choices were based on the previous reference [2] but also included aqueous buffers comprising potas-

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sium phosphate or sodium borate (high pH) as part of the screen. Using non-volatile buffers can lead to further development later in the development cycle if investigations need to occur using LC–MS. Additionally, this strategy worked well for this specific molecule and related isomers, but was not proven for a large variety of different structures.

Another application of a method development strategy was published by Krisko et al. entailed the use of simple mass spectrometric compatible buffers, varied pH, and a wide range of 16 different stationary phases selected using the Column Match™ program. However, acetonitrile was the only organic modifier tested. This screening strategy was thoroughly tested with a variety of analytes, including a challenging mixture of acid, base, and neutral compounds. Data was imported to DryLab™ software for prediction optimization; however, the large number of stationary phases resulted in large amounts of data to be processed and analyzed [4].

Van Gyseghem et al. applied chemometrics to evaluate 28 stationary phases based on eight chromatographic parameters, including hydrophobicity, steric selectivity, efficiency, silanol activity, H-bonding capacity, and ion-exchange capacity. Two test mixtures each containing three analytes were used to generate the data. The authors ranked the stationary phases that should provide the most efficient and selective separations for impurities in drugs, although an actual drug impurity case was not described. In another publication, the same authors evaluated eight silica-based stationary phases with four mobile phase systems (acetonitrile as the only organic modifier) at various pH values in an attempt to provide the maximum orthogonal system. There were 68 drugs (mostly basic) used in groups as the test mixtures, as well as 15 proprietary drug substances as a test mixture. The number of chromatographic systems evaluated by this approach was rather high; however, the results led to a reduction in the number of systems needed to 40% from the initial starting point [5,6].

Although automation and software packages have been shown in several cases to provide efficiency in implementation, they often produce a larger volume of information that needs to be analyzed and processed because more experiments can be conducted within a similar timeline due to the efficiency gains. The use of a large number of columns, although providing more data-driven opportunities to find selectivity differences, also results in a large volume of information. Additionally, peak tracking and data compilation for a sample with many peaks can become overwhelming as more column and mobile phase combinations are investigated.

This paper reports a strategy that has used a mathematical approach (Column Match™) to find optimum selectivity for a strategy that employs only a two-column approach to enable rapid early method development along with a four-column approach for commercial method development. Also described are simple MS-compatible mobile phases with UV detection which can easily be followed by practically all lab scientists without additional training, and also allow easy transfer to skilled LC–MS experts.

Although software packages can greatly improve efficiency, they can also complicate operations with the need for additional training. Since the strategy reported here is simplified by the small number of experiments generated with the two- and four-column choices, software packages were not required to analyze and process data due to the lower volume of information compared to other approaches. Prior to developing this approach, the authors surveyed internal method development groups to gauge the variability in selection of stationary phase, organic modifier, and pH modifier. The results encompass 44 methods for a total of 31 drug candidates for which 15 stationary phases were employed to complete these methods.

Implementing a simple and efficient approach to reversed-phase method development, reduced expenses, eliminated random or non-scientific based column selection, improved efficiencies, and provided consistency during the entire HPLC development process. In this report, the success of this reversed-phase HPLC method screening strategy is discussed and illustrated with proprietary small molecule pharmaceutical drug candidate examples and a system suitability test mixture composed of commercially available compounds.

2. Experimental

2.1. Materials and supplies

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were obtained from EMD Chemicals Inc. (Gibbstown, NJ). ACS reagent-grade ammonium hydroxide solution (28.0–30.0%, w/w) was obtained from J.T. Baker (Phillipsburg, NJ). The 99.5% purity trifluoroacetic acid (TFA) was supplied by Acros Organics and obtained through Fischer Scientific (Pittsburgh, PA). House de-ionized water was used without further treatment.

TFA solutions were prepared at 0.1% (v/v) by adding 1 mL of acid to 1 L of water or organic modifier and mixing well. The ammonium hydroxide solution was prepared at a final concentration of about 9 mM by weighing 1.75 g of ammonium chloride into a 250 mL Erlenmeyer flask. Approximately 240 mL of de-ionized water was added and mixed until the NH_4Cl was dissolved. While stirring, the pH of the solution was adjusted to 10.0 with NH_4OH (28.0–30.0%, w/w). Ten mL of this solution was diluted to 1 L using de-ionized water and organic modifier as needed.

The Zorbax SB C-8 (75 mm × 4.6 mm, 3.5 μm) and Zorbax Bonus RP (75 mm × 4.6 mm, 3.5 μm) were obtained from Agilent Technologies (Palo Alto, CA). The Xterra MS C18 (75 mm × 4.6 mm, 2.5 μm) was obtained from Waters (Milford, MA) and the ACE Phenyl (75 mm × 4.6 mm, 3 μm) was purchased through Mac-Mod (Chadds Ford, PA). The ACE CN (75 mm × 4.6 mm, 3 μm) can be obtained from Mac-Mod (Chadds Ford, PA), the Waters Atlantis dC18 (75 mm × 4.6 mm, 3 μm) can be obtained from Waters (Milford, MA), the Varian Polaris C-8 Ether (75 mm × 4.6 mm, 3 μm) can be obtained from Varian (Palo Alto, CA), and the Alltech Alltima C-18 (75 mm × 4.6 mm, 3 μm) can be obtained from Grace (Deerfield, IL).

2.2. System suitability and sample preparation

Atenolol, naproxen sodium, propanolol hydrochloride, warfarin, pindolol, indoprofen, terfenadine, and retinoic acid were all commercially available from Sigma–Aldrich (St. Louis, MO). A stock solution was individually prepared for each analyte of the test mixture. Atenolol and retinoic acid were prepared at a concentration of 2 mg/ml, while the other compounds were prepared at 1 mg/ml. The sample solvent used to prepare the stock solution for each compound was different due to solubility differences: atenolol, naproxen sodium, and propanolol hydrochloride are soluble in water; warfarin, pindolol, and indoprofen are soluble in 50/50 acetonitrile/water; terfenadine is soluble in 75/25 acetonitrile/water; and retinoic acid is soluble in acetonitrile. One-hundred μl of each stock was then transferred to an amber HPLC vial and then the contents were hand-shaken to mix. The test mixture was frozen until use.

The drug substance Compound A impurity and degradant mixture was prepared at a concentration of approximately 0.005 mg/ml of each component in 50/50 acetonitrile/water. The drug substance Compound B impurity and degradant mixture was prepared at a concentration of approximately 0.1 mg/ml of each component in 50/50 acetonitrile/water.

Table 1
Mobile phase gradient conditions for HPLC screening.

Organic Modifier	Gradient	Equilibration Time (min)	Initial organic composition (%)	Gradient Slope (%/min)	Gradient Time, Tg (min)	Final organic composition (%)	Hold Time (min)
Acetonitrile	1	5.7	4.0	7.7	9.5	77.0	2.6
	2	5.7		1.9	38.1		
Methanol	1	5.7	5.0	9.5	9.5	95.0	2.6
	2	5.7		2.4	38.1		

2.3. Instrumentation

A Hewlett-Packard 1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA) equipped with a vacuum degasser, binary pump, autosampler, and variable wavelength detector was used.

Method parameters used for the screening process are outlined in Tables 1 and 2.

2.4. Software

JMP from the SAS Institute Inc., SAS Campus Drive, Building S, Cary, NC, 27513.

Column Match™ from Rheodyne LLC, Rohnert Park, CA, 94928.

Marvin from ChemAxon, Budapest, 1037 Hungary.

DryLab™ from LC Resources, Walnut Creek, CA, 94596.

3. Results and discussion

3.1. Column selection

Screening is defined as the process of identifying suitable starting conditions from which the final method can be established through further optimization. For screening experiments, maximizing the differences in the columns explored, but also minimizing the number of columns employed to cover as much of the development space as possible, is a practical approach.

Column characterization approaches have been thoroughly investigated and reported in literature [7–9]. These approaches often use a set of test solutes as probes to identify different properties of the column. Snyder and co-workers identified the parameters that impact column selectivity in an HPLC separation [10]. Column selectivity, according to Snyder, can be characterized by five different parameters: hydrophobicity, steric hindrance, acidity, basicity, and cation-exchange ability. Each of these parameters relate both to the stationary phase and the analyte. In recent years, the selectivity data generated on numerous reversed-phase columns based on these five factors were used with a software program (Column Match™) that allows the user to determine if two columns have differences in selectivity (orthogonality) or provide equivalency. Column selectivity has been discussed in much detail in a series of papers by Snyder and co-workers [10–15]. A factor (described as F_s) combining the five selectivity parameters is used in the software to determine equivalency or orthogonality between two columns.

Historically, this approach has been used to determine the selectivity factor to facilitate comparison of two columns based on their properties. Rather than comparing columns pair-wise, a different approach was selected here. A cluster analysis using Ward's method [16] was performed on the column-specific coefficients (H, S^*, A, B, C) using JMP statistical software. The intent was to logically group columns based on similarity so that a subset of columns could be selected that exhibit maximally different properties.

Beyond selectivity differences, column selection should also be influenced by (1) the probability of success based on experience, (2) peak symmetry (i.e., absence of adverse interactions with the stationary phase), (3) the reliability of the vendor and column manufacturing processes, (4) the stability of the column, (5) global availability, and (6) the availability of column configurations (column dimensions and particle size) that facilitate screening. The columns evaluated were limited to those prepared using Type B silica and only those columns available with particles sizes $\leq$3.5 μm were considered.

Results from the cluster analysis of data available in Column Match™ are shown in Fig. 1. It is interesting to note that in several cases, columns from the same vendor are often clustered together despite differences in the bonded phase. This is likely the result of common properties of the underlying silica, implying that maximum differences in column properties may only be achieved by using columns from different vendors. Also note that the Zorbax Bonus RP column is unique, possibly due to residual amines on the surface of the silica gel.

Using this approach, seven clusters were identified, although within clusters there were clearly differences among the columns. Because the goal of this work was to cover as much of the space as efficiently as possible, a set of four columns from different clusters, designated as first tier (Table 3), was selected.

The Zorbax SB C8 column was selected due to its reliability and frequency of use based on previous methods captured during the internal survey. It also is a sterically protected phase which allows for enhanced pH stability at low pH.

The ACE Phenyl column was selected to allow for a difference in selectivity and particularly to enhance pi–pi interactions with aromatic molecules. Based on previous experiences, the ACE Phenyl column also provides highly symmetric peaks and in some cases improved analyte loadability.

Table 2
Column and mobile phase components for HPLC screening.

Sequence	Column Identification	Organic Modifier	pH modifier
1 5	Zorbax SB C-8 75 mm × 4.6 mm id/3.5 μm	ACN MeOH	0.1% (v/v) TFA
2 6	Zorbax Bonus 75 mm × 4.6 mm id/3.5 μm	ACN MeOH	0.1% (v/v) TFA
3 7	ACT Ace Phenyl 75 mm × 4.6 mm id/3 μm	ACN MeOH	0.1% (v/v) TFA
4 8	Xterra MS C-18 75 mm × 4.6 mm id/2.5 μm	ACN MeOH	pH 10 ammonium hydroxide buffer

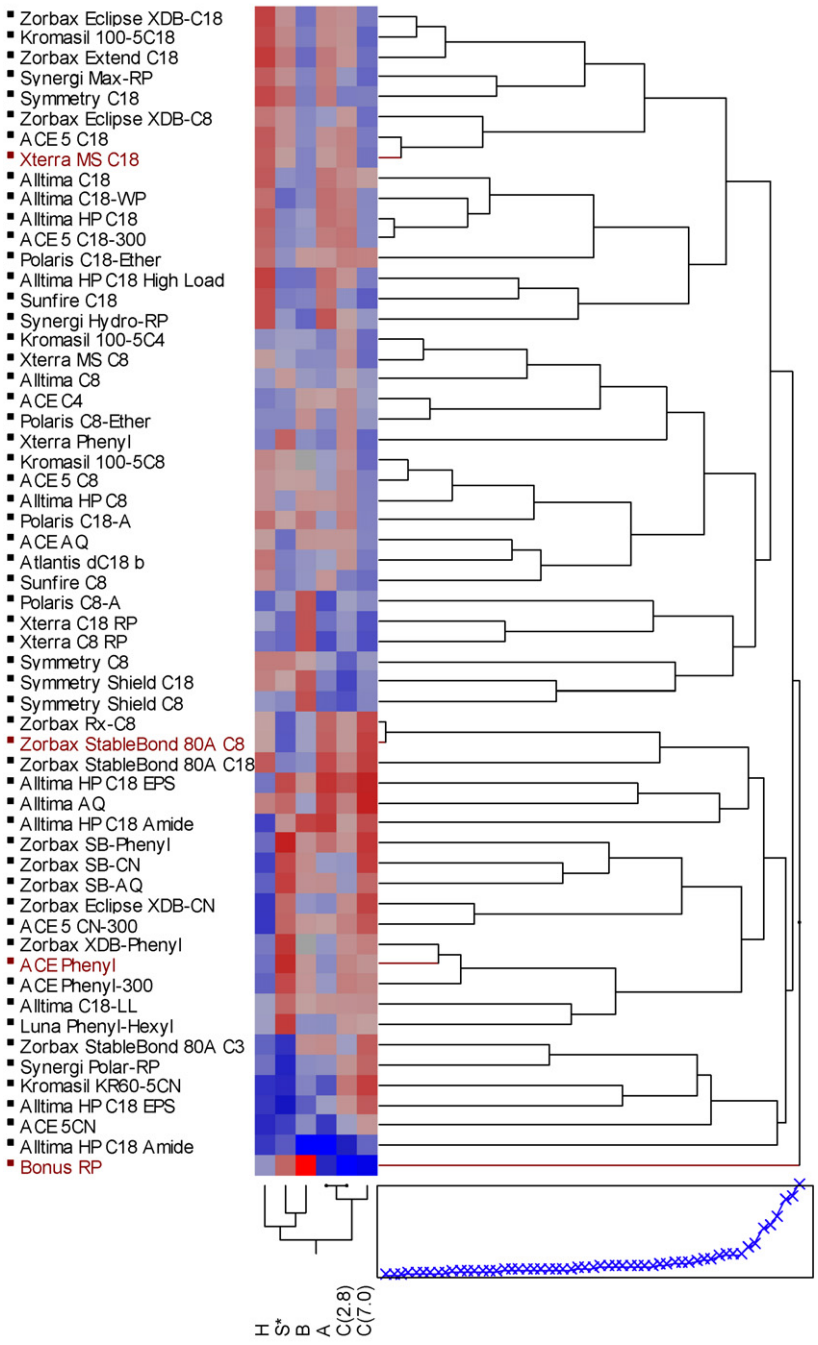


Fig. 1. Dendogram showing groupings of columns based on approach of Snyder et al.

Table 3
Orthogonal columns determined from cluster analysis.

Column Identification	Column Chemistry	Vendor-Reported pH range	Chemical interaction with solute
Zorbax SB C8	Sterically protected, Non-endcapped	pH 1–6	Hydrophobic, dispersive forces
Zorbax Bonus RP[a]	Sterically protected, Embedded Polar Group, Endcapped	pH 2–8	Basic and dipolar interactions
HiChrom ACT Ace Phenyl	Endcapped	pH 1.8–11	π–π interactions, dispersive
Waters Xterra MS C18	Non-polar, Most stable Xterra column	pH 1–12	Hydrophobic, dispersive forces

[a] Tailing peaks are observed for acidic analytes using the Zorbax Bonus RP column; consequently, a different selection would be appropriate when the test solutes include acidic compounds.

The Zorbax Bonus RP, a polar embedded phase, was selected due to its unique selectivity (see dendogram in Fig. 1), a column that may help provide a solution to a difficult separation. This uniqueness in selectivity compared to other reversed-phase stationary phases is a result of the mixed mode separation that this phase provides. Mixed mode is defined as two potential types of interactions of the analyte with a given stationary phase (e.g., hydrophobic interactions and electrostatic interactions). The surface of the stationary phase can dictate this type of interaction.

The Waters Xterra MS C18 was chosen primarily to provide an option for high pH screening. Few stationary phases are available for high pH application and this phase was able to provide longer term stability compared to other phases.

A second set of four columns was also selected to cover the remaining clusters. These include the columns: HiChrom ACT Ace CN, Waters Atlantis dC18, Varian Polaris C-8 Ether, and Alltech Alltima C-18. However, after screening over 40 different pharmaceutical compounds, the second set was not needed, demonstrating that a first tier column is sufficient in most cases. Table 3 shows the predominant solute–stationary phase interactions that would be expected based on column chemistry [17]. It is important to note that other columns from within a given cluster may have given similar results to the columns selected.

The screening strategy employed short columns (75 mm × 4.6 mm id) packed with stationary phase particles 3.5 μm or smaller, thus limiting the time required to acquire the data. Because the purpose of the screening is to evaluate selectivity, a shorter column length (75 mm rather than 150 mm) was selected because the difference in efficiency is not sufficient to have a significant impact if peaks are poorly resolved.

3.2. Selectivity as a function of organic modifier, pH, and temperature

Although column selection is very important, it also has been reported that the greatest changes in selectivity are often afforded by changes of organic modifier [17]. Typical organic modifiers for reversed-phase include acetonitrile, methanol, and in some cases, tetrahydrofuran. However, the use of acetonitrile is preferred because of the low UV cutoff and low viscosity compared to isoeluotropic solutions containing either methanol or tetrahydrofuran. For this screening strategy, acetonitrile was used first, followed by methanol. In this study isoelutropic mobile phases were used to find differences in band spacing rather than difference in absolute retentiveness.

The mobile phase pH can be a significant factor that drives the selectivity of the method due to differences in the pK_a of analytes. Usually, acidic conditions are the first choice for the chromatography of both acids and bases. Under these conditions, acids are non-ionized whereas bases are ionized. Particularly for bases, an advantage of using low pH mobile phases is that the silanol groups are not ionized, leading to a more favorable peak shape for basic solutes. A disadvantage of using acidic conditions for basic analytes is that the protonated basic analyte will be less retained than the non-ionized form.

For early phase projects in our laboratory, volatile pH modifiers are preferred. A volatile modifier permits the development of MS-compatible chromatographic conditions. A commonly used modifier for this purpose is TFA, although it has limitations regarding ionization suppression in MS detection and high absorbance at low wavelengths [18]. Typically, TFA is used at a concentration of about 0.1% (v/v) in both the strong and weak solvents. Under basic conditions, ammonium hydroxide may be used.

The purpose of screening is not to study the effect of pH on a single column, but rather to maximize the pH range explored to determine if there is value in pursuing pH as a variable. Regardless of the identity of the buffer salt, it is important to realize that the actual pH of the organic containing solutions will be different from the measured buffer pH [19]. This effect can cause the pH to be much closer to the stability limits of a column than expected.

Temperature is not a variable for the screen, but it is maintained above ambient conditions (in this case at a fixed temperature of 35 °C) to reduce viscosity, improve efficiency, and minimize drift due to temperature change. The impact of temperature can be investigated once the primary method variables (column, organic modifier, and pH) are established.

3.3. System suitability

A system suitability test can verify that the HPLC system is suitable for screening experiments by demonstrating the adequate functioning of the instrument, electronics, column, and mobile phase. The system suitability test adds a level of confidence that reproducible results can be attained, especially with regard to column age and condition. In this report, the system suitability test mixture was used to verify that the system set-up was functioning properly prior to initiation of screening experiments. This test mixture, consisting of eight commercially available acidic and basic compounds, was developed to capture variations in analyte polarity and molecular structure.

Table 4 shows the names and structures of the compounds in the system suitability solution. The recommended preparation procedure was described in the experimental section. Since retinoic acid is unstable to light, an amber vial was required to minimize degradation.

Different sample solvents were needed to initially dissolve stock solutions of each compound (due to differences in polarity); hence, a smaller injection volume of 2 μl is required to avoid disturbances in the chromatography. For example, atenolol can result in a split peak at high injection volumes due to the sample solvent composition of the final system suitability test mixture. Freeze/thaw experiments were also conducted to demonstrate that stock solutions could be prepared and stored in the freezer to minimize the frequency of preparation.

Prior to developing methods using this strategy, a system suitability test mixture was injected to ensure the HPLC system was functioning properly. Figs. 2 and 3 show the system suitability test mixture chromatograms for each column used in the screen with acetonitrile and methanol, respectively.

The system suitability test mixture was also used to demonstrate the orthogonality of the experimental design. Figs. 2 and 3 indicate that differences in selectivity were observed across the eight different screening experiments. Recall that three of the columns (Zorbax SB-C8, Ace Phenyl, and Zorbax Bonus RP) were evaluated at low pH with TFA as the modifier and that only one column, the Xterra MS-C18, was evaluated at high pH with hydroxide as the modifier. From a column perspective, differences in retention order were observed when comparing the Zorbax SB-C8 and Zorbax Bonus RP based separations. The Zorbax SB-C8 and Ace Phenyl separations provided similar retention orders.

Organic modifier impacted selectivity, as noted by differences in peak pair retention orders. For example, the peak order switched for indoprofen/naproxen on the Ace Phenyl and retinoic acid/propanolol on the Xterra MS-C18. The impact of pH was apparent through the observed differences in retention order between the Zorbax SB-C8 (low pH separation) and the Xterra MS-C18 (high pH separation). These differences were greater than those expected between a C8- and C18-based separation; therefore, they were attributed to the change in mobile phase pH.

3.4. Screening experiment examples

To demonstrate the utility of the screening approach, two examples from the 40 pharmaceutical compounds screened are discussed. When performing the screening experiments we typically use mixtures of the compounds of interest, all prepared at approximately the same concentration. Of course, impurities in actual samples would be at low levels, typically <0.1%, compared to the main component. The screening chromatograms, even though not equivalent to those from actual pharmaceutical samples, are useful in assessing selectivity, the primary goal of the screening experiments. Given the relative concentrations of impurities in actual samples, we set higher expectations for the separation of impurity peaks near the main component relative to expectations for the separation of impurities from each other. In the first example, the complete experimental set was performed to sufficiently map the chromatographic design space. The compound set was a basic drug substance, Compound A, with related impurities and degradation products. The results from the chromatographic experiments are shown in Figs. 4 and 5.

In Fig. 4, evaluating the eleven-component mixture with acetonitrile and the 9.5 min gradient across the four different columns

Table 4
Listing of the components in the system suitability solution.

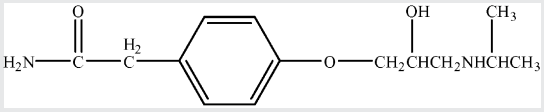
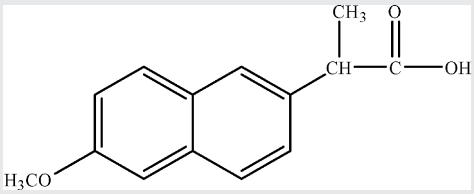
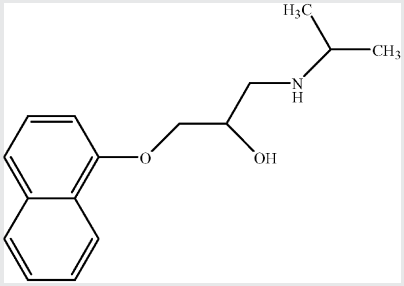
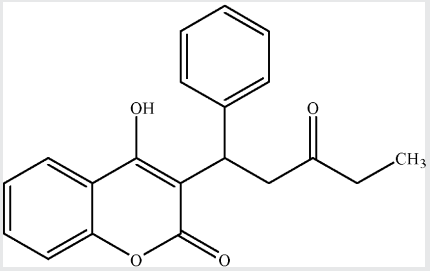
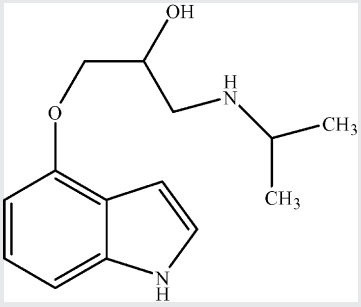
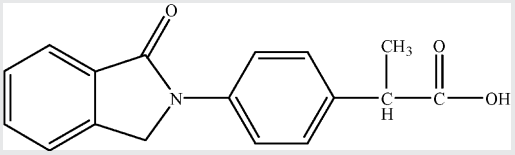
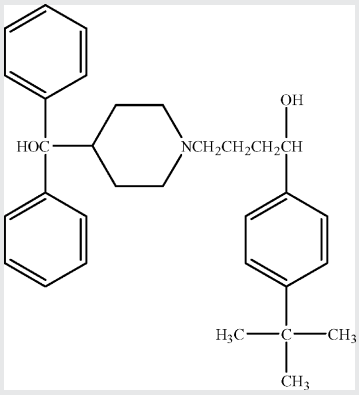
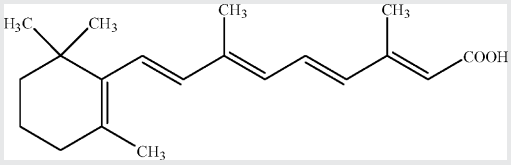
Compound	Structure	Log P^1	pK_a^2
Atenolol		0.56	9.87
Naproxen		2.99	4.19
Propanolol		2.80	9.87
Warfarin		3.80	4.38
Pindolol		1.90	9.87
Indoprofen		2.80	3.74

Table 4 (*Continued*)

Compound	Structure	Log P^1	pK_a^2
Terfenadine		7.05	10.20
Retinoic Acid		4.73	5.00

[1]Marvin Calculated.
[2]Calculated.

is shown, demonstrating the capability of the experimental design. Differences in selectivity are apparent across the four experiments as demonstrated by contrasting band spacing and retention order.

The same experiments were repeated with methanol as the organic modifier, and the results are shown in Fig. 5. This example demonstrates that key experimental parameters of stationary phase, organic modifier, and pH have a significant effect on the selectivity of the separation. In addition, the example shows that through a very limited number of experiments, a set of conditions can be identified that are appropriate for further optimization. For the Compound A example, the Zorbax Bonus RP with acetonitrile was selected as the best solution and these conditions were then further optimized.

The second example demonstrates the utility of the approach when rapidly developing a chromatographic method to enable initial clinical trials for a drug candidate. For this example, the compound set was a weakly acidic drug substance, Compound B, with related impurities and degradation products. Since this case required rapid method development, only a partial set of experiments was performed, namely the evaluation of the Zorbax SB-C8 and Ace Phenyl stationary phases with acetonitrile and methanol at low pH. The results from these chromatographic experiments can be found in Fig. 6. It is interesting to note that both column and organic modifier have a significant impact on the selectivity of the method. From this data set, the Zorbax SB-C8 with acetonitrile was selected as the best solution.

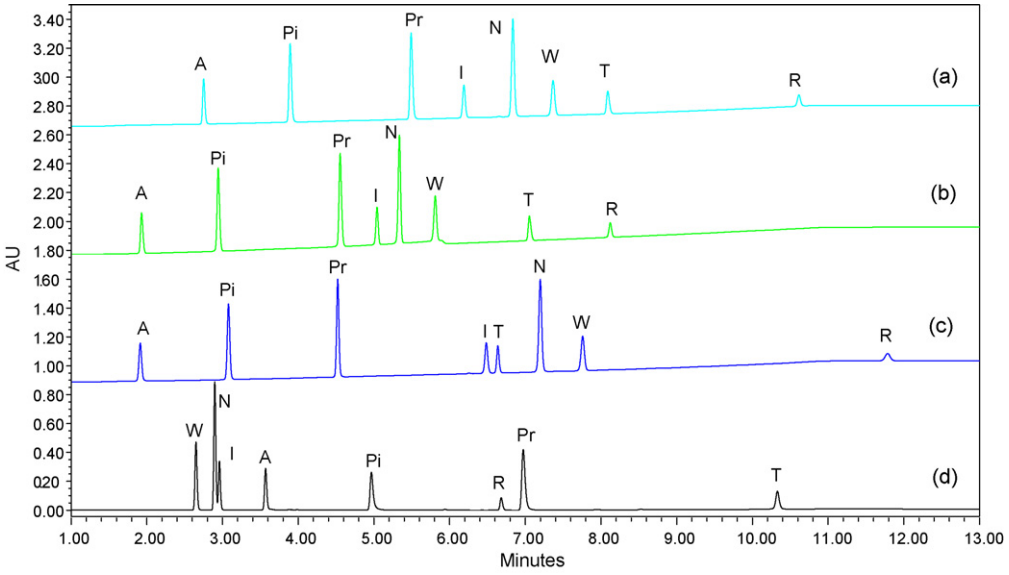


Fig. 2. Demonstration of selectivity differences generated with the system suitability solution and all four columns with acetonitrile as the organic modifier. The chromatograms correspond to (a) Zorbax SB-C8, (b) Ace Phenyl, (c) Zorbax Bonus RP, and (d) Xterra MS-C18. Peak identification is the following: A = atenolol, Pi = pindolol, Pr = propanolol, I = indoprofen, N = naproxen, W = warfarin, T = terfenadine, R = retinoic acid.

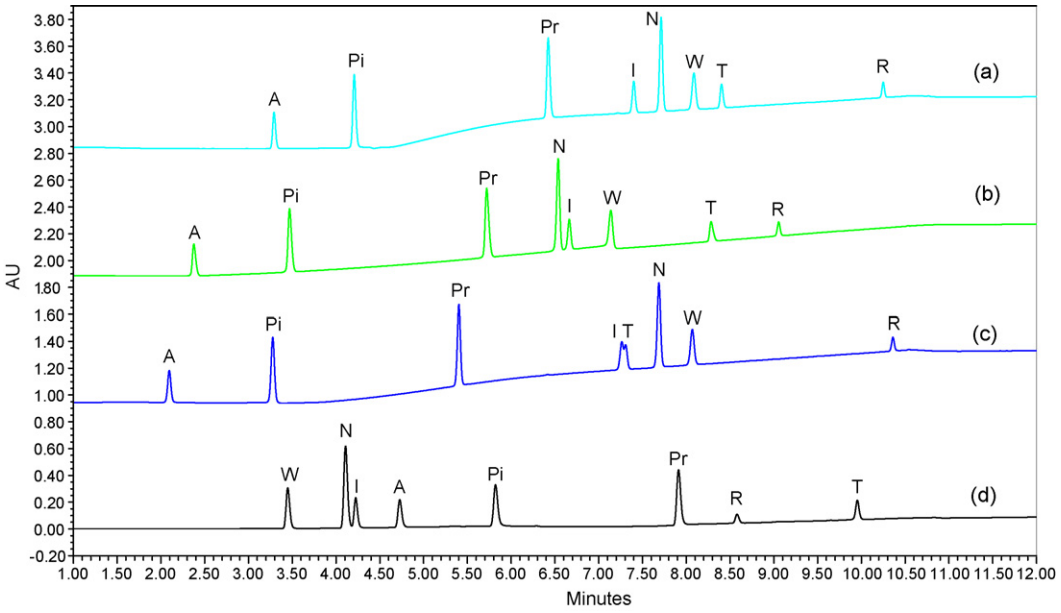


Fig. 3. Demonstration of selectivity differences generated with the system suitability solution and all four columns with methanol as the organic modifier. The chromatograms correspond to (a) Zorbax SB-C8, (b) Ace Phenyl, (c) Zorbax Bonus RP, and (d) Xterra MS-C18. Peak identification is the following: A = atenolol, Pi = pindolol, Pr = propanolol, I = indoprofen, N = naproxen, W = warfarin, T = terfenadine, R = retinoic acid.

3.5. Orthogonality assessment

Beyond the visual approach, several more quantitative approaches for determining if a separation is orthogonal when compared to an alternative separation have been published. One such approach from Snyder et al. consists of extracting information from plots that compare the retention of a series of analytes produced by one separation with the retention of the same series of analytes generated by a second separation [20]. An indication of orthogonality is the $|\delta \log \alpha|_{avg}$ value which is determined according to the following equation:

$$\left|\delta \log \alpha\right|_{avg} = 1.4\left(\frac{4\,\Delta\phi F}{t_G}\right) \mathrm{SE}$$

where $\Delta\phi$ is the change in volume fraction of the organic modifier during the gradient, F is the eluent flow rate in ml/min, t_G is the gradient time in minutes, and SE is the standard error from the linear least squares regression of the two data sets. Snyder has suggested that a $|\delta \log \alpha|_{avg}$ value of >0.10 is needed to establish that one set of separation conditions is orthogonal to a second set of separation conditions.

In order to more quantitatively compare the results presented earlier, the $|\delta \log \alpha|_{avg}$ values for the separations evaluated for the system suitability mixture and the Compound A set were determined by comparing each set of separation conditions to the designated standard separation conditions: Zorbax SB-C8 column, acetonitrile/TFA mobile phase. The resulting $|\delta \log \alpha|_{avg}$ values are listed in Table 5.

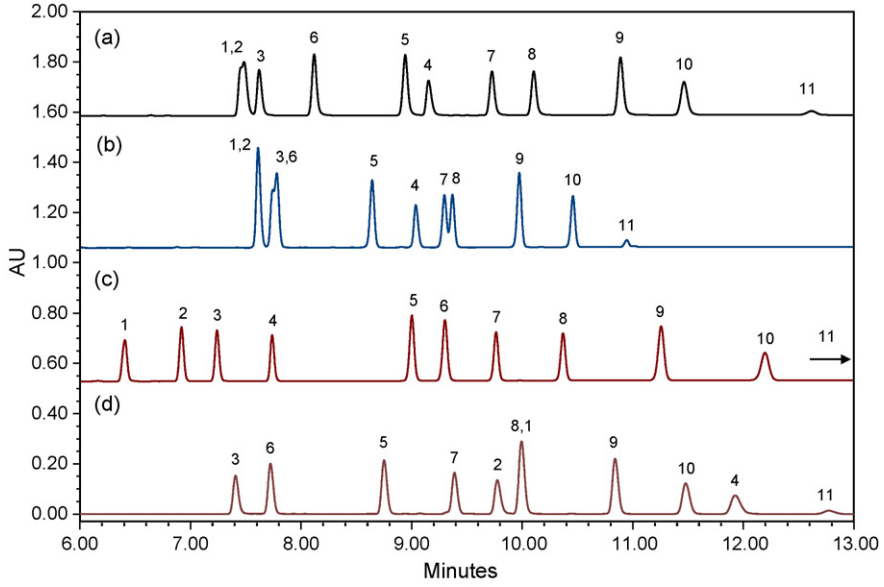


Fig. 4. Demonstration of selectivity differences generated for drug substance Compound A (peak 2) and its related impurities and degradants with all four columns and acetonitrile as the organic modifier. The columns evaluated were (a) Zorbax SB-C8, (b) Ace Phenyl, (c) Zorbax Bonus RP, and (d) Xterra MS-C18.

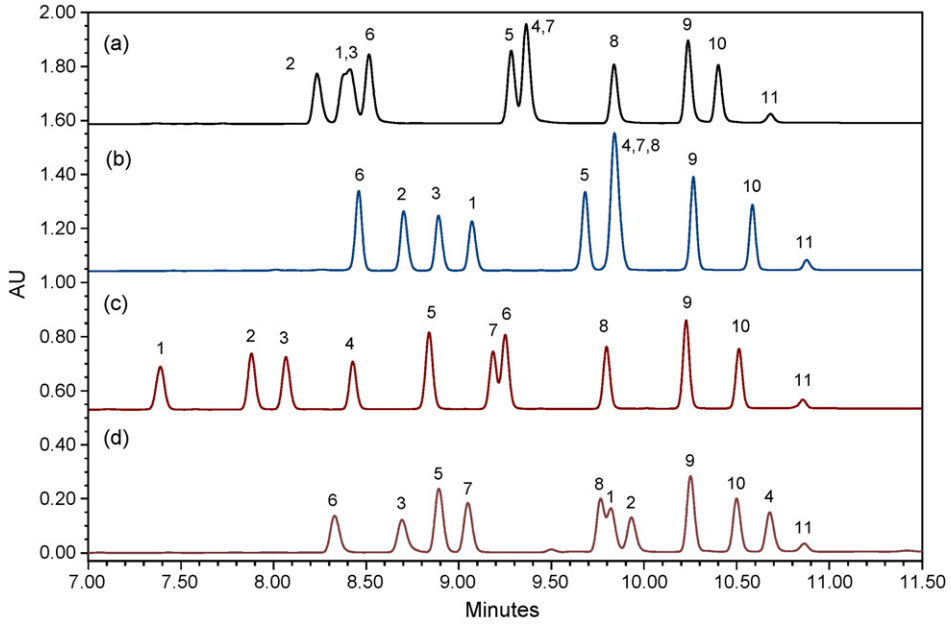


Fig. 5. Demonstration of selectivity differences generated for drug substance Compound A (peak 2) and its related impurities and degradants with all four columns and methanol as the organic modifier. The columns evaluated were (a) Zorbax SB-C8, (b) Ace Phenyl, (c) Zorbax Bonus RP, and (d) Xterra MS-C18.

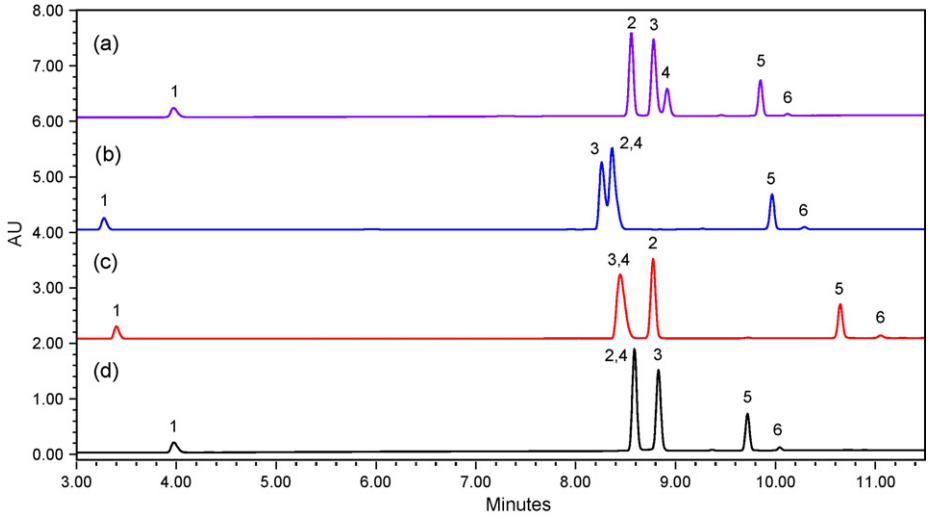


Fig. 6. Demonstration of selectivity differences generated for drug substance Compound B (peak 3) and its related impurities and degradants with two columns and acetonitrile and methanol as the organic modifier. The conditions evaluated were (a) Zorbax SB-C8 with acetonitrile, (b) Zorbax SB-C8 with methanol, (c) Ace Phenyl with acetonitrile, and (d) Ace Phenyl with methanol.

A review of the values indicates that all evaluated separation conditions were determined to be orthogonal relative to the standard conditions (Zorbax SB-C8 column, with acetonitrile, and TFA as the pH modifier). It is interesting to note that the more elevated $|\delta \log \alpha|_{avg}$ values came from a change in column and also from a change in pH.

Table 5
Listing of the $|\delta \log \alpha|_{avg}$ values for the Compound A and system suitability examples.

Column	Organic modifier	Compound A $\delta \log \alpha$	System suit $\delta \log \alpha$
Zorbax SB C8	MeOH	0.42	0.37
Zorbax Bonus RP	ACN	0.47	0.52
Zorbax Bonus RP	MeOH	0.55	0.63
ACE Phenyl	ACN	0.20	0.21
ACE Phenyl	MeOH	0.53	0.49
Waters Xterra	ACN	0.74	1.76
Waters Xterra	MeOH	0.57	1.86

After implementation of this systematic approach to method development, forty analyte sets related to drug candidates were evaluated. In summary, 35% of the final methods utilized the Zorbax SB-C8 column, 32% utilized the ACE Phenyl column, 20% utilized the Xterra MS-C18 column, and 12% utilized the Zorbax Bonus RP column. The method solutions were greater for the Zorbax SB-C8 and ACE Phenyl, partially due to the fact that the two-column approach was employed for 27 out of the 40 analyte sets. For the 13 analyte sets that were evaluated with the four-column approach, eight of these had more than one method solution.

4. Conclusions

A simple and efficient approach to reversed-phase HPLC method screening using only two columns for early develop-

ment and four columns through late stage development has been described. The applicability of this strategy was demonstrated with two pharmaceutical compounds and their impurities, as well as a system suitability test mixture consisting of eight commercially available acids and bases of varying polarities. Excellent selectivity using the four columns was achieved using simple MS-friendly mobile phases consisting of acetonitrile and methanol with TFA for low pH and ammonium hydroxide for high pH screening. The practicality of the screening strategy is not limited to the examples in this report, as our laboratory has successfully developed methods for over 40 pharmaceutical small molecule compounds using this approach. Implementing this RP HPLC strategy has reduced expenses, improved efficiencies, and provided consistency during the entire HPLC development process. Future revisions to this strategy will incorporate sub-3 μm based phases to further reduce method run time, which will continue to enhance laboratory efficiency.

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References

[1] K.P. Xiao, Y. Xiong, F. Liu, A. Rustum, J. Chromatogr. A 1163 (2007) 145–156.
[2] G. Xue, A.D. Bendick, R. Chen, S.S. Sekulic, J. Chromatogr. A 1050 (2004) 159–171.
[3] E.F. Hewitt, P. Lukulay, S. Galushko, J. Chromatogr. A 1107 (2006) 79–87.
[4] R.M. Krisko, K. McLaughlin, M.J. Koenigbauer, C.E. Lunte, J. Chromatogr. A 1122 (2006) 186–193.
[5] E. Van Gyseghem, M. Jimidar, R. Sneyers, D. Redlich, E. Verhoeven, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 1042 (2004) 69–80.
[6] E. Van Gyseghem, M. Jimidar, R. Sneyers, D. Redlich, E. Verhoeven, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 1074 (2005) 117–131.
[7] M.R. Euerby, P. Petersson, J. Chromatogr. A 994 (2003) 13–36.
[8] D. Visky, E. Haghedooren, P. Dehouck, Z. Kovacs, K. Kóczián, B. Noszál, J. Hoogmartens, E. Adams, J. Chromatogr. A 1101 (2006) 103–114.
[9] T. Baczek, R. Kaliszan, K. Novotna, P. Jandera, J. Chromatogr. A 1075 (2005) 109–115.
[10] J.W. Dolan, L.R. Snyder, T.H. Jupille, N.S. Wilson, J. Chromatogr. A 960 (2002) 51–67.
[11] N.S. Wilson, J.W. Dolan, L.R. Snyder, P.W. Carr, L.C. Sander, J. Chromatogr. A 961 (2002) 217–236.
[12] J.J. Gilroy, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 1000 (2003) 757–778.
[13] J.J. Gilroy, J.W. Dolan, P.W. Carr, L.R. Snyder, J. Chromatogr. A 1000 (2003) 77–89.
[14] N.S. Wilson, J. Gilroy, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 1026 (2003) 91–100.
[15] N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2003) 171–193.
[16] J.H. Ward Jr., J. Am. Stat. Assoc. 58 (1963) 236–244.
[17] J.J. Kirkland, J. Chromatogr. A 1060 (2004) 9–21.
[18] U.A. Mirza, B.T. Chait, Anal. Chem. 66 (1994) 2898–2904.
[19] E. Bosch, S. Espinosa, M. Soses, J. Chromatogr. A 824 (1998) 137–146.
[20] J. Pellett, P. Lukulay, Y. Mao, W. Bowen, R. Reed, M. Ma, R. Munger, J. Dolan, L. Wrisley, K. Medwid, N. Toltl, C. Chan, M. Skibic, K. Biswas, K. Wells, L. Snyder, J. Chromatogr. A 1101 (2006) 122–135.