

## Looking Beyond C18 Bonded Phase Chemistry When Developing HPLC Methods

Most chromatographers generally begin method development with a C18 stationary phase. If, after altering variables like the pH, percent organic, ionic strength and temperature, the separation is not suitable, the analyst has a number of choices. Frequently, another C18 column from a different vendor is chosen. While C18 phases from different sources can provide differences in retention and selectivity, these differences are frequently small and not sufficient to produce improved separation. An alternative to changing to a different C18 phase is to choose a stationary phase chemistry that provides different or enhanced mechanisms for retention. The result likely will be an improved, more acceptable separation than with another C18.

We recently needed to separate two compounds under isocratic conditions and found the desired separation was difficult to obtain using a C18 column. The compounds of interest were Feldene® (piroxicam) and its synthetic intermediate 2-aminopyridine (Figure A). Piroxicam serves as an analgesic in the treatment of osteoarthritis and rheumatoid arthritis. Piroxicam is sparingly water soluble, exhibits a weakly acidic 4 hydroxy proton, ( $pK_a=5.1$ ) and is relatively non-polar. A good starting column would be a C18.

Starting with an acidic mobile phase to reduce potential tailing (some potential for ionic interactions, hydrogen bonding with silanol surface) we see in Figure B that the piroxicam is well retained on the C18 column but the 2-aminopyridine elutes at the void volume. Decreasing the percent acetonitrile results in excessive piroxicam retention and the 2-aminopyridine is still unretained (Figure C).

We then increased the pH to 6.8. The 2-aminopyridine is retained, as expected, yet the piroxicam is still excessively retained (Figure D).

At this point there are three possible solutions to attain the desired retention and resolution of these compounds. First, we could use gradient elution. Gradients can add complexity to the separation, may not be reproducible and require time for re-equilibration.

Second, we could add an ion-pair reagent to increase the 2-aminopyridine retention. However, ion-pair reagents could make the method less robust, add to reproducibility problems, and complicate method development and validation.

Third, we could change the stationary phase. As shown in Figure E, we have changed from a C18 to a Discovery HS F5 column. The

(continued on page 4)

Figure A. Structures of Piroxicam and 2-Aminopyridine

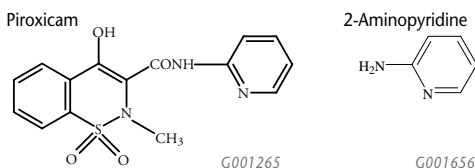


Figure B. 2-Aminopyridine is Unretained on C18 Using Mobile Phase to Assay Piroxicam

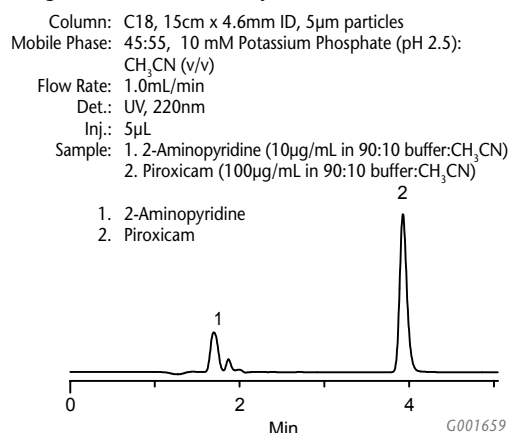


Figure C. Decreasing % Acetonitrile Results in Excessive Piroxicam Retention, No Improvement in Retention of 2-Aminopyridine

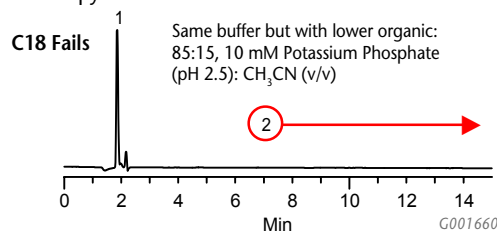
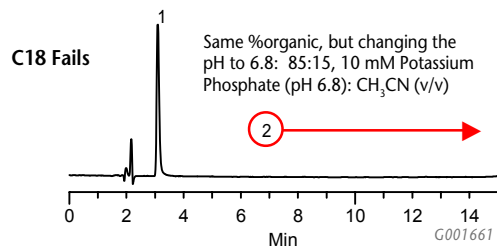


Figure D. A Change in pH Retains 2-Aminopyridine, Still Excessive Piroxicam Retention



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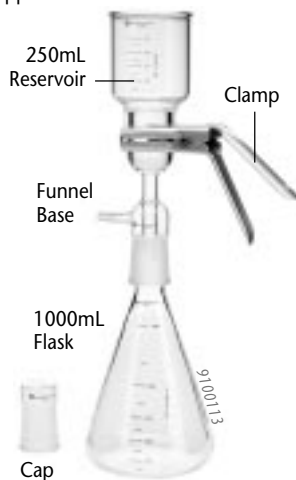
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Upfront Column Screening Facilitates Method Development: Separation of Corticosteroids

Apparatus 1



Flasks sold separately

Apparatus 2



Flasks sold separately

## NEW PRODUCTS

### Supelco Mobile Phase Filtration Apparatus

Your HPLC column is valuable. To maintain the highest column efficiency over its lifetime, protect it by removing particles and gasses from solvents, buffers and other mobile phase components by using the Supelco Mobile Phase Filtration Apparatus. These units come with Nylon 66 membrane filters that are compatible with all solvents commonly used in HPLC. Apparatus 1 connects to a standard 1000mL sidearm flask (sold separately) and any standard vacuum source, while Apparatus 2, with its tapered funnel base, directly connects to a standard vacuum source, as well as an aspiration line.

**Filtration Apparatus 1** (connects to 1000mL sidearm flask) - Includes 250mL glass reservoir, funnel base and stopper, clamp, stainless steel holder and screen, 10 Teflon gaskets, 50 Nylon 66 filters (47mm, 0.45µm pores).

**Filtration Apparatus 2** (connects to aspiration line) - Includes 250mL glass reservoir, (T) 34/45 tapered funnel base, (T) 34/45 tapered 1000mL flask and glass cap, clamp, stainless steel holder and screen, 10 Teflon gaskets, 50 Nylon 66 filters (47mm, 0.45µm pores).

For more information, request T100826.

Description	Cat. No.
<b>Supelco Mobile Phase Filtration Apparatus</b>	
Filtration Apparatus 1	58061
Filtration Apparatus 2	58062-U
<b>Replacement Glass Parts</b>	
For Filtration Apparatus 1	
Reservoir, 250mL	58063
Reservoir, 500mL	58074
Funnel Base and Stopper	58064
For Filtration Apparatus 2	
Tapered Funnel Base	58068
Tapered Flask, 1000mL	58070-U
Tapered Flask, 2000mL	58075
Cap for Flask	58071
<b>Replacement Filter Parts for Both Apparatus</b>	
Filter Holder and Screen, SS	58065
Gaskets, Teflon (pk. of 10)	58066
Filter, Nylon 66, 47mm (pk. of 50)	
0.45µm pores	58067
0.20µm pores	58060-U
Clamp, Spring Action	58053
Vacuum Flask, 1000mL, with Sidearm	Z290459-1EA

## LIQUID CHROMATOGRAPHY PERFORMANCE TIP

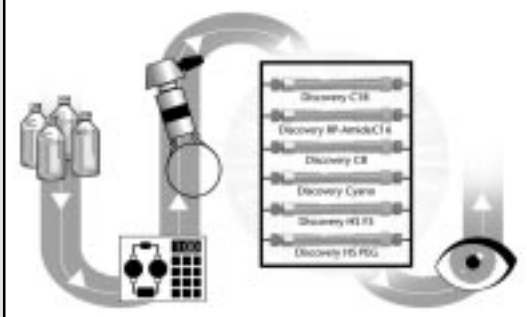
### Screening Multiple Functionalized Reversed-Phases

While C18 columns from different suppliers can provide some differences in retention and selectivity, these differences are frequently small and not sufficient to produce valuable, improved separations. Functionalized reversed-phases are designed to provide separations based on unique combinations of polar and hydrophobic retention. Screening several functionalized reversed-phases will often show significantly different selectivities for a separation and yield far more valuable results. While screening multiple columns can be accomplished with a simple, manual HPLC system, an automated, multi-solvent system with a programmable, temperature controlled column selector valve is highly recommended (Figure H).

So the basic steps for column screening are: 1) Scout; 2) Screen; 3) Evaluate; 4) Optimize.

For more information, request T402075.

Figure H. Automated HPLC and Column Selector Valve Containing Up To Six Columns that may be Screened.



Steps to choosing a functionalized reversed-phase column:

- Step 1.** Run your separation on a C18 using your preferred mobile phase, adjusting % organic to obtain a  $k'$  between 2 and 10. Discovery C18 is a good choice for classic selectivity with high efficiency.
- Step 2.** Under the same conditions, screen Discovery RP-AmideC16 and Discovery HS F5 columns. Reduce the organic concentration by 20% and screen Discovery Cyano and Discovery HS PEG columns.
- Step 3.** Evaluate the results of the screening runs to determine which column gives the desired balance between resolution and retention.
- Step 4.** Optimize the separation on the most promising column(s) using standard reversed-phase mobile phase adjustment techniques.

All literature mentioned in this issue can be obtained from the website, [sigmaaldrich.com/TheReporter](http://sigmaaldrich.com/TheReporter), by completing the Literature Request section on the reply card, or by calling our Technical Service Dept.

## NEW APPLICATIONS

### Functionalized Reversed-Phases for Impurity Analysis

It is exceedingly important to test for, identify, and quantify impurities in pharmaceutical therapeutics. The discovery of impurities late in the drug development process may result in substantial costs to modify the production process. Therefore, it is desirable to determine early in the research and development process whether synthetic and subsequent purification processes result in a product free from impurities.

Quinidine is an antimalarial schizonticide and an antiarrhythmic agent. It is the d-isomer of quinine and its molecular weight is 324.4.

Neat quinidine was assayed on a C18 HPLC column under a variety of conditions. Condition A in Figure I was the initial test condition and yielded an acceptable retention ( $k' \cong 3$ ). A single peak was observed, suggesting the quinidine was pure. The % organic was decreased to increase the retention in condition B. The result was a desirable increase in retention ( $k' \cong 6.5$ ), but with a concomitant deterioration in peak shape. The % organic was then increased to decrease retention ( $k' \cong 1.5$ ) in condition C. A partially resolved front shoulder peak was observed. Additional modifications to the mobile phase and chromatographic conditions (flow rate, temperature, etc.) did not allow for resolution of the impurity.

The separation mechanism of a typical C18 column is based on the differences in hydrophobicity between the analytes.

Therefore, if two molecules have very similar hydrophobicities, it is likely that they will not be adequately resolved. However, the Discovery HS F5 is a unique, functionalized reversed-phase. The pentafluorophenylpropyl group allows for typical hydrophobic as well as polar interactions with the analyte. Since the retention mechanism of the HS F5 is based on both polar and hydrophobic interactions, not only will resolution of analytes with differing hydrophobicities be achieved, but also analytes with differing polarities yet similar hydrophobicities. The impurity in the quinidine sample is clearly resolved with the HS F5 column, as shown in Figure I, condition (D).

Additional analysis with a mass spectrometer determined the molecular weight of the impurity to be 326.4. It was identified as dihydroquinidine. Figure J shows the structures of quinidine and dihydroquinidine.

The Discovery HS F5 uncovered a trace impurity in quinidine that was missed with a C18. The ability to resolve, identify, and quantitate the impurity resulted in the potential saving of valuable time. During method development, a quick screen using unique, functionalized reverse-phases such as HS F5 greatly increases the chances of finding trace impurities early in the drug development process, before they can become large problems. Even if a separation appears acceptable with your laboratory's preferred C18 column, a column with significantly different stationary phase chemistry makes an excellent confirmational column.

For more information, request T402075.

Figure J. Structures of Quinidine and Dihydroquinidine

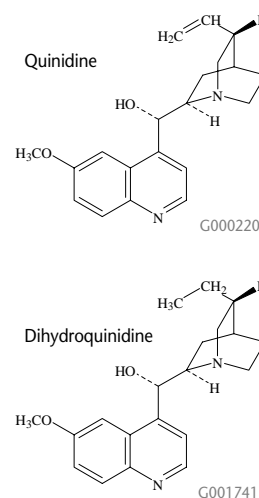
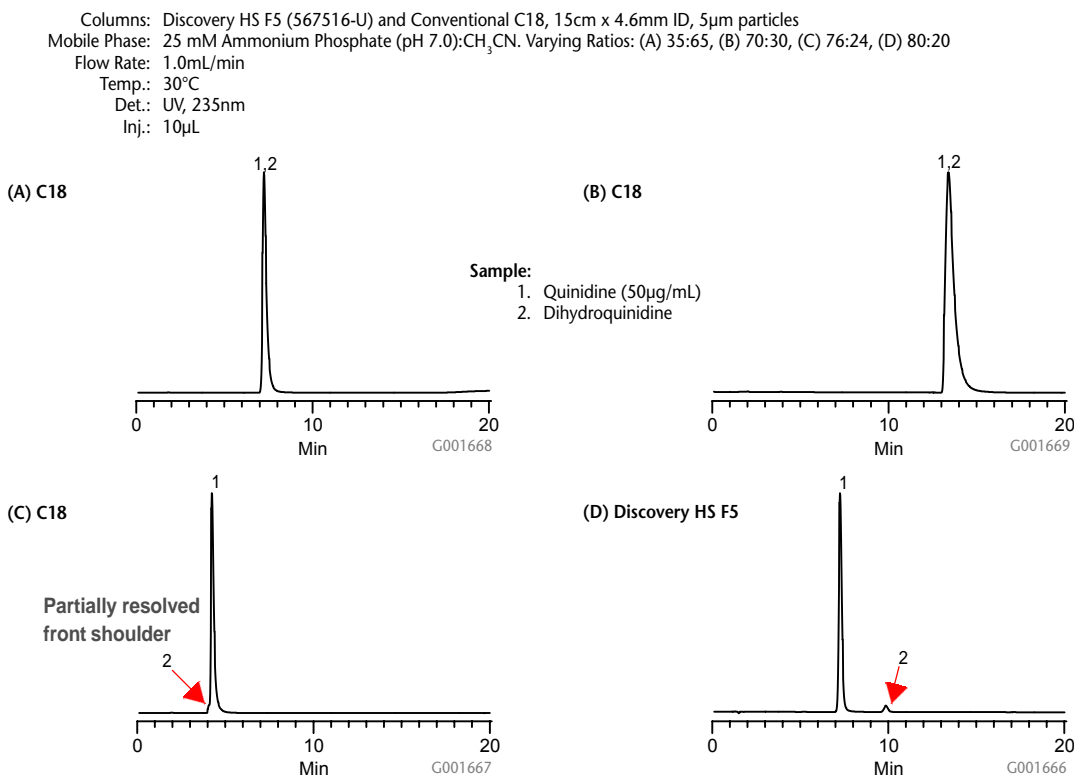


Figure I. Discovery HS F5 Resolves Trace Impurity in Quinidine that C18 Column Does Not



#### New Literature:

Request these documents on the literature request portion of the survey.

**Three NEW Product Profiles** are available for Discovery BIO Wide Pore C18, C8 and C5 HPLC Columns.  
Request T401097, T401098, T401099.

**Four NEW Application Notes** using Discovery BIO HPLC Columns are also available.  
Request T302166, T302167, T302168, T302169.

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Feldene - Pfizer  
Teflon - E.I. du Pont de Nemours & Co., Inc.

## Looking Beyond C18 Bonded Phase...

(continued from page 1)

HS F5 phase was chosen because it has greater retention for certain polar compounds than a C18. Note that both the 2-aminopyridine and the piroxicam elute in a reasonable time using either pH 2.5 or 6.8.

In this example using piroxicam and the intermediate 2-aminopyridine, we were not able to get a good separation on C18, but by simply changing to a stationary phase that incorporated a polar functionality, we were able to provide a simple and elegant solution to what seemed like a complicated problem using C18.

The next time you are faced with a challenging separation and C18 does not give you the desired results, consider changing the stationary phase to something other than another C18.

For more information request T402075.

## CASE STUDY 4

### Upfront Column Screening Facilitates Method Development: Separation of Corticosteroids

The goal of the study was to develop HPLC conditions suitable for the separation of five corticosteroids (hydrocortisone, prednisolone, prednisone, corticosterone and hydrocortisone acetate). Method development began by screening several stationary phase chemistries for selectivity toward the analytes. Discovery C18, Discovery Cyano, Discovery HS PEG, and Discovery HS F5 were chosen. A mixture of the five analytes was run on the C18 and HS F5 columns using a mobile phase consisting of water:methanol (50:50, v/v) and on Cyano and HS PEG with a mobile phase consisting of water:methanol (80:20, v/v) and the chromatograms were evaluated.

As shown in Figure G, the HS F5 stationary phase was found to exhibit greater selectivity toward the analytes. This phase was chosen for further optimization. Following just a few iterations, successful separation of all analytes was obtained in under 15 minutes (Figure G).

Method development scientists often choose a single stationary phase for development. If the chosen phase is not the best chemistry to affect a given separation, many hours may be spent studying mobile phase compositions that may or may not yield a suitable separation. Screening several stationary phase chemistries upfront during method development and choosing the best phase for further optimization can save many precious hours. In addition, the use of a more effective stationary phase chemistry often eliminates the need for mobile phase additives that can greatly complicate separation conditions.

For more information, request T402075.

Figure E. Unique Retention and Selectivity of Discovery HS F5 Phase Yields Successful Separation at pH 2.5 and pH 6.8

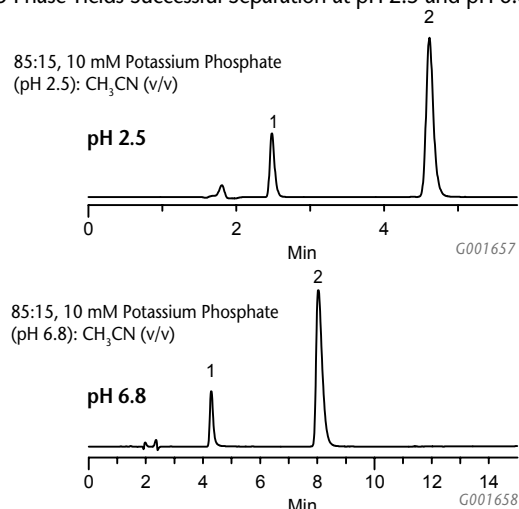


Figure F. Column Screening Results

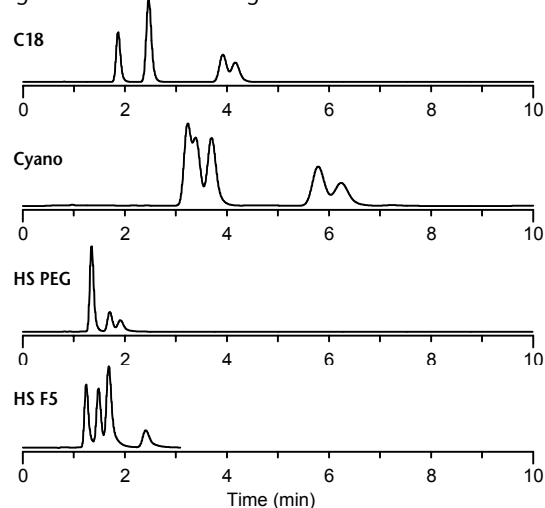
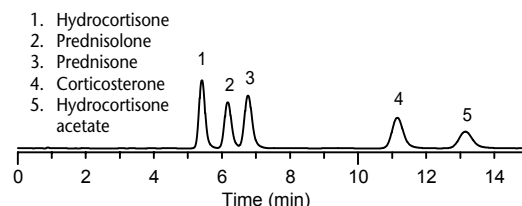


Figure G. Optimized Separation of Corticosteroids on Discovery HS F5

Column: Discovery HS F5, 5cm x 4.6mm ID, 5µm  
Mobile Phase: Water:Methanol (60:40, v/v)  
Flow Rate: 1.5mL/min  
Temperature: 60°C  
Detection: UV, 240nm  
Injection Volume: 5µL  
Sample: 10mg/mL mixture of corticosteroids in mobile phase



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