RESTEK
Chromatography Products

Pharmaceutical Applications

Systematic Transfer of HPLC Methods to UHPLC

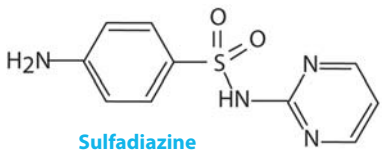
- Methods on Pinnacle™ DB columns are easily transferred from 3µm and 5µm to 1.9µm particle sizes, allowing faster analysis without losing separation quality.
- Pinnacle™ DB columns are 100% Restek manufactured–from base silica to final packed column.
- Restek offers the widest selection of stationary phases for UHPLC—more choices mean better selectivity for your analytes.

Higher Efficiencies Mean Increased Sample Throughput

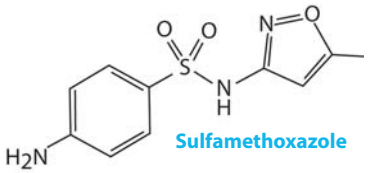
Ultra High Pressure Liquid Chromatography (UHPLC) is based on a modification to traditional liquid chromatographic systems that makes possible the use of increased operating pressures. Higher maximum operating pressures mean that packing materials consisting of small (<2µm) particle sizes can now be used. By decreasing the particle size of the packing material, the analyst can maximize the number of theoretical plates, making shorter column lengths possible. At the same time, by decreasing the particle size, the optimum range of mobile phase linear velocities is greatly expanded. As a result, higher linear velocities can be used without sacrificing separation quality. Both of these factors result in faster analytical run times and increased sample throughput.

UHPLC is commonly used in two ways. First, to increase sample throughput, a conventional HPLC analysis can be scaled down to UHPLC. Alternatively, to reduce time spent in method development, a preliminary separation can be developed in UHPLC and then scaled up to conventional HPLC for routine analysis. In both cases, when transferring an analysis from conventional HPLC to UHPLC, or from UHPLC to HPLC, comparable method parameters must be used to maintain equivalent separations. First and foremost, to maintain selectivity while scaling an analysis, some column properties and operating conditions should remain consistent, while other parameters are optimized. For the analytical column, the pore size, carbon load, and support material must remain the same. Differences in selectivity can be seen when not using equivalent columns. However, when decreasing particle size and column dimensions, it is equally important that certain operating conditions be adjusted properly. To provide guidance, we will identify important method parameters that need to be adjusted and illustrate with an example of method transfer.

Figure 1 Example chemical structures for sulfonamides.



Sulfadiazine



Sulfamethoxazole

In this example, we will perform a scale-down method transfer for sulfonamides from conventional HPLC to UHPLC. The initial analysis was conducted on a standard analytical scale 150mm x 4.6mm ID x 5µm HPLC column using gradient elution. To speed the analysis, we decreased the internal diameter of the column to 2.1mm and reduced the particle size to 1.9µm.

Optimize Selectivity for Enhanced Separations

To ensure that we used the optimum stationary phase for this application, we first compared the selectivity of a biphenyl column to a C18 column. Sulfonamides are commonly assayed on alkyl phases; however, their sulfone-containing ringed structures (Figure 1) may show enhanced selectivity with a biphenyl phase that is capable of π-π interactions. When comparing C18 and biphenyl columns of identical dimensions under identical conditions, the biphenyl column showed better selectivity towards the early eluting sulfonamides (Figure 2). Therefore, for better selectivity and faster analysis times, we used a biphenyl stationary phase in this method.

Adjusting the Column Dimensions

When performing a scale-down procedure, a few simple calculations can be used to determine equivalent run conditions. The first calculation to make is the determination of the appropriate column length. Keeping the same column length while decreasing the particle size will increase the number of theoretical plates in that given column length. Therefore, when decreasing particle size, column length can be shortened without losing resolution. By adjusting the column length properly, using Equation 1, we can maintain the same separation.

Adjusting the Injection Volume

Once we have determined the proper column length, we can determine the appropriate injection volume. Decreasing the column internal diameter and length, decreases the overall column volume and sample capacity. Therefore, we must alter the injection volume as described in Equation 2. Please note that since overall column volume has decreased, it is important to match the sample solvent to the starting mobile phase composition. Mismatched sample solvents can cause irreproducible retention times, efficiencies, and even changes in selectivity. If using a larger injection volume than calculated, check for peak abnormalities and irreproducibility that could result from phase overload.

Adjusting the Flow Rate

Next, when decreasing the internal diameter of the columns from 4.6mm to 2.1mm, the new column flow rate needs to be determined. Linear velocity is defined as the distance mobile phase travels over time (cm/min.), whereas flow rate is the volume of mobile phase that travels over time (mL/min.). To maintain the same linear velocity through a column with a smaller internal diameter, the flow rate must be decreased proportionally to the column internal diameter. Equation 3 can be used to determine the adjusted flow rate needed to maintain equivalent mobile phase linear velocities when changing column internal diameter.

Thus far, our example has focused on establishing a UHPLC method with equivalent linear velocity. However, a significant advantage of using a <2µm particle column is the ability to use higher linear velocities without detrimental effects on separation quality. When scaling down an isocratic analysis, a higher linear velocity, or relative flow rate, can easily be substituted to further shorten the analytical run. However, when using a gradient elution, a direct substitution of a higher flow rate is not practical and may require a more detailed adjustment. Gradients work by changing the mobile phase composition over time and, therefore, create a more dynamic relationship between the mobile and stationary phases. Maintaining a separation under gradient conditions requires maintaining a comparable relationship between gradient time, mobile phase composition, and column linear velocity and geometry. If the mobile phase linear velocity is increased to further shorten analysis time under gradient conditions, to maintain the same separation and gradient profile, the gradient times must also be adjusted accordingly. We will look at time program adjustment in the next section.

Adjusting Time Program

After we have determined the proper column length, injection volume, and flow rate, we can find the equivalent times needed for gradient or step elutions. As previously discussed, when an analytical method is scaled down, the time program also needs to be scaled down to keep the phase interactions the same. Time can be adjusted for each step using Equation 4. If an increased flow rate is desired, when calculating the gradient time program, we can substitute the increased flow rate into the same equation to find the comparable time program needed to maintain the same gradient profile, or mobile phase conditions. It is important to keep in mind system limitations, as mobile phase change rates can be limited by dwell volume, mixing chamber capability, and pump accuracy.

Equation 1 Adjusted column length can easily be calculated when scaling from HPLC to UHPLC.

$$L_{C2} = \frac{L_{C1} \cdot dp_2}{dp_1}$$

Example:

$$L_{C2} = \frac{150\text{mm} \cdot 1.9\mu\text{m}}{5\mu\text{m}}$$

$$L_{C2} = 57\text{mm}$$

L_C = Column Length
dp = Particle Size

*Note: use closest available size (50mm).

Equation 2 Changing column dimensions requires an adjusted injection volume.

$$V_{I2} = V_{I1} \bullet \left(\frac{d_{C2}^2 \bullet L_{C2}}{d_{C1}^2 \bullet L_{C1}} \right)$$

Example:

$$V_{I2} = 10\mu\text{l} \cdot \left(\frac{2.1\text{mm}^2 \cdot 50\text{mm}}{4.6\text{mm}^2 \cdot 150\text{mm}} \right)$$

$$V_{I2} = 0.69\mu\text{l}$$

V_I = Injection Volume
L_c = Column Length
d_c = Column Diameter

*Note: use closest possible volume (1µl).

Equation 3 Changing column internal diameter requires using an adjusted flow rate.

$$F_{C2} = \left(\frac{d_{C2}}{d_{C1}} \right)^2 \bullet F_{C1}$$

Example:

$$F_{C2} = \left(\frac{2.1\text{mm}}{4.6\text{mm}} \right)^2 \bullet 1.0\ \text{ml/min}$$

$$F_{C2} = 0.208\ \text{ml/min}$$

F_c = Column Flow
d_c = Column Diameter

Equation 4 When scaling down a gradient method, the time program needs to be adjusted.

$$t_{g2} = t_{g1} \bullet \left(\frac{F_{C1}}{F_{C2}} \right) \bullet \left(\frac{d_{C2}^2}{d_{C1}^2} \right) \bullet \left(\frac{L_{C2}}{L_{C1}} \right)$$

Example:

$$t_{g2} = 5\text{min.} \bullet \left(\frac{1.0\text{ml/min}}{0.2\text{ml/min}} \right) \bullet \left(\frac{2.1\text{mm}^2}{4.6\text{mm}^2} \right) \bullet \left(\frac{50\text{mm}}{150\text{mm}} \right)$$

$$t_{g2} = 1.7\ \text{min}$$

t_g = Gradient Time
F = Column Flow
L_c = Column Length
d_c = Column Diameter

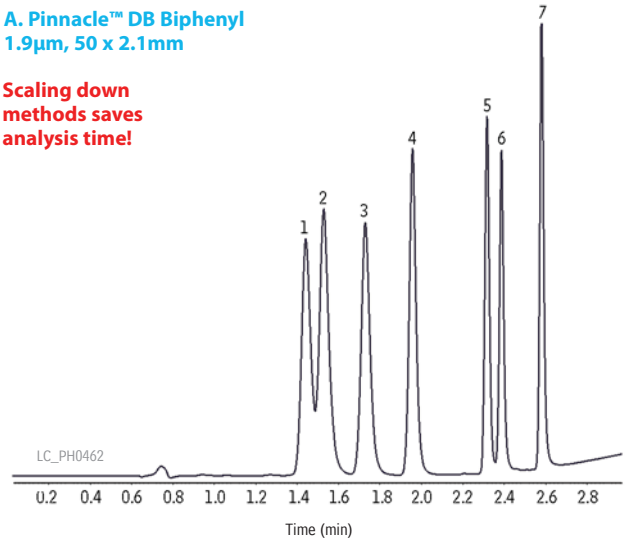
Conclusion

After determining the equivalent conditions for scaling down the analysis of sulfonamides, we can see that the separations are equivalent, while the analysis time was greatly reduced (Figure 2). Under conventional HPLC the last compound eluted at 7.2 minutes and under UHPLC the last compound eluted at 2.6 minutes. By following the procedure described here to ensure that the columns are equivalent, scaling analytical procedures from HPLC to UHPLC, or vice versa, can easily be accomplished.

Figure 2 Converting the conventional HPLC method to UHPLC, using the calculations given in Equations 1-4, reduces analysis time.

A. Pinnacle™ DB Biphenyl
1.9µm, 50 x 2.1mm

Scaling down methods saves analysis time!



Peak List:
1. sulfadiazine
2. sulfathiazole
3. sulfamerazine
4. sulfamethazine
5. sulfachlorpyridizine
6. sulfamethoxazole
7. sulfamethoxine

Sample:
Inj.: 1µL
Conc.: 100µg/ml
Sample diluent: starting mobile phase (80:20 A:B)

Column: **1.9µm Pinnacle™ DB Biphenyl**
Cat. #: 9409252
Dimensions: 50 x 2.1mm
Particle size: 1.9µm
Pore size: 140Å

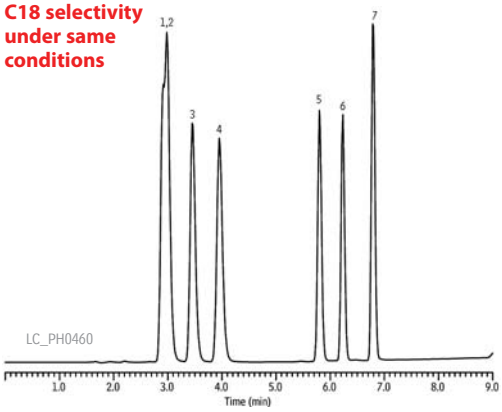
Conditions:
Mobile phase: A: 0.1% formic acid in water
B: 0.1% formic acid in acetonitrile

Time(min.)	%B
0.0	20
0.4	20
2.1	80
2.8	80

Flow: 0.2mL/min.
Temp.: 30ºC
Det.: UV @ 254nm

B. Pinnacle™ DB Biphenyl
5µm, 150 x 4.6mm

C18 selectivity under same conditions



Peak List:
1. sulfadiazine
2. sulfathiazole
3. sulfamerazine
4. sulfamethazine
5. sulfachlorpyridizine
6. sulfamethoxazole
7. sulfamethoxine

More selectivity for early eluting compounds.

Sample:
Inj.: 10µL
Conc.: 100µg/mL
Sample diluent: starting mobile phase (80:20 A:B)

Column: **Pinnacle™ DB Biphenyl**
Cat.#: 9409565
Dimensions: 150mm x 4.6mm
Particle size: 5µm
Pore size: 140Å

Conditions:
Mobile phase: A: 0.1% formic acid in water
B: 0.1% formic acid in acetonitrile

Time(min.)	%B
0.0	20
1.0	20
6.0	80
8.0	80

Flow: 1.0mL/min.
Temp.: 30ºC
Det.: UV @ 254nm

Pinnacle™ DB 1.9µm HPLC Columns

New phases now available!
Cyano • IBD • C8

1.9µm Pinnacle™ DB HPLC Columns

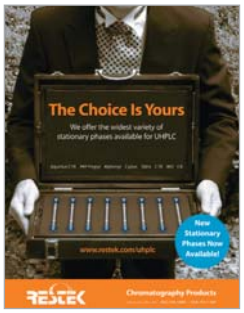
Ruggedness and reproducibility are guaranteed, as we control every step in the process, from base silica to bonded phase to final packed column. The silica particles are classified and selected to give an exceptionally tight distribution around 1.9µm, while eliminating <1µm particles that can contribute to a poorly packed bed. Highly base-deactivated Pinnacle™ DB stationary phases are an excellent choice when analyzing a wide range of compounds, from acidic to basic. To optimize your ultra high pressure HPLC methods, reach for Restek small particle HPLC columns!

Physical Characteristics:

particle size: 1.9µm
pore size: 140Å
endcap: yes
pH range: 2.5 to 7.5
temperature limit: 80°C

Chromatographic Properties:

Highly base-deactivated spherical silica manufactured by Restek.



UHPLC Columns Flyer
(lit. cat.# PHFL1009)

Length	2.1mm ID cat.#
Pinnacle™ DB C18 1.9µm Columns	
30mm	9414232
50mm	9414252
100mm	9414212
Pinnacle™ DB Silica 1.9µm Columns	
30mm	9410232
50mm	9410252
100mm	9410212
Pinnacle™ DB PFP Propyl 1.9µm Columns	
30mm	9419232
50mm	9419252
100mm	9419212
Pinnacle™ DB Biphenyl 1.9µm Columns	
30mm	9409232
50mm	9409252
100mm	9409212

Length	2.1mm ID cat.#
Pinnacle™ DB Aqueous C18 1.9µm Columns	
30mm	9418232
50mm	9418252
100mm	9418212
Pinnacle™ DB IBD 1.9µm Columns	
30mm	9425232
50mm	9425252
100mm	9425212
Pinnacle™ DB Cyano 1.9µm Columns	
30mm	9416232
50mm	9416252
100mm	9416212
Pinnacle™ DB C8 1.9µm Columns	
30mm	9413232
50mm	9413252
100mm	9413212

All phases also available in 3µm & 5µm particle sizes using Restek manufactured Pinnacle™ DB Silica!

www.restek.com/uhplc

RESTEK

Lit. Cat.# GNAN1033

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