The Secrets of Rapid HPLC Method Development

Choosing Columns for Rapid Method Development and Short Analysis Times

Agilent Technologies
Rapid Analysis Is More Than Run Time

• It is developing a method to meet a goal and developing and validating it quickly.

• The final method should minimize analysis time for the greatest sample throughput.

• It must be reproducible and robust.
Four Critical Aspects of Rapid Method Development and Analysis

• Rapid Sample Preparation – minimum steps for maximum effectiveness, use updated tools (combination filters) and multi-sample preparation equipment (SPE 96-well plates)

• Choose best bonded phases for high resolution – selecting from typical C18 and C8 bonded phases or those targeted to special sample types

• Choose the best column configuration for minimum analysis time with high efficiency and resolution – best column length, internal diameter, particle size

• Using your HPLC instrument to further reduce analysis time and increase sample throughput – optimizing the HPLC and using new features effectively
Bonded Phase Choice Drives Resolution

- Changing selectivity ($\alpha$) influences resolution the most
- Bonded phase is the column choice that controls selectivity

$$Rs = \frac{1}{1 + \frac{\alpha - 1}{\alpha}} \left( \frac{k}{\frac{1}{1+k}} \right)^{\frac{1}{2}}$$

$\alpha =$ selectivity – increase by changing bonded phase and mobile phase

$k =$ retention – increase by changing bonded phase and mobile phase
does not improve $Rs$ above $k \approx 10$
Selecting a Bonded Phase

- Choose columns known to have long lifetimes at operating mobile phase pH.

- Choose bonded phases on high purity, low acidity silica for best peak shape.

- Select a C18 or C8 bonded phase first for good retention and resolution with typical acidic, basic and neutral samples.

- If sample is expected to be difficult (i.e. very polar, difficult to retain, very basic) then select targeted bonded phases available for these types of samples.
Rapid Method Development Scheme – Low pH

- Start at low pH for best peak shape, retention and long-term reproducibility
  - Select starting conditions
    - StableBond C18 or C8 for maximum lifetime – Rapid Resolution columns
    - pH 1 – 3 with 20 – 50 mM buffer for best peak shape
    - Acetonitrile or methanol – start high to scout
    - Adjust organic for maximum resolution and retention
    - Change organic if resolution not achieved – MeOH or ACN
    - Change bonded phase if resolution not achieved – SB-CN, SB-Phenyl, SB-C3
  - Use elevated temperature to reduce analysis time further
Rapid Method Development Scouting
Chromatograms on StableBond-C18
Rapid Resolution Columns

A : B  20 : 80  40 : 60  60 : 40

Columns: Zorbax Rapid Resolution
StableBond SB-C18
4.6 x 75 mm, 3.5 µm

Phase:
A: 25 mM NaH₂PO₄, pH 3.0
B: ACN

Flow Rate: 2.0 mL/min.
Temperature: 35°C
UV Detection: 254 nm

Sample: Cardiac Drugs

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Rapid Method Development
Scouting Chromatograms
Change Organic Modifier

Column: Zorbax Rapid Resolution SB-C18, 4.6 x 75 mm, 3.5 µm
Mobile Phase: A: 25 mM NaH2PO4, pH 3.0 B: MeOH
Flow Rate: 2.0 mL/min
Temperature: 35°C
Detection: UV 254 nm

A : B
20 : 80 30 : 70 40 : 60

• Changing organic modifier can alter selectivity and improve peak shape.
Rapid Method Development Process Optimizes Separation on StableBond-C18 at Low pH

A Rapid Resolution SB-C18 column at low pH was used to develop this thorough and rapid analysis of steroids and impurities following the rapid method development process.

- Column: Zorbax Rapid Resolution SB-C18, 4.6 x 75 mm, 3.5 µm
- Mobile Phase: 50% ACN, 50% 20 mM NaH2PO4, pH 2.8
- Flow Rate: 1.0 mL/min
- Temperature: RT
- Detection: UV 254 nm
- Sample:
  1. Estradiol
  2. Ethynylestradiol
  3. Dienestrol
  4. Norethindrone
Increase Temperature to Reduce Analysis Time

Method Optimization

Low Temperature

High Temperature

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Agilent instruments provide reliable temperature control to 80 - 90°C allowing method development flexibility.

Column: Zorbax SB-C18, 4.6 x 75 mm, 5 µm
Mobile Phase: 74% 7.4 mM hexane sulfonate and 0.07% phosphoric acid, 26% MeOH
Flow Rate: 1 mL/min
Detection: UV 280 nm
Sample: B Vitamins

min.
Rapid Method Development Scheme – Mid pH

- Try pH 7 (pH 6 – 9) with Eclipse XDB-C18 or C8 and follow same process, selecting Eclipse XDB-Phenyl as alternate bonded phase

- Use temperature to reduce analysis time further
Eclipse XDB-C18 is Bonded Phase Choice for High Resolution at Mid pH

Mobile Phase: 20% Methanol: 80% 20 mM phosphate buffer + (10 mM TEA @ pH 7)  
Flow Rate: 1.0 mL/min  
Temperature: RT  
Detection: UV 254 nm  

- This sample is only resolved at mid pH on Eclipse XDB-C18 following rapid method development process.
Choose Special Bonded Phases for Difficult Samples

- **Sample 1 – Highly basic compounds**
  - May exhibit poor peak shape
  - May be difficult to retain
  - Column choice 1 – Bonus-RP for better peak shape
  - Column choice 2 – Extend-C18 at high pH for better retention and peak shape

- **Sample 2 – Highly polar compounds**
  - May be difficult to retain
  - May require high aqueous mobile phases
  - Column choice 1 – SB-Aq for better retention with high aqueous mobile phases
  - Column choice 2 – Bonus-RP for use with high aqueous mobile phases
Sample 1: Highly Basic Compounds
Select Bonus-RP for Improved Peak Shape

Column: 4.6 x 150 mm 5 µm
Mobile Phase: A: 75% 25 mM NH₄Ac, pH 5.5 : 25% ACN
B: 80% 25 mM NH₄Ac, pH 5.5 : 20% ACN
Flow Rate: 1.5 mL/min
Detection: UV 254 nm
Sample: 1. Doxylamine 2. Chlorpheniramine 3. Triprolidine

A. Alkyl C8

B. Bonus-RP
Sample 1: Highly Basic Compounds
Extend-C18 Improves Retention and Efficiency of Procainamides at High pH

Column: Zorbax Extend-C18, 4.6 x 150 mm, 5 µm
Mobile Phase: See Below
Flow Rate: 1.0 mL/min
Detection: UV 254 nm
Temperature: RT

50% 25 mM Na₂HPO₄, pH 7.0: 50% MeOH
50% 20 mM TEA, pH 11: 50% MeOH

Plates
1. 4903
2. 7621
3. 7567

Plates
1. 9475
2. 8823
3. 8794

Time (min)

High efficiency improves resolution and column lifetime and the increase in retention results in a more rugged method.
Sample 2: Highly Polar Compounds
Select New ZORBAX SB-Aq Columns for Method Development in High Aqueous Mobile Phases

- Good retention for polar compounds in high aqueous mobile phases
- Reproducible retention without “phase collapse”
- Different selectivity vs. conventional C18 columns
- Highly, stable at low pH and high temperature (up to 80°C)
Sample 2: Highly Polar Compounds
ZORBAX SB-Aq Provides Good Retention of Polar Compounds

- These small polar compounds are difficult to retain on most columns.
- The SB-Aq provides excellent retention with a 90% aqueous mobile phase.
Sample 2: Highly Polar Compounds
ZORBAX SB-Aq Provides Good Retention of Water Soluble Vitamins without Ion Pairing

- The SB-Aq column provides good retention of these polar compounds without ion pairing.
- The result is a simpler method without the reproducibility problems associated with ion pairing.
Sample 2: Highly Polar Compounds
ZORBAX SB-Aq Provides Good Retention of NADH/NAD\(^+\) with LC/MS Compatible Mobile Phase

- These coenzymes are difficult to retain and analyze by LC/MS.
- The SB-Aq provides baseline resolution without ion pairing.
Rapid Resolution Columns Reduce Method Development and Analysis Time

• Rapid Resolution columns (3.5 µm) are available in many configurations
  • Available in analytical, narrow bore, microbore, and capillary internal diameters for compatibility with any sample size.
  • Semi-preparative and preparative columns use 5 µm particles.
• Rapid Resolution columns reduce isocratic and gradient run times with:
  • Shorter column lengths
  • Higher flow rates
  • Optimized HPLC instrument
## Choose Column Configuration for Application

<table>
<thead>
<tr>
<th>Column Type</th>
<th>I.D. (mm)</th>
<th>Lengths (mm)</th>
<th>Particle Sizes (µm)</th>
<th>Flow Rate Ranges</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary</td>
<td>0.3, 0.5</td>
<td>35 – 250</td>
<td>3.5, 5</td>
<td>1 – 10 µL/min</td>
<td>Max sensitivity LC/MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Higher sensitivity LC/MS</td>
</tr>
<tr>
<td>MicroBore</td>
<td>1.0</td>
<td>30 – 150</td>
<td>3.5, 5</td>
<td>30 – 60 µL/min</td>
<td>LC/MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High sensitivity LC/MS</td>
</tr>
<tr>
<td>Narrow Bore</td>
<td>2.1</td>
<td>15 – 150</td>
<td>3.5, 5</td>
<td>0.1 – 0.3 mL/min</td>
<td>LC/MS</td>
</tr>
<tr>
<td>Solvent Saver</td>
<td>3.0</td>
<td>150, 250</td>
<td>3.5, 5</td>
<td>0.3 – 1.0 mL/min</td>
<td>High sensitivity LC/MS</td>
</tr>
<tr>
<td>Analytical</td>
<td>4.6</td>
<td>15 – 250</td>
<td>3.5, 5</td>
<td>1 – 4 mL/min</td>
<td>Analytical</td>
</tr>
<tr>
<td>Semi-prep</td>
<td>9.4</td>
<td>50 – 250</td>
<td>5</td>
<td>4 – 10 mL/min</td>
<td>Analytical</td>
</tr>
<tr>
<td>Preparative</td>
<td>21.2</td>
<td>50 – 250</td>
<td>5, 7</td>
<td>20 – 60 mL/min</td>
<td>Small scale prep (mg) Large scale prep</td>
</tr>
</tbody>
</table>
High Resolution with Rapid Resolution Columns

**Isocratic Separation of Aspartame**

**Column:** Zorbax StableBond SB-C18  
**Mobile Phase:** 85% Water with 0.1% TFA : 15% Acetonitrile  
**Flow Rate:** 1.0 mL/min  
**Temperature:** 35°C  
**Sample:** 1. Phenylalanine  
2. 5-Benzyl-3,6-dioxo-2-piperazine acetic acid  
3. Asp-phe  
4. Aspartame

**Dimensions:**
- 4.6 x 150 mm, 5 µm
- 4.6 x 75 mm, 3.5 µm
- 4.6 x 50 mm, 3.5 µm
- 4.6 x 30 mm, 3.5 µm
- 4.6 x 15 mm, 3.5 µm

**Time (min):** 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12

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Rapid Resolution Columns Reduce Isocratic Analysis Time by 50% or More

StableBond SB-C18
4.6 x 150 mm, 5 µm

Mobile Phase: 45% 25 mM NaH₂PO₄, pH 3.0
55% MeOH
Flow Rate: 2.0 mL/min
Temperature: 35°C
Detection: UV 254 nm
Sample: Cardiac Drugs
1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. Lidoflazine
5. Flunarizine

Analysis Time: 11.7 min

StableBond SB-C18
4.6 x 75 mm, 3.5 µm

Analysis Time: 5.2 min
Higher Flow Rates Reduce Analysis Time with Rapid Resolution Columns

Column: Zorbax Rapid Resolution StableBond SB-C18, 4.6 x 30 mm, 3.5 µm

Pressure

90 bar

133 bar

181 bar

2650 psi

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Gradient Re-equilibration Times are Minimal on Short Rapid Resolution Columns

Column Volume (Vm) and Equilibration Time

<table>
<thead>
<tr>
<th>Column Dimension (mm)</th>
<th>Internal Volume (Vm)</th>
<th>Equilibration Time at 1.0 mL/min (Vm x 10 x F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6 x 50</td>
<td>0.5 mL</td>
<td>5 min</td>
</tr>
<tr>
<td>4.6 x 30</td>
<td>0.3 mL</td>
<td>3 min</td>
</tr>
<tr>
<td>4.6 x 15</td>
<td>0.15 mL</td>
<td>1.5 min</td>
</tr>
<tr>
<td>4.6 x 150</td>
<td>1.54 mL</td>
<td>15 min</td>
</tr>
</tbody>
</table>

Gradient Analysis Time = Run Time + Equilibration Time (single step return)

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Short, Rapid Resolution Columns Reduce Gradient Analysis Time

Gradient Time & Column Length

*Column:* Zorbax Eclipse XDB-C8, 4.6 mm i.d.  
*Gradient:* 45-90% B in $t_G$ minutes  
*Mobile Phase:* A: 25 mM Na$_2$HPO$_4$, pH 3  
B: Methanol  
*Temperature:* 35°C  
*Flow Rate:* 1.0 mL/min  
*Sample Cardiac Drugs:* 1. Diltiazam  
2. Dipyridamole  
3. Nifedipine  
4. Lidoflazine  
5. Flunarizine

**Length = 75 mm, 3.5 µm**

- $t_G = 9$ min

**Length = 50 mm, 3.5 µm**

- $t_G = 6$ min

Time (min)

0.0  2.5  5.0  7.5

0.0  2.5  5.0
Rapid Gradient CombiChem Analysis
1.5 min Injection-to-Injection

Column: Zorbax SB-C18, 2.1 x 30 mm, 3.5µm
Flow Rate: 0.832 mL/min
Temperature: 20°C

Mobile Phase Gradient: A = 0.1% Formic Acid, H₂O; B = 0.1% Formic Acid, ACN; 30-98% B in 0.6 min

Well-Plate Position
P1-A-02
P1-A-03
P1-A-04
P1-A-05
P1-A-07
P1-A-08
P1-A-09
P1-A-10
P1-B-01
P1-B-02

Agilent Technologies
Dial 1-904-779-4740 for e-Seminar Audio
Increasing Flow Rate Reduces Gradient Analysis Time Further

Column: Zorbax Eclipse XDB-C8 4.6 x 50 mm, 3.5 µm
Gradient: 45-90% B in tG minutes
Mobile Phase: A: 25 mM Na2HPO4, pH 3, B: MeOH
Flow Rate: 1.0 mL/min
Temperature: 35°C

If $t_G \times F = \text{constant}$
then the elution pattern is unchanged

F = 2.0 mL/min
$t_G = 3$ min

F = 3.0 mL/min
$t_G = 2$ min

Dial 1-904-779-4740 for e-Seminar Audio
HPLC Instrument Optimization Increases Efficiency and Decreases Method Development and Analysis Time

• Choose the best column configuration for your instrument type and application (i.e. Capillary LC for highest sensitivity or compatibility with LC/MS)

• Instrument optimization can improve efficiency for isocratic and gradient separations

• New instrument features can help you reduce analysis time
## Choose HPLC Instrument for Application

<table>
<thead>
<tr>
<th>Agilent 1100 Type</th>
<th>Compatible Columns</th>
<th>Flow Rate Range</th>
<th>Applications</th>
<th>Why?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary LC</td>
<td>Capillary, MicroBore, Narrow-bore, Solvent Saver, Analytical</td>
<td>1 μL/min – 2.5 mL/min</td>
<td>1. Sample limited, 2. Most MS compatible</td>
<td>5 μL dwell volume, best low volume gradient reproducibility</td>
</tr>
<tr>
<td></td>
<td>Narrow-bore, Solvent Saver, Analytical, Semi-prep</td>
<td>0.05 – 5 mL/min* binary</td>
<td>1. Analytical MS compatible</td>
<td>Standard analytical more semi-prep than low volume</td>
</tr>
<tr>
<td></td>
<td>Semi-prep, Prep</td>
<td>0.2 – 10 mL/min* isocratic/quaternary</td>
<td>2. MS compatible</td>
<td>Work at 3- 4mL/min</td>
</tr>
<tr>
<td>Prep LC</td>
<td></td>
<td>1 - 100 mL/min</td>
<td>1. Purification</td>
<td>Preparative and CombiChem prep</td>
</tr>
</tbody>
</table>

* Optimum range

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Optimizing Results with Rapid Resolution Columns

Isocratic Separations

- Minimize extra column volume to minimize band broadening
  - Keep the injection volume small (< 5 µL)
  - Use semi-micro or micro detector cells (5 µL or less)
  - Use 0.12 mm i.d. tubing (0.005”)
- Prepare the sample in an injection solvent with the same or weaker solvent strength than the mobile phase
- Overlap injections
- Select the Agilent 1100 Capillary HPLC for capillary and microbore columns
- Select correct detector response time for rapidly eluting peaks
Effect of Detector Cell Volume on Peak Width
4.6 x 75 mm, 3.5 µm

Column: Zorbax StableBond SB-C18
Mobile Phase: 85% H₂O with 0.1% TFA : 15% ACN
Temperature: 35°C
Sample: 1. Phenylalanine 2. 5-benzyl-3,6-dioxo-2-piperazine acetic acid 3. Asp-phe 4. Aspartame

Flow Rate: 1.0 mL/min

- Standard Flow Cell - 8 µL
- Microflow Cell - 2.5 µL

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6200</td>
<td>-29%</td>
</tr>
<tr>
<td>2</td>
<td>6700</td>
<td>-23%</td>
</tr>
<tr>
<td>3</td>
<td>7000</td>
<td>-16%</td>
</tr>
<tr>
<td>4</td>
<td>9250</td>
<td>-7%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8700</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8700</td>
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</tr>
<tr>
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<td>8300</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10,000</td>
<td></td>
</tr>
</tbody>
</table>

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Optimizing Results with Rapid Resolution Columns
Gradient Separations

- Minimize dwell volume – includes mixing volume and injector volume
  - Use high pressure mixing and reduce mixer volume
  - Run autosampler in bypass – reduce dwell volume by 300 µL
- Minimize extra column volume
- Overlap injections
- Select correct detector response time for rapidly eluting peaks
- Select Agilent 1100 Capillary HPLC with 5 µL dwell volume for capillary and microbore columns
Reproducible, Fast Gradient Analysis with High Pressure Mixing

<table>
<thead>
<tr>
<th>Compound</th>
<th>rsd RT for 10 runs</th>
<th>rsd Area for 10 runs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylphthalate</td>
<td>0.09</td>
<td>0.98</td>
</tr>
<tr>
<td>Diethylphthalate</td>
<td>0.10</td>
<td>0.87</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>0.08</td>
<td>1.83</td>
</tr>
<tr>
<td>o-Terphenyl</td>
<td>0.07</td>
<td>0.90</td>
</tr>
<tr>
<td>Dimethylphthalate</td>
<td>1.09</td>
<td>5.91</td>
</tr>
<tr>
<td>Diethylphthalate</td>
<td>0.55</td>
<td>5.64</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>0.52</td>
<td>5.30</td>
</tr>
<tr>
<td>o-Terphenyl</td>
<td>0.43</td>
<td>6.87</td>
</tr>
</tbody>
</table>

Agilent 1100, binary pump  Low pressure mixing, Brand X

Column: Zorbax StableBond C18, 2.1 x 50 mm

Dial 1-904-779-4740 for e-Seminar Audio
Reduce Gradient Analysis Time and Dwell Volume with Bypass of the Sample Loop

Column: Zorbax SB-C18, 4.6 x 30 mm, 3.5µm  
Flow Rate: 0.832 mL/min  
Temperature: 20°C  
Mobile Phase Gradient: A = 0.1% Formic Acid, H2O; B = 0.1% Formic Acid, ACN; 30-98% B in 0.6 min

Agilent 1100 WPS  
Auto Bypass By-Pass Off

Agilent 1100 WPS  
Auto Bypass By-Pass On

Dial 1- 904-779-4740 for e-Seminar Audio
Overlapped Injection Reduces Analysis Time

- Overlapped injections – sample is drawn up during previous injection – reduce gradient and isocratic run times.

**Overlapped Injection**
Cycle time is ~2min 12sec
Total time for 20 runs: 44 min 22 sec

**Standard Injection**
Cycle time is ~2min 57sec
Total time for 20 runs: 58 min 20 sec

Dial 1-904-779-4740 for e-Seminar Audio
Effect of Detector Response Time on Ultra-Fast Gradient Analyses

- You may have to adjust the response rate of your detector for rapid peak detection.

Agilent 1100 DAD
Agilent 1100 WPS with ADVR

Column: Poroshell 300SB-C18
2.1 x 75 mm, 5 µm

Mobile Phase:
A: 95% H₂O, 5% ACN with 0.1% TFA
B: 5% H₂O, 5% ACN with 0.1% TFA

Flow Rate: 2 mL/min
Temperature: 70°C
Detector: UV 215 nm
Piston stroke: 20

Sample:
1. Neurotensin
2. RNase A
3. Lysozyme
4. Myoglobin

Sample: 1. Neurotensin  3. Lysozyme
2. RNase A  4. Myoglobin

Response Time

0.1 sec
1st peak = 1.2 sec
At 20 pts/sec = 24 pts/sec

0.2 sec

0.5 sec
1st peak = 1.2 sec
At 5 pts/sec = 6 pts/sec

1.0 sec

2.0 sec

1st peak = 1.2 sec
At 20 pts/sec = 24 pts/sec

0.1 sec

0.2 sec

0.5 sec
1st peak = 1.2 sec
At 5 pts/sec = 6 pts/sec

1.0 sec

2.0 sec
Summary

- C18 and C8 bonded phases are the best for initial rapid method development with typical sample types.
- Choosing the most sample appropriate bonded phase and using special, targeted bonded phases, such as SB-Aq for polar, difficult to retain compounds can decrease method development time.
- Rapid Resolution columns are needed to reduce method development time.
- Rapid Resolution columns reduce both gradient and isocratic analysis time and permit high throughput rapid analysis.
- Rapid Resolution columns can work effectively with your HPLC.
- HPLC instruments may have additional capabilities to speed up method development and reduce analysis time.