New Developments in Hybrid Particle Technology: Stability, Selectivity, Separation and SPEED

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AAPS 2002 Toronto
Abstract

Pharmaceutical companies have been driven to bring products to market faster by increasing sample throughput. To meet these goals, this has required new chromatographic approaches which expand the limits currently imposed by silica gel based reversed-phase separations. The development of hybrid particles for HPLC columns has set new limits for speed of analysis, operating temperatures and a wider useable pH range (1-12).

Because of the mandate to run faster separations, researchers have tried to move away from hour-long separations on 15 or 25 cm columns. Many have turned to the monolith technology due to claims of reductions in separation time and increased sample throughput. However, running at such high flow rates (up to 10 mL/min) increases solvent consumption dramatically. Smaller i.d. monoliths have been promised, but are not yet available. Additionally, monolith technology is not yet available in preparative dimensions which creates a barrier to direct scale-up possibilities.

New column hardware in 4.6 x 20 mm dimensions has been developed and packed with 2.5 µm and 3.5 µm XTerra® MS C18 particles. We have measured the peak capacities of these columns and found them to be comparable to results obtained with the monolith columns. We have developed several applications with total run times of 5 minutes or less, using reasonable flow rates. We will show how an application run on a long column can be transferred to these shorter columns resulting in optimum chromatographic performance in a much shorter run time. We also show 1000 injections of a protein precipitation sample on a 4.6 x 20 mm column packed with 2.5 µm particles.
Organic vs. Inorganic Packings

<table>
<thead>
<tr>
<th>Inorganic (C&lt;sub&gt;18&lt;/sub&gt; -Silica)</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mechanically strong</td>
<td>Tailing peaks for bases</td>
</tr>
<tr>
<td></td>
<td>High efficiency</td>
<td>Limited pH range</td>
</tr>
<tr>
<td>Organic (Poly-styrene)</td>
<td>Wide pH range</td>
<td>Low efficiency</td>
</tr>
<tr>
<td></td>
<td>No tailing for bases</td>
<td>Mechanically weak</td>
</tr>
</tbody>
</table>

Hybrid Organic/Inorganic Technology: Combining the Best of Inorganic and Organic Particles
Hybrid packing materials bring new capabilities to HPLC

- Classical surface chemistry with high-pH and higher temperature stability than silica-gel based columns
  - Provides new selectivity options in method development
  - Provides options for faster separations
  - Basic compounds in non-ionic form give improved peak shape
  - Basic compounds in non-ionic form give high retention
  - Basic compounds in non-ionic form high preparative loadability

- Lower silanol content than classical silica-based packings
  - Less tailing for basic compounds
  - Simpler retention mechanism
Synthesis of Hybrid Particles

Tetraethoxysilane + Organofunctional triethoxysilane → Polyethoxyoligosiloxane Polymer → Porous Hybrid Particles

- First generation Hybrid, $R = \text{CH}_3$
- Characterized by %C, SEM, TGA, BET, NMR

Patent Pending

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>140 – 330 m$^2$/g</td>
</tr>
<tr>
<td>TPV</td>
<td>0.4 – 1.0 cc/g</td>
</tr>
<tr>
<td>MPD</td>
<td>90 – 300 Å</td>
</tr>
</tbody>
</table>
**First Generation Hybrid: \( R = \text{CH}_3 \)**

**Methyl Hybrid Attribute** | **RP-HPLC Consequence**
--- | ---
Surface \( \text{CH}_3 \) groups reduce surface silanol concentration and the pKa of surface silanols | Reduced USP peak tailing factors
Internal \( \text{CH}_3 \) groups provide hydrophobicity | Increased base stability of column

**Bonded methyl hybrid packings show significantly increased base stability vs. bonded silica packings!**

*We can use these particles to achieve FAST separations...*
Achieving Faster Separations

- To help decrease sample run times, we know that we can move to shorter length columns run at higher flow rates
  - Many chemists have already moved from 25 or 30 cm columns to 10 and 15 cm columns to decrease run times

- In recent years, fast separations (under 5 min) have been developed on the monolith column technology, but at extremely high (i.e. 10 mL/min) flow rates
  - Many researches seem to believe that fast separations can only be performed on silica-rod technology

  **This is a misconception!**

- The development of 4.6 x 20 mm **column** hardware can also give us shorter run times on columns packed with spherical particles.
Peak Capacity ($P$) in Gradients

Number of peaks ($P$) with a peak width ($w$) that can be separated in a given Gradient Time ($t_g$)

$$P = 1 + \frac{t_g}{w}$$

We can use Peak Capacity to compare column performance.
Chromatographic Conditions for Measuring Peak Capacities

**Conditions**
- **Columns**: XTerra® MS C\(_{18}\), 4.6 x 20 mm, 2.5 µm
  - XTerra® MS C\(_{18}\), 4.6 x 20 mm, 3.5 µm
  - Monolith Column, 4.6 x 50 mm
- **Mobile Phase A**: H\(_2\)O
- **Mobile Phase B**: ACN
- **Mobile Phase C**: 100 mM NH\(_4^+\)CH\(_3\)COO\(^-\), pH 5.0
- **Flow Rates**: As Indicated
- **Gradient**:
  - Time Profile
  - (min) %A %B %C
  - 0 85 5 10
  - 4 20 70 10
- **Injection Volume**: 20.0 µL
- **Temperature**: Ambient
- **Detection**: UV @ 238 nm
- **Instrument**: Alliance™ 2695 with 2996 PDA

**Analytes**
1. Lidocaine (40 µg/mL)
2. Prednisolone (10 µg/mL)
3. Naproxen (3 µg/mL)
4. Amitriptyline (10 µg/mL)
5. Ibuprofen (40 µg/mL)
Peak Capacities for XTerra® MS C$_{18}$ 4.6 x 20 mm, 2.5 µm

- 2 mL/min
  - 1550 psi
  - P = 31

- 3 mL/min
  - 2400 psi
  - P = 44

- 4 mL/min
  - 3350 psi
  - P = 42

- 5 mL/min
  - 4330 psi
  - P = 47
Peak Capacities for XTerra® MS C₁₈ 4.6 x 20 mm, 3.5 µm

- 2 mL/min, 790 psi: P = 32
- 3 mL/min, 1200 psi: P = 39
- 4 mL/min, 1650 psi: P = 38
- 5 mL/min, 2100 psi: P = 44
Peak Capacities for Monolith Technology

P = 30  2 mL/min  345 psi

P = 35  3 mL/min  520 psi

P = 41  4 mL/min  700 psi

P = 44  5 mL/min  865 psi

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Typical Reversed-Phase Separation on a 4.6 x 150 mm Column

**Conditions**
- **Column:** XTerra® MS C₁₈, 4.6 x 150 mm, 5.0 µm
- **Mobile Phase A:** Water
- **Mobile Phase B:** Acetonitrile
- **Mobile Phase C:** 100 mM NH₄HCO₃, pH 10
- **Flow Rate:** 1.4 mL/min
- **Gradient:**

```
(min) | %A  | %B  | %C
0     | 90  | 0   | 10
50    | 60  | 30  | 10
```

- **Injection Volume:** 10.0 µL
- **Sample concentration:** 20 µg/mL
- **Temperature:** 30 ºC
- **Detection:** UV @ 254 nm
- **Instrument:** Alliance™ 2795 w/996 PDA

Six peaks fully resolved in almost ONE HOUR.

How can we improve our sample throughput?

1. Caffeine
2. Aniline
3. N-Methylaniline
4. 2-Ethylaniline
5. 4-Nitroanisole
6. N-N-Dimethylaniline

P = 52
PSI = 1575
Step One: First Pass at a Gradient Run on a 4.6 x 20 mm Column

Conditions
Column: XTerra® MS C<sub>18</sub>, 4.6 x 20 mm, 3.5 µm
Mobile Phase A: Water
Mobile Phase B: Acetonitrile
Mobile Phase C: 100 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 10
Flow Rate: 3.0 mL/min
Gradient: Time Profile
(min) %A %B %C
0 90 0 10
7 60 30 10
Injection Volume: 10.0 µL
Sample concentration: 20 µg/mL
Temperature: 30 °C
Detection: UV @ 254 nm

Ratio of column length to gradient time:
150 mm/ 50 min = 3
Try: 20 mm/ 7 min = 2.8
Results: Total run time of 7 min – much better than the 50 minutes!
We know that we can go even FASTER.

1. Caffeine
2. Aniline
3. N-Methylaniline
4. 2-Ethylaniline
5. 4-Nitroanisole
6. N-N-Dimethylaniline

P = 26
PSI = 1224
Step Two: Reduce Gradient Time to 4 Minutes

Conditions

- **Column**: XTerra® MS C₁₈, 3.5 µm 4.6 x 20 mm, 3.5 µm
- **Mobile Phase A**: Water
- **Mobile Phase B**: Acetonitrile
- **Mobile Phase C**: 100 mM NH₄HCO₃ pH 10
- **Flow Rate**: 3.0 mL/min
- **Gradient**:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

- **Injection Volume**: 10.0 µL
- **Sample concentration**: 20 µg/mL
- **Temperature**: 30 °C
- **Detection**: UV @ 254 nm
- **Instrument**: Alliance™ 2795 w/996 PDA

Analytes

1. Caffeine
2. Aniline
3. N-Methylaniline
4. 2-Ethylaniline
5. 4-Nitroanisole
6. N-N-Dimethylaniline

P = 23
PSI = 1235
Reduction in Total Run Time by a Factor of 12!

4.6 x 150 mm
1.4 mL/min, 50 min

4.6 x 20 mm
3 mL/min, 4 min
**Data Comparison:**

**Monolith vs. X Terra® MS C_{18} 3.5 \mu m**

X Terra® at pH 10 makes it possible to separate 6 analytes in 4 minutes and operate at a lower flow rate.

<table>
<thead>
<tr>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column: Monolith, 4.6 x 100 mm</td>
</tr>
<tr>
<td>Mobile Phase A: 20 mM Phosphate buffer pH 7.7</td>
</tr>
<tr>
<td>Mobile Phase B: Acetonitrile</td>
</tr>
<tr>
<td>Flow Rate: <strong>7.0 mL/min</strong></td>
</tr>
<tr>
<td>Gradient: Time Profile</td>
</tr>
<tr>
<td>(min)</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
</tr>
<tr>
<td>3.0</td>
</tr>
<tr>
<td>Injection Volume: 10.0 \mu L</td>
</tr>
<tr>
<td>Temperature: Ambient</td>
</tr>
<tr>
<td>Detection: UV @ 254 nm</td>
</tr>
</tbody>
</table>

1. Caffeine
2. Aniline
3. N-Methylaniline
4. 2-Ethylaniline
5. 4-Nitroanisole
6. N-N-Dimethylaniline

**Conditions**

Column: X Terra® MS C_{18}, 3.5 \mu m 4.6 x 20 mm, 3.5 \mu m

Mobile Phase A: Water

Mobile Phase B: Acetonitrile

Mobile Phase C: 100 mM NH_{4}HCO_{3} pH 10

Flow Rate: **3.0 mL/min**

Gradient: Time Profile

(min) | %A | %B | %C |
<table>
<thead>
<tr>
<th></th>
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<tr>
<td>4</td>
<td>60</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

Injection Volume: 10.0 \mu L

Sample concentration: 20 \mu g/mL

Temperature: 30 °C

Detection: UV @ 254 nm

http://www.merck.de/english/services/chromatographie/hplc/chromolith/pdf/appl_05.pdf
Application: Separation of 9 Analytes in 4 Minutes on a 4.6 x 20 mm Column

Conditions
Column: XTerra® MS C₁₈, 4.6 x 20 mm, 3.5 µm
Mobile Phase A: 0.1% Formic Acid in Water
Mobile Phase B: 0.1% Formic Acid in ACN
Flow Rate: 3.0 mL/min
Gradient: Time Profile
(min) %A %B
0 100 0
4 0 100
Injection Volume: 10.0 µL
Sample concentration: 10 µg/mL
Temperature: 30 °C
Detection: UV @ 254 nm
Instrument: Alliance™ 2795 w/996 PDA

Compounds
1. Acetanilide
2. Triamcinolone
3. Hydrocortisone
4. 2-Amino-7-chloro-5-oxo-5H-[1]benzopyrano[2,3-b]pyridine-3-carbonitrile
5. 6α-Methyl-17α-hydroxyprogesterone
6. 3-Aminofluoranthene
7. 2-Bromofluorene
8. Perylene
9. Naphtho(2,3-a)pyrene

P = 23
PSI = 1440
**Application: Sulfonamides**

**Conditions**
- **Column:** XTerra™ MS C<sub>18</sub> 4.6 x 20 mm, 3.5 µm
- **Mobile Phase A:** 0.1% FA in Water
- **Mobile Phase B:** 0.1% FA in MeOH
- **Flow Rate:** 3.0 mL/min
- **Gradient:**
  - (min) %A %B
  - 0 100 0
  - 4 50 50
- **Injection Volume:** 10.0 µL
- **Sample concentration:** 20 µg/mL
- **Temperature:** 30 °C
- **Detection:** UV @ 254 nm
- **Instrument:** Alliance™ 2795 w/996 PDA

**Analytes**
1. Sulfadiazine
2. Sulfathiazole
3. Sulfamerazine
4. Sulfamethoxazole
5. Sulfisoxazole
6. Sulfadimethoxine

P = 23
PSI = 2045
Column Lifetime: Protein Precipitation Samples

Injection 1
Maximum Backpressure = 2500 psi

Injection 1000
Maximum Backpressure = 2560 psi

1000 injections on a 2.5 µm particle column with only a slight increase in backpressure!
Protein Precipitation Procedure:

**Analytes**: atenolol (10 mg/mL in MeOH)
- pindolol (5 mg/mL in MeOH/H₂O)
- metoprolol (10 mg/mL in MeOH)

*prepared in strong conc. to spike into plasma

**Spiked Plasma Sample**:
- 50 µL of atenolol
- 50 µL of metoprolol
- 50 µL of pindolol
- 4750 µL of Rat Plasma
- 100 µL of H₃PO₄
- 5000 µL total sample volume

**Protein Precipitation**:
- Multiple samples prepared from 5000 µL spiked plasma
  - 350 µL of Spiked Plasma sample
  - 1000 µL of acetonitrile
  - 1350 µL total sample volume
- Samples were centrifuged at 3000 RPM for 30 minutes.
- Supernatent transferred to culture tube and evaporated.
- Sample reconstituted in 350 µL water and injected.

**Conditions**
- Column: XTerra® MS C₁₈, 4.6 x 20 mm, 2.5 µm
- Mobile Phase A: 0.1% TFA in H₂O
- Mobile Phase B: ACN
- Flow Rate: 3.0 mL/min
- Gradient: Time Profile %A %B
  - 0 100 0
  - 4 20 80
- Injection Volume: 20.0 µL
- Sample Concentrations: 0.1 mg/mL of atenolol,
  0.05 mg/mL of pindolol
  0.1 mg/mL of metoprolol
- Temperature: Ambient
- Detection: UV @ 220 nm
- Instrument: Alliance™ 2695 w/2996 PDA

**Peaks**
1. Atenolol
2. Pindolol
3. Metoprolol
Conclusions

- Many different hybrid organic/inorganic packing materials can be designed for HPLC

- C\textsubscript{18}-bonded hybrid packing materials show:
  - Low silanol activity = improved peak shape compared to the best silica-based packings
  - Improvements in high-pH stability compared to silica-based materials

- These new 4.6 x 20 mm columns packed with XTerra\textsuperscript{®} offer a better advantage over the monolith technologies by providing:
  - Better peak shapes for basic analytes
  - Ability to run at high pH and high temperature
  - Available in MS C18, MS C8, RP18 and RP8 chemistries and
  - Availability of Preparative dimensions for direct scale-up
  - Lower flow rates allow for less solvent waste and easier split into a mass spectrometer without a loss in sensitivity

- Chemists do not need the monolith technology
  - To achieve faster separations
  - To run 100’s of protein precipitation samples
    - This can all be accomplished on the new 4.6 x 20 mm columns