High performance liquid chromatography

Robust Particles for HPLC & Scale-up for Peptide Purification

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Outline of the presentation

- Introduction of Kromasil Eternity
- Product characteristics
- Applications/Comparisons
  - Analytical HPLC
  - Preparative HPLC
- Preparative HPLC
- R&D Peptide Purification Case Study
- Conclusion
Controlling and utilizing pH

- Controlling and utilizing pH as a method development tool will greatly enhance your chromatography

- pH strongly influences the efficiency of separation of ionizable compounds

- Ideally, mobile phase pH should be at least 1 pH unit away from the analyte’s pKₐ

- The ability to choose the optimal pH from a wide range pH 1 – 12 is very beneficial
Kromasil Eternity platform

- Particle sizes: 2.5 µm & 5 µm
- Pore Size: 100 Å
- Surface Area (porous): 330 m²/g
Kromasil Eternity C18 & PhenylHexyl

- **Ligand:** C18
- **Endcapping:** Proprietary
- **pH Range:** 1 - 12
- **USP:** L1

Eternity platform

Eternity C18
### Product characteristics:

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand:</td>
<td>C18</td>
</tr>
<tr>
<td>Particle sizes:</td>
<td>2.5 µm &amp; 5 µm</td>
</tr>
<tr>
<td>Pore Size:</td>
<td>100 Å</td>
</tr>
<tr>
<td>Surface Area:</td>
<td>330 m²/g</td>
</tr>
<tr>
<td>Carbon Load:</td>
<td>14 %</td>
</tr>
<tr>
<td>Endcapping:</td>
<td>Proprietary</td>
</tr>
<tr>
<td>pH Range:</td>
<td>1 - 12</td>
</tr>
<tr>
<td>USP:</td>
<td>L1</td>
</tr>
</tbody>
</table>

Kromasil®
Long term high pH stability – a comparison

**Amitriptyline**

- Percentage of initial efficiency
- Comparison between Kromasil Eternity and Waters XBridge

**Prednisolone**

- Percentage of initial efficiency
- Comparison between Kromasil Eternity and Waters XBridge

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**Test conditions**

- **Columns:**
  - Kromasil Eternity-5-C18 4.6 x 250 mm
  - Waters X-Bridge, 5 μm, C18, 4.6 x 250 mm
- **Mobile Phase A:** 10 mM ammonium bicarbonate, pH 10.5/acetonitrile (90/10)
- **Mobile Phase B:** 10 mM ammonium bicarbonate, pH 10.5/acetonitrile (10/90)
- **Flow rate:** 1 mL/min
- **Temperature:** 45°C
- **Gradient**
  - 0 min 100% A
  - 10 min 100% B
  - 15 min 100% B
  - 16 min 100% A
  - 20 min 100% A
- **Test Amitriptyline:** 10 mM ammonium bicarbonate pH 10.5/acetonitrile (30/70)
- **Test Prednisolone:** 10 mM ammonium bicarbonate pH 10.5/acetonitrile (70/30)
- **Test Cycle:** 6 x gradient + tests = 172 min/cycle

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Kromasil®

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Tomorrow’s Answers Today
pH variation to control selectivity

Test conditions
Column: Kromasil Eternity-2.5-C18 4.6 x 50 mm
Mobile phase: acetonitrile/20 mM sodium phosphate
pH 2.1, 7.2 and 11.0 respectively
Gradient: 0-0.5 min: 10%, 5.5 min: 50% acetonitrile
Flow rate: 1.5 ml/min
Temperature: 25°C
Detection: UV 254 nm
Scale-up or Scale-down

Kromasil Eternity,
5 µm, 21.2 x 50 mm

Kromasil Eternity,
5 µm, 4.6 x 50 mm

Kromasil Eternity,
2.5 µm, 4.6 x 50 mm

Test conditions:
- Sample: Mix of beta-blockers
  1 = ursalil
  2 = atenolol
  3 = pindolol
  4 = metoprolol
  5 = propranolol
  6 = alprenolol
- Mobile Phase: acetonitrile/50 mM triethylamine acetate, pH 7.5 (40/60)
- Flow rate: 0.43 ml/min and 9.0 ml/min for 4.6 and 21.2 mm i.d. columns, respectively
- Temperature: 20°C
- Detection: UV 230 nm
- Stationary Phase: Kromasil Eternity (2.5 and 5 µm)
- Column length: 50 mm
Increase efficiency – Save time (1/3)

Save time without resolution loss by decreasing particle size

Stationary phase: Kromasil Eternity-2.5-C18
Column size: 4.6 x 100 mm
Flow rate: 1.6 ml/min
Mobile phase: ACN/H₂O/formic acid (25/75/.01)

1. Uracil
2. suplathiazole
3. sulphamerazin
4. sulphamethoxazole
Increase efficiency – Save time (2/3)

1) Save even more time with shorter column (1) and increased flow rate (2)

Stationary phase: Kromasil Eternity-2.5-C18
Column size: 4.6 x 50 mm
Flow rate: 2.7 ml/min

N = 123 000 / m
Rs1 = 2.9
Rs2 = 13
ΔP = 366 bar

2) Increase efficiency – Save time (2/3)

Stationary phase: Kromasil Eternity-2.5-C18
Column size: 4.6 x 50 mm
Flow rate: 1.7 ml/min

N = 153 000 / m
Rs1 = 3.6
Rs2 = 15
ΔP = 234 bar

Mobile phase: ACN/H₂O/formic acid (25/75/0.01)
Increase efficiency – Save time (3/3)

Stationary phase: Kromasil Eternity-5-C18
Column size: 4.6 x 250 mm
Flow rate: 1.0 ml/min
Mobile phase: ACN/H₂O/formic acid (25/75/0.01)

N = 77,000 / m
Rs₁ = 6.9
Rs₂ = 26
ΔP = 125 bar

Run at 1/14 of original analysis time

Stationary phase: Kromasil Eternity-2.5-C18
Column size: 4.6 x 50 mm
Flow rate: 2.7 ml/min

N = 123,000 / m
Rs₁ = 2.9
Rs₂ = 13
ΔP = 366 bar
Reduce solvent consumption – An example

Stationary phase: Kromasil Eternity-5-C18
Column size: 4.6 x 250 mm
Flow rate: 1.0 ml/min

N = 77 000 / m
Rs1 = 6.9
Rs2 = 26
ΔP = 125 bar

Stationary phase: Kromasil Eternity-2.5-C18
Column size: 4.6 x 100 mm
Flow rate: 1.6 ml/min

N = 194 000 / m
Rs1 = 5.8
Rs2 = 25
ΔP = 383 bar

Save solvent without resolution loss

<table>
<thead>
<tr>
<th>Particle size (µm)</th>
<th>Col. Dim. (mm x mm)</th>
<th>Flow rate (ml/min)</th>
<th>Cycle time (min)</th>
<th>Mobile phase volume/ cycle (ml)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4,6 x 250</td>
<td>1,0</td>
<td>12</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>2,5</td>
<td>4,6 x 100</td>
<td>1,6</td>
<td>3</td>
<td>4,8</td>
<td>40</td>
</tr>
<tr>
<td>2,5</td>
<td>3,0 x 100</td>
<td>0,7</td>
<td>3</td>
<td>2,1</td>
<td>18</td>
</tr>
<tr>
<td>2,5</td>
<td>2,1 x 100</td>
<td>0,33</td>
<td>3</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>
Applications at high pH

Separation of some classical histamine antagonists

**Test conditions:**

- **Stationary Phase:** Kromasil Eternity-5-C18
- **Column size:** 4.6 x 250 mm
- **Mobile Phase:** methanol/water, 50 mM triethyl-amine acetate buffer, pH 11 (70/30)
- **Flow rate:** 1.4 ml/min
- **Temperature:** 25°C
- **Detection:** UV 254 nm

1 = Maleic acid  
2 = Doxylamine  
3 = Chlorpheniramine  
4 = Diphenhydramine  
5 = Tripolidine
" – The long-lasting phase when alternative selectivity is required"
Eternity PhenylHexyl provides alternate selectivity

Common chromatographic conditions
Column size: 2.1 x 50 mm
Sample: Vitamins: 1 = p-aminobenzoic acid, 2 = biotin, 3 = vitamin B₁₂, 4 = vitamin B₂
Mobile Phase: methanol/potassium phosphate buffer, 20 mM, pH 2.5
gradient: methanol: 0 min 20%, 5 min 37%.

Flow rate: 0.35 ml/min
Temperature: 25°C
Detection: UV @ 210 nm
Kromasil Eternity – Analytical scale

- 2.5 µm particle size
- UHPLC and HPLC (> 200,000 pl/m)
- Easy to scale up to 5µm
Preparative Chromatography

- Need to purify milligram to gram amounts of compound for further research

- Huge difference in the loadability of ionized vs. un-ionized species
  - The loadability difference can be as much as 50-fold

- Un-ionized compounds can be loaded at a much higher concentration thus there is a need for robust stationary phase at all pH’s
Prep application at low and high pH [1(3)]

Relationship of 1-phenylpiperazine retention with mobile phase strength and pH

1-(2-chlorophenyl) piperazine

\[ k' = \frac{t_R - t_0}{t_0} \]

- \( k' \): retention factor
- \( t_R \): retention time
- \( t_0 \): retention time of non-retained compound
Prep application at low and high pH [2(3)]

Development of preparative separation methods for two basic crude compounds at acidic and basic pH

1-(2-chlorophenyl) piperazine

Chromatographic conditions
Sample: crude tryptamine and 1-(2-chlorophenyl)piperazine
Injection: 2.5 mg (0.5 ml of 5 mg/ml sample solution)
Column: Kromasil Eternity 5-C18 4.6 x 150 mm
Mobile phase: Acetonitrile/0.1 M potassium phosphate pH 2.5 or pH 11.0
Flow rate: 1 ml/min
Detection: UV @ 300 nm and 280 nm
Prep application at low and high pH [3(3)]

Scale up of column dimension followed by 10 times load increase without reaching touching bands

**Chromatographic conditions**
Sample: crude 1-(2-chlorophenyl)piperazine
Injection: 10 ml of 5, 20, and 50 mg/ml sample, sample dissolved in acetonitrile/0.1 M potassium phosphate pH 13 30/70
Column: Kromasil Eternity 5-C18 21.2 x 150 mm
Mobile phase: Acetonitrile/0.1 M potassium phosphate pH 11 30/70 v/v
Flow rate: 21 ml/min
Detection: UV @ 280 nm
Purification of crude amitriptyline

Initial Purity: 62%
Load: 420 mg (36 mg/g)

- Column: Kromasil Eternity 5 μm C18 (21.2 x 50 mm),
  Mobile phase: 25 mM NH4HCO3 pH 10.5/ACN,
  Gradient: 45-80% ACN/10 min.
  Flow: 21 mL/min, 25°C, 254 nm

- Purity assessment of fraction pool:
  Column: Kromasil Eternity 2.5 μm C18 (4.6 x 50 mm),
  Mobile phase: 10 mM NH4HCO3 pH 10.5/MeCN (45/55)
  Flow rate: 1.7 mL/min, 25°C, 254 nm
# Product Assortment

<table>
<thead>
<tr>
<th></th>
<th>2.5 μm Eternity™</th>
<th>5 μm Eternity™</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 x 50 mm</td>
<td>Eternity-2.5-C18 2.1 x 50</td>
<td>Eternity-5-C18 2.1 x 50</td>
</tr>
<tr>
<td>2.1 x 100 mm</td>
<td>Eternity-2.5-C18 2.1 x 100</td>
<td>—</td>
</tr>
<tr>
<td>2.1 x 150 mm</td>
<td>—</td>
<td>Eternity-5-C18 2.1 x 150</td>
</tr>
<tr>
<td>4.6 x 50 mm</td>
<td>Eternity-2.5-C18 4.6 x 50</td>
<td>Eternity-5-C18 4.6 x 50</td>
</tr>
<tr>
<td>4.6 x 100 mm</td>
<td>Eternity-2.5-C18 4.6 x 100</td>
<td>Eternity-5-C18 4.6 x 100</td>
</tr>
<tr>
<td>4.6 x 150 mm</td>
<td>—</td>
<td>Eternity-5-C18 4.6 x 150</td>
</tr>
<tr>
<td>4.6 x 250 mm</td>
<td>—</td>
<td>Eternity-5-C18 4.6 x 250</td>
</tr>
<tr>
<td>10 x 50 mm</td>
<td>—</td>
<td>Eternity-5-C18 10 x 50</td>
</tr>
<tr>
<td>10 x 150 mm</td>
<td>—</td>
<td>Eternity-5-C18 10 x 150</td>
</tr>
<tr>
<td>10 x 250 mm</td>
<td>—</td>
<td>Eternity-5-C18 10 x 250</td>
</tr>
<tr>
<td>21.2 x 50 mm</td>
<td>—</td>
<td>Eternity-5-C18 21.2 x 50</td>
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<tr>
<td>21.2 x 150 mm</td>
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<td>Eternity-5-C18 21.2 x 150</td>
</tr>
<tr>
<td>21.2 x 250 mm</td>
<td>—</td>
<td>Eternity-5-C18 21.2 x 250</td>
</tr>
<tr>
<td>30 x 50 mm</td>
<td>—</td>
<td>Eternity-5-C18 30 x 50</td>
</tr>
<tr>
<td>30 x 150 mm</td>
<td>—</td>
<td>Eternity-5-C18 30 x 150</td>
</tr>
<tr>
<td>30 x 250 mm</td>
<td>—</td>
<td>Eternity-5-C18 30 x 250</td>
</tr>
</tbody>
</table>

1. Other column dimensions available upon request.
2. Also available in columns for UHPLC use.
3. Guard columns are available for each column dimension.
Kromasil Eternity – The Benefits

Modified silica ➞ Long-lasting columns ➞ Better economy

Small particles (2.5 μm) ➞ High efficiency ➞ Faster analyses

Modified silica ➞ pH 1 to 12 ➞ Easier method development
Analytical vs. preparative HPLC

**Analytical HPLC:**

Objective: Identification and/or quantification of compounds

Output: Information

Compound destination: waste

**Preparative HPLC:**

Objective: Purification and isolation of compounds

Output: purified compound

Compound destination: Fraction collection

Column dimension is irrelevant, it is the SCOPE of the separation determines whether it is analytical or prep HPLC
HPLC vs. LPLC

- HPLC: High Performance Liquid Chromatography
- LPLC: Low Pressure Liquid Chromatography

- HPLC: Faster analysis time
- LPLC: Slower analysis time
Chromatographic Modes

MW (Da)

Polarity

RP-bonded
IEC
RP-HIC
GPC
GF
CSP
NP

org. soluble
water soluble

MW (Da)

10^2 10^3 10^4 10^5 10^6
# Working areas of preparative HPLC

<table>
<thead>
<tr>
<th>Compound amount</th>
<th>Working area</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>- Isolation of e.g. enzymes for research purpose</td>
</tr>
</tbody>
</table>
| mg              | - Biological and biochemical testing  
|                 | - Structure elucidation and characterization of side products, metabolites, natural products |
| g               | - Reference compounds (standards)  
|                 | - Compounds for toxicological screenings |
| kg - tons       | - Industrial scale, APIs, drugs |
Column types

Fixed- bed, pre-packed
- 2.1 – 50 mm ID
  - Semi-prep
  - Combi-Chem
  - R&D

Self-packers
- 25 – 100 mm ID
  - Development
  - Pilot Plant

DAC-columns
- 50 – 1000 mm ID
  - Development
  - Pilot Plant
  - Production
Linear vs. Non-linear chromatography

Typical injection: 0.005 mg/g

Typical injection: 10 mg/g X 2500
Volume – vs. concentration overload

a) Analytical Injection

b) Volume Overload

Not detector signal saturation

Volume overload should be avoided

c) Concentration Overload

Concentration overload can lead to displacement effects……
Compression

Analytical injection

Preparative injection (individual profiles) - 98% purity, 60% yield

Preparative injection (real profiles) - 98% purity, 75% yield
Tag-along

Analytical injection

Preparative injection (individual profiles)
98% purity
90% yield

Preparative injection (real profiles)
Case Study

- Peptide Purification– R&D
  (Example of a Reversed Phase Application Development)
Peptide Purification R&D

Characteristics of R&D Application:

• Only very small amount of material to work with

• Free hands → no restrictions concerning e.g. mobile phase

• Short time frame

Cost Distribution:
Objectives:

• 9 amino acid peptide has to be purified from ca 75% to 98% purity

• The peptide is stable below pH 9

• 1 g of peptide has to be purified per injection

• Recovery > 30%
Peptide Purification R&D

Strategies:

1. Compare different pH values $\rightarrow \alpha_{\text{max}}$
2. Compare different buffer systems $\rightarrow \alpha_{\text{max}}$, preferences
3. Compare different stationary phases $\rightarrow \alpha_{\text{max}}$
4. Overload $\rightarrow$ fraction analysis $\rightarrow$ purity / recovery calculations
5. Calculation of productivity $\rightarrow$ required column dimension
Peptide Purification R&D

1. Comparing pH value

- 50 mM potassium phosphate / MeCN
- 28 – 31 % MeCN / 30 min
- KR100-5-C18, 4,6 x 250 mm
- 1 mL/min
- 230 nm

**Better selectivity at higher pH**

Small peptide (< 10 amino acids) → no need for gradient elution
Peptide Purification R&D

2. Try TRIS as buffer
3. Run under isocratic conditions
4. Compare C18 with C4

0.1M Tris pH 8 / MeCN 73/27 (v/v)
1 mL/min
230 nm
5. Overloaded injection $\rightarrow$ fraction analysis $\rightarrow$ purity / recovery assessment

0.1M Tris pH 8 / MeCN 73/27 (v/v)
KR100-10-C4 (4.6 x 250 mm)
10 mg injection
1 mL/min
230 nm

Purity: 98.4%
Recovery: 69.4%

OK
6. Productivity calculation → required column dimension

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$m_{\text{inj}}$</td>
<td>10 mg</td>
</tr>
<tr>
<td>$m_{\text{packing}}$</td>
<td>2.36 g</td>
</tr>
<tr>
<td>relative load</td>
<td>4.24 mg/g</td>
</tr>
<tr>
<td>initial purity</td>
<td>75%</td>
</tr>
<tr>
<td>recovery</td>
<td>69%</td>
</tr>
<tr>
<td>cycle time</td>
<td>45 min (incl. washing and re-equilibration)</td>
</tr>
<tr>
<td>productivity</td>
<td>$5.65 \text{ mg}<em>{\text{crude}} \cdot h^{-1} \cdot g</em>{\text{packing}}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$2.92 \text{ mg}<em>{\text{product}} \cdot h^{-1} \cdot g</em>{\text{packing}}^{-1}$</td>
</tr>
</tbody>
</table>

Scale-up factor: 100  
$m_{\text{packing}}$: 236 g → 414 ml column volume → I.D. column = 2.3 cm

Required column dimension: 30 x 250 mm
Conclusions

- **Reversed phase HPLC is a powerful, versatile tool for peptide purification**
  - Adequate for difficult separations
  - Fast separation → high productivity
  - Concentrated product fraction → cost effective work-up

- **Kromasil Eternity**
  - 2.5µm particle size for efficient analytical chromatography
  - Broad pH range (1-12) provides maximum method development flexibility
  - 5µm particle size provides robust choice for small scale purification
  - C18 & PhenylHexl phases
Thank you for your attention!

www.kromasil.com
www.sigmaaldrich.com/kromasil