DEVELOPMENT OF AFFINITY MICROCOLUMNS FOR HIGH-THROUGHPUT BIOINTERACTION ANALYSIS

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European Bioanalysis Forum 2011
17 November 2011
Drug-Protein Interactions

**Target Tissue**

Drug + Receptor → Drug-Receptor Complex → Response

**Kidneys**

Drug (+ Carrier) → Excretion into Urine

**Blood/Plasma**

Drug + Protein ↔ Drug-Protein Complex

**Liver**

Drug → Enzymes → Metabolites → Excretion into Urine or Bile

High-Performance Affinity Chromatography (HPAC)

Monolithic Supports

- Higher external porosity than particle-based supports
  - Higher permeability
  - Lower backpressures
  - Better efficiency

- Two types of pores
  - Flow-through pores (macropores)
  - Smaller “diffusion” pores (mesopores)

Silica Particles vs. Silica Monoliths

**Silica Particles**
- Good mechanical strength
- High surface area
- Total porosity ~65%

**Silica Monoliths**
- Single piece of porous silica
- Total porosity ~80%
- Lower backpressure
- Better mass transfer
- Faster separations
- Shrinkage

Affinity Microcolumns

Advantages
- Require small amounts of ligand
- Reduced surface area, less non-specific binding
- Reduced analysis times

Challenges
- Need new approaches for preparing and packing columns
- Need new immobilization methods to increase the activity of ligand
- Need better understanding of properties and limitations

Frontal Analysis Measurements

- Breakthrough times: 4.2-9.5 min
- Breakthrough volume: 2.8 mL

- Breakthrough times: 30-45 s
- Breakthrough volume: 0.29 mL

Frontal Analysis Measurements

\[ \frac{1}{m_{\text{Lapp}}} = \frac{1}{K_A m_L [A]} + \frac{1}{m_L} \]

Frontal Analysis Results

<table>
<thead>
<tr>
<th>Column Length (mm)</th>
<th>$m_L$ (nmol)</th>
<th>Relative Activity (versus 20 mm column)</th>
<th>$K_a$ ($x 10^5$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>27.6 (± 0.2)</td>
<td>1.00</td>
<td>2.0 (± 0.1)</td>
</tr>
<tr>
<td>3</td>
<td>3.8 (± 0.3)</td>
<td>0.9 (± 0.1)</td>
<td>2.3 (± 0.1)</td>
</tr>
<tr>
<td>2</td>
<td>2.6 (± 0.3)</td>
<td>0.9 (± 0.1)</td>
<td>2.1 (± 0.2)</td>
</tr>
<tr>
<td>1</td>
<td>1.2 (± 0.1)</td>
<td>0.8 (± 0.1)</td>
<td>2.6 (± 0.1)</td>
</tr>
</tbody>
</table>

Literature $K_a$ values: 2.1 – 2.6 x 10$^5$ M$^{-1}$

• Binding capacity decreased in proportion to column size
• Decrease in precision in $K_a$ values (2 cm: ±5%, 1 mm: ±25%) due to decrease in amount of time and stationary phase available for drug binding

Preparation of HSA Silica Monoliths

- Chromolith™ Performance Si column was donated by Merck KGaA (4.6 mm i.d. x 10 cm)

1. Columns cut to lengths from 1 to 5 mm
2. Converted into diol silica monolith
3. Immobilized HSA by Schiff base method

Warfarin Retention on HSA Silica Monoliths

- Longer HSA columns (4-10 cm): 25-150 min
- HSA silica monolith microcolumn (3 mm): 1.4-9.5 min

Silica Particles vs. Silica Monoliths

**Graphs:**
- **Silica particles** vs. **Silica monolith**
  - Plate height (mm) vs. Linear velocity (cm/s)
- **Silica monolith** vs. Flow rate (mL/min)
  - Retention factor
- **Silica particles** vs. Flow rate (mL/min)
  - Retention factor

Rapid Determination of Drug-Protein Dissociation Rates


![Graph showing response over time for Control Column and HSA Column.](image)

**Control Column**
- Washing away of non-bound analyte
- Release of any non-specifically bound analyte

**HSA Column**
- Release of the analyte from the immobilized HSA
Typical Results

<table>
<thead>
<tr>
<th>Drug</th>
<th>( K_a ) (M(^{-1}))</th>
<th>Binding Site on HSA</th>
<th>Measured Value ( k_d ) (s(^{-1}))</th>
<th>Literature Value ( k_d ) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin</td>
<td>2.1-2.6 ( \times 10^5 )</td>
<td>I</td>
<td>0.41 (± 0.06)</td>
<td>0.35 - 0.66</td>
</tr>
<tr>
<td>Imipramine</td>
<td>1.6 ( \times 10^5 )</td>
<td>II</td>
<td>0.29 (± 0.11)</td>
<td>0.41 - 0.67</td>
</tr>
<tr>
<td>Diazepam</td>
<td>2.2 ( \times 10^5 )</td>
<td>II</td>
<td>0.44 (± 0.11)</td>
<td>--</td>
</tr>
<tr>
<td>Acetohexamide</td>
<td>0.43-1.3 ( \times 10^5 )</td>
<td>I, II</td>
<td>0.58 (± 0.02)</td>
<td>--</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>5.3-5.5 ( \times 10^4 )</td>
<td>I, II</td>
<td>0.49 (± 0.15)</td>
<td>--</td>
</tr>
</tbody>
</table>

Same general behavior and types of plots were seen for the other drugs that were examined.

Analysis of Free Drug Fractions

- HSA microcolumns were used in ultrafast extraction and free drug fraction measurements
  - HSA is most abundant serum protein and important binding agent for a variety of drugs-use for more than analyte
  - Lower association equilibrium constants of drugs for HSA ($10^3$-$10^5$) versus antibodies ($10^6$-$10^{12}$) allows for the use of isocratic conditions
  - Less expensive to perform compared to using columns based on immobilized antibodies

Analysis of Free Drug Fractions

Analysis times of only 40 s per injection at 4.5 mL/min

## Analysis of Free Drug Fractions

<table>
<thead>
<tr>
<th>Immobilization Method and Sample</th>
<th>HSA Microcolumns (Ultrafast Extraction)</th>
<th>Ultrafiltration (Reference Method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schiff Base Method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Warfarin + HSA</td>
<td>0.20 (± 0.03)</td>
<td>0.20 (± 0.02)</td>
</tr>
<tr>
<td>R-Warfarin + HSA</td>
<td>0.31 (± 0.05)</td>
<td>0.28 (± 0.02)</td>
</tr>
<tr>
<td>SMCC Method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Warfarin + HSA</td>
<td>0.23 (± 0.02)</td>
<td>0.21 (± 0.03)</td>
</tr>
<tr>
<td>S-Ibuprofen + HSA</td>
<td>0.28 (± 0.02)</td>
<td>0.25 (± 0.07)</td>
</tr>
<tr>
<td>Imipramine + HSA</td>
<td>0.92 (± 0.02)</td>
<td>0.86 (± 0.07)</td>
</tr>
</tbody>
</table>

Summary

• Affinity microcolumns provide comparable results to those obtained with longer columns and can be used in rapid analysis of drug-protein binding
  – Smaller amount of protein (low nmol to upper pmol) needed for column preparation
  – Faster analysis times (net retention times are 100 times faster than traditional 10 cm column)
  – Affinity silica monolith microcolumns have better efficiency (higher flow rates, larger number of theoretical plates)

• Ultrafast extraction based on HSA microcolumns can be used for measuring the free fractions of some drugs in drug-protein mixtures.
  – Good agreement with reference method
  – Fast analysis times (40 s per injection)
References


Acknowledgements

- Bioanalysis editorial board and readers
- Waters Corporation
- Prof. David S. Hage
- National Institutes of Health (R01 GM044931)
- Merck KGaA