LC Method Development in Bioanalysis

Successfully Developing HILIC Methods
Challenges in Bioanalysis: Business Impact

- **Throughput**
  - Ranking of compound in discovery phase to ensure fast and accurate decision-making
  - Need to return results quickly to clinic
  - Faster time to market
  - Faster service for client

- **Productivity**
  - Cost-effective use of time and assets
  - Maximize use of analyst’s time
  - Accessible technology
    - Usable by anyone
    - Yields consistent high quality data

- **Right first time**
  - Reduce the occurrence of questionable results
  - Reduce the occurrence of transcription errors
  - Carry out sufficient experiments to understand both analytes and matrix
Matrix Effects
   - Recent Crystal City meeting provided guidance on the determination and acceptability of matrix effects as a result of sample preparation

Incurred Sample Reanalysis (ISR)
   - Recommendation that a subset of patient samples are re-analyzed and compared to original
   - Direct impact on productivity
   - Increased costs

Metabolite Identification in Safety Testing (MIST)
   - Recent FDA guidelines require that all metabolites with an exposure >10% of the active must be quantified and identified
• **Sensitivity** is required to accurately monitor drug candidate and metabolites
  — More potent New Chemical Entities (NCE), lower dosing levels, smaller sample volumes, extended release medicines

• High **throughput** is not just desirable, it is a necessity
  — Hundreds of samples analyzed that possess diverse physiochemical properties
  — Fast turnaround time from sample receipt to Pharmacokinetic (PK) report is critical

• Method **ruggedness** and **reliability** is essential for the generation of an accurate PK profile
  — Co-eluting endogenous materials and metabolites can result in reduced assay accuracy

• **Data quality** must be maintained
  — Better, more informed decisions
Typical Approach

- LC/MS/MS is the analytical platform of choice
  - Multiple Reaction Monitoring (MRM) provides a high degree of selectivity
  - Belief that MRM minimizes the impact on MS response from endogenous materials or metabolites

- Compromises in sample preparation and chromatography are often made due to MS/MS selectivity
  - Sample preparation
    - Minimal use of solid phase extraction (SPE) for sample preparation due to cost and method development time
    - Protein precipitation (PPT) is often the sample preparation method of choice
  - Chromatography
    - Universal reversed-phase (RP) methods employed
    - Rapid gradients used with short (20 – 50 mm) C_{18} columns
Process for Developing a Successful Bioanalytical Method

- Develop MRM Method
  - Mass Spectrometry instrument platform
  - Conventional procedure
  - Consideration of parameters requiring optimization
  - Novel approach to automated MRM method development

- Develop LC Method
  - LC instrument platform
  - LC column choice
  - Basic approach to fast method development
  - Focused gradient method optimization

- Assessment of Method as “Fit for Purpose”
  - Sensitivity
  - Specificity/matrix interference
  - Additional steps?
Process for Developing a Successful Bioanalytical Method

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- Develop LC Method
  - LC instrument platform
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- Assessment of Method as “Fit for Purpose”
  - Sensitivity
  - Specificity/matrix interference
  - Additional steps?
### Column Selection in Method Development

- **Three particle substrates [130Å and 300Å BEH, HSS]**
  - All are available in HPLC and UPLC particle sizes
- **Wide and growing selection of column chemistries**
  - 11 stationary phases
    - BEH 130Å C₁₈, C₈, Shield RP₁₈, Phenyl, HILIC, Amide
    - BEH 300Å C₁₈ and C₄
    - HSS C₁₈, T₃, C₁₈ SB
- **Proven application-based solutions**
  - AAA, OST, PST, PrST and Glycan
- **Hybrid Particle Technology**
  - Widest pH range for easier method development
  - Increased mass loading / loadability
  - Pressure tolerance
  - Choice of 130Å (small molecules) & 300Å (large molecules) pore sizes
- **Scalability with XBridge and HSS HPLC columns**
- **VanGuard Pre-columns**
- **eCord Technology**

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**Column List**

<table>
<thead>
<tr>
<th>Column</th>
<th>Image</th>
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</thead>
<tbody>
<tr>
<td>BEH C₁₈</td>
<td>![Image](BEH C₁₈)</td>
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<td>BEH C₈</td>
<td>![Image](BEH C₈)</td>
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<td>BEH Phenyl</td>
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<td>BEH Shield RP₁₈</td>
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<td>BEH HILIC</td>
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<td>BEH Amide</td>
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</tr>
<tr>
<td>HSS C₁₈ SB</td>
<td>![Image](HSS C₁₈ SB)</td>
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</table>

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Benefits of High pH Mobile Phase in Mass Spectrometry

1. 9-OH Risperidone
2. Risperidone
3. Clozapine (I.S.)

Independent of which organic modifier is used, these basic compounds exhibit a higher MS response in a high pH mobile phase

Why?
Benefit of High pH Separations: Enhanced Retention of Basic Compounds

Silica-based materials dissolve at high pH

Analytes retain more when they are un-ionized

Amitriptyline

Nortriptyline

pH 2

pH 8

pH 10

Silica-based materials dissolve at high pH

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Choosing an LC column that has extended pH utility (1-12) allows the flexibility to investigate analyte response

- Manipulate retention of acids and bases
- Improve the retention of polar metabolites
- Elution in higher organic concentration leads to improved desolvation in the MS source
- Analytes eluting in their un-ionized form combined with proper choice of mobile phase buffer/concentration can lead to significant increase in MS response*

2nd Generation Hybrid Particles: Ethylene Bridged Hybrid (BEH) Particles

**Surface**

- Reduced Silanols
- Bridging Group
- Hydrophobic Group

**Wall**

- Internal bridging groups provide high interconnectivity
- Internal hybrid groups provide hydrophobicity

### Hybrid Particle Attribute

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface hybrid groups reduce surface silanol concentration</td>
<td>Improved USP tailing factors</td>
</tr>
<tr>
<td>Internal bridging groups provide high interconnectivity</td>
<td>Increased chemical and mechanical stability</td>
</tr>
<tr>
<td>Internal hybrid groups provide hydrophobicity</td>
<td>Increased high pH stability</td>
</tr>
</tbody>
</table>
Improved Retention and Sensitivity: High pH Mobile Phase

**HIGH pH**
0.1% NH₄OH

**Compounds**
1. 10-hydroxymorphine
2. Morphine-3β-D-glucuronide
3. Morphine-6β-D-glucuronide
4. Morphine
5. Morphine N-oxide
6. 6-acetylmorphine

**LOW pH**
0.1% HCOOH

10-hydroxymorphine
Morphine-3β-D-glucuronide
Morphine-6β-D-glucuronide
Morphine N-oxide
6-acetylmorphine
BEH Technology in Bioanalysis: High pH Case Study

- Mobile phase pH can impact retention, selectivity and sensitivity if the analytes are ionizable.
- Selectively elute analytes away from phospholipids.
- Basic analytes are unionized at high pH, and therefore, retain greater on the stationary phase, eluting in higher organic.

Phospholipid: 1-Stearoyl-2-Hydroxy-sn-Glycero-3-Phosphocholine

Molecular Weight: 523.34
Molecular Formula: C_{26}H_{54}NO_{7}P

BEH Technology in Bioanalysis: High pH Case Study

Column: ACQUITY UPLC® BEH C$_{18}$, 2.1 x 50 mm 1.7 µm

Fig. 4. MRM transitions for all phosphatidylcholine-containing phospholipids (top) from plasma samples prepared by ACN PPT analyzed by UPLC$^\text{®}$/MS/MS under (A) pH 2.7 and (B) pH 9 mobile phase conditions. MRM transitions for 1 ng/mL terfenadine (middle) and amitriptyline (bottom) under the same mobile phase conditions.
Benefits:

- Higher sensitivity
  - *Abundant protonated molecules can be produced in high pH conditions even though basic analytes are un-ionized*
  - *Greater retention for un-ionized molecules, resulting in elution in a higher organic mobile phase (easier desolvation)*
  - *Selectivity can be manipulated to selectively elute analytes away from residual phospholipids*
  - *Highly efficient, symmetrical peaks resulting in narrow chromatographic bands*
Typical UPLC® Method Scouting Conditions

- ACQUITY UPLC® BEH C\textsubscript{18}, 2.1 x 50 mm, 1.7 \mu m
  - Wide pH range (1-12)
  - Lower silanol activity than silica

- Low and high pH mobile phases
  - 0.1% Formic Acid (pH ~2.7)
  - 0.1% Ammonium Hydroxide in H\textsubscript{2}O (pH ~11.3)

- Two organic modifiers
  - Methanol and acetonitrile

- Generic gradient: 2% to 98% B in 2 min

- Best chromatography chosen as a starting point for optimization
  - Good peak shapes
  - Sensitivity
  - Resolution/selectivity
Alprazolam is a very weak base and exhibits very little change in retention at the different pHs.

MeOH is a weaker eluting solvent resulting in increased retention of the analyte.

Note the significant difference in MS sensitivity when the molecule is analyzed under high pH mobile phase conditions.
Polar Compounds and Metabolites:

- Often overlooked due to their elution in the void space of the column

- Co-elute with endogenous species present within biological matrices, adversely effecting assay precision and accuracy

- Require high aqueous mobile phases that may adversely effect MS response

- Ion-pairing reagents aid in retention, however, often suppress MS response
What is HILIC?

- **HILIC** - *Hydrophilic Interaction Chromatography*
  - Term coined in 1990 to distinguish from normal-phase*

- HILIC is a *variation* of normal-phase chromatography without the disadvantages of using solvents that are not miscible in water
  - “Reverse reversed-phase” or “aqueous normal-phase” chromatography

- Stationary phase is a POLAR material
  - Silica, hybrid, cyano, amino, diol, amide

- The mobile phase is highly organic (> 80% ACN) with a smaller amount of aqueous mobile phase
  - Water (or the polar solvent(s)) is the strong, eluting solvent

**Benefits of HILIC**

- **Retention** of highly polar analytes not retained by reversed-phase
  - Less interference from non-polar matrix components

- Complementary **selectivity** to reversed-phase
  - Polar metabolites/impurities/degradants retain more than parent compound

- Enhanced **sensitivity** in mass spectrometry
  - High organic mobile phases (> 80% ACN) promotes enhanced ESI-MS response
  - Direct injection of PPT supernatant without dilution
  - Facilitates use of lower volume samples

- Improved sample **throughput**
  - Direct injection of high organic extracts from PPT, LLE or SPE without the need for dilution or evaporation and reconstitution
**HILIC-MS/MS Sensitivity**

**0.178 nmol/L metanephrine**

LLOQ (S:N ≥ 10) calculated by extrapolation = **0.012 nmol/L**

**0.525 nmol/L normetanephrine**

LLOQ (S:N ≥ 10) calculated by extrapolation = **0.06 nmol/L**

Direct injection of SPE Eluate
When to Use HILIC:

- Need improved retention of hydrophilic or ionizable compounds
- Need improved MS response for polar or ionizable compounds
- Need improved sample throughput for assays using organic extraction
Outline

- Overview of HILIC
- Retention mechanisms and characteristics
  - Retention mechanisms
  - Retention and selectivity matrix
    - Organic modifier
    - Stationary phase
    - Mobile phase pH
- Practical considerations
- HILIC method development strategy
- Conclusions
Multi-modal Retention Mechanisms in HILIC

Combination of partitioning, ion-exchange and hydrogen bonding

- Polar analyte partitions between bulk mobile phase and partially immobilized polar layer on material surface
- Secondary interactions between surface silanols and/or functional groups with the charged analyte leading to ion-exchange
- Hydrogen bonding between positively charged analyte and negatively charged surface silanols

Influence of Organic Solvent Composition on Retention

- **Hydrophobic retention on siloxane bonds**
- **Liquid-liquid partitioning**

Retention Factor ($k$) vs. % ACN in mobile phase

*Nicotinic acid*  
$pK_a = 2.2, 4.8$

*Nortriptyline*  
$pK_a = 2.2, 4.8$

Retention and Selectivity Matrix

Stationary Phase

HILIC Retention and Selectivity

Mobile Phase pH

Organic Modifier
Influence of % Acetonitrile on Retention

Silica Particle

Exponential retention increase with mobile phases containing more than 90% acetonitrile

Nicotinic acid
Nortriptyline
Cytosine
Methacrylic acid

Nicotinic Acid
pKa = 2.2, 4.8

Nortriptyline
pKa = 10

Cytosine
pKa = 12.2

Methacrylic Acid
pKa = 4.58
Weakest

Solvent Selectivity and Elution Strength

Strongest

Primary [Weak] Solvents
Acetone
Acetonitrile
Isopropanol
Ethanol
Methanol
Water

Elution [Strong] Solvents

Use a less polar solvent to Increase retention of polar analytes
Influence of Polar Modifier on Retention and Selectivity

10 mM ammonium acetate with 0.02% acetic acid

Analytes:
1: methacrylic acid
2: cytosine
3: nortriptyline
4: nicotinic acid

Retention increases with decreasing solvent polarity

- 90:10 ACN:H₂O
- 90:5:5 ACN:H₂O:MeOH
- 90:5:5 ACN:H₂O:EtOH
- 90:5:5 ACN:H₂O:IPA
Retention and Selectivity Matrix

Stationary Phase

HILIC Retention and Selectivity

Mobile Phase pH

Organic Modifier
Stationary Phases for HILIC separations

Hybrid HILIC Columns (pH range 1 – 11)

Silica HILIC Columns (pH range 1 – 5)

ACQUITY UPLC® BEH Amide
XBridge™ Amide

ACQUITY UPLC® BEH HILIC
XBridge™ HILIC

Atlantis® HILIC Silica
Influence of Stationary Phase on Retention

ACQUITY UPLC BEH HILIC  
2.1 x 50 mm, 1.7 µm  
Unbonded hybrid with low silanol activity

ACQUITY UPLC BEH Amide  
2.1 x 50 mm, 1.7 µm  
Bonded hybrid

Atlantis HILIC Silica  
2.1 x 50 mm, 3 µm  
Unbonded silica with high silanol activity

(1) acenaphthene (2) thymine (3) 5-fluoroorotic acid (4) adenine (5) cytosine; UV 254 nm
Stationary phase functional groups can influence retention.

Nicotinic Acid
pKa = 2.2, 4.8

Methacrylic Acid
pKa 4.58

Nortriptyline
pKa = 10

Cytosine
pKa = 12.2

Stationary phase functional groups can influence retention.
Retention and Selectivity Matrix

HILIC Retention and Selectivity

Stationary Phase

Mobile Phase pH

Organic Modifier
Influence of Mobile Phase pH on Retention and Selectivity

ACQUITY UPLC BEH Amide, 2.1 x 50 mm, 1.7 µm

Compounds
1. Methacrylic acid
2. Nortriptyline
3. Nicotinic acid
4. Cytosine

Methacrylic Acid
pKa = 4.58

Nortriptyline
pKa = 10

Cytosine
pKa = 12.2

Nicotinic Acid
pKa = 2.2, 4.8

Methacrylic Acid
pKa 4.58
Influence of Mobile Phase pH on Retention and Selectivity

**ACQUITY UPLC BEH Amide, 2.1 x 50 mm, 1.7 µm**

**Compounds**

1. Nicotinamide
2. Pyridoxine
3. Riboflavin
4. **Nicotinic acid**
5. Thiamine
6. **Ascorbic Acid**
7. B12
8. **Folic Acid**

**Folic acid**

**Ascorbic acid**

**Nicotinic acid**
Influence of Mobile Phase pH on MS Signal Intensity

Ion counts

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 9</th>
<th>pH 3</th>
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<tbody>
<tr>
<td>Morphine</td>
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</tr>
<tr>
<td>Procainamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nortriptyline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acyclovir</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzylamine</td>
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<td></td>
</tr>
<tr>
<td>MMPA</td>
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<td></td>
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<tr>
<td>CMPA</td>
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<td>PMPA</td>
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<tr>
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<tr>
<td>EMPA</td>
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<td></td>
</tr>
<tr>
<td>2-naphthalenesulfonic acid</td>
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<td></td>
</tr>
<tr>
<td>4-aminosalicylic acid</td>
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<td></td>
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<tr>
<td>salicylic acid</td>
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<td></td>
</tr>
<tr>
<td>5-fluorouracil</td>
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<td></td>
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<tr>
<td>Thymine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
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</tbody>
</table>

ES+

ES−
Influence of Mobile Phase pH on MS Signal Intensity

**Low pH**

**High pH**

**ACQUITY UPLC BEH HILIC**
Injection solvent 95/5 ACN/H2O + 0.2% HCOOH

MRM of 3 Channels ES+
- gabapentin d10: 182.1 > 164, 1.20e5
- gabapentin: 172 > 153.9, 1.20e5
- pregabalin: 160 > 141.9, 1.20e5
Retention and Selectivity Summary

- Decreasing solvent polarity increases retention
- Screen multiple columns to maximize retention and selectivity differences
- Analytes have greater retention when they are ionized [acids at high pH, bases at low pH]

Stationary Phase

HILIC Retention and Selectivity

Mobile Phase pH

Organic Modifier
Outline

- Overview of HILIC
- Retention mechanisms and characteristics
- Practical considerations
  - Common buffers and additives
  - Mobile phase preparation
  - Column equilibration
  - Sample diluent
- HILIC method development strategy
- Conclusions
Before You Start:
Common HILIC mobile phases

- Common buffers/additives*
  - Ammonium formate, ammonium acetate
  - Formic acid, ammonium hydroxide, acetic acid
  - Phosphate salt buffers **ARE NOT** recommended due to precipitation in the highly organic mobile phase (phosphoric acid is OK)

- Recommended buffer concentration: 10 – 20 mM ON-COLUMN

- Recommended additive concentration: 0.2% ON-COLUMN

*The actual pH of the mobile phase may be 1 pH unit closer to neutral due to the highly organic mobile phase

Effect of Buffer Concentration on Retention: pH 3.0

All contain 90:10 MeCN:H₂O

1. Methacrylic acid
2. Nicotinic acid
3. Nortriptyline
4. Cytosine

Methacrylic Acid
pKa 4.58

Nicotinic Acid
pKa = 2.2, 4.8

Cytosine
pKa = 12.2

Nortriptyline
pKa = 10

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Effect of Buffer Concentration on Retention: pH 9.0

All contain 90:10 MeCN:H₂O

0 mM ammonium acetate

2.5 mM ammonium acetate

5.0 mM ammonium acetate

10 mM ammonium acetate

20 mM ammonium acetate

1. Methacrylic acid
2. Nicotinic acid
3. Nortriptyline
4. Cytosine

Methacrylic Acid
pKa 4.58

Nicotinic Acid
pKa = 2.2, 4.8

Nortriptyline
pKa = 10

Cytosine
pKa = 12.2
Additives vs. Buffered Mobile Phases: Impact on Retention and Peak Shape

**pH 9 Observations**

Acids are unretained without a buffered mobile phase
Selectivity shifts for basic compounds

**pH 3 Observations**

Poor peak shape and retention for bases without a buffered mobile phase
Selectivity shifts for acidic compounds

All contain 90:10 MeCN:H₂O
**Influence of Buffer Concentration on Peak Shape**

**ACQUITY UPLC BEH HILIC**

Injection solvent 75/25 ACN/MeOH + 0.2% HCOOH
Buffer: ammonium formate (pH 3)
Before You Start:
Mobile Phase Preparation

- **Additives**
  - Replace 0.2% of mobile phase volume with additive [2 mL out of 1 L]

- **Buffers**
  - Prepare a stock buffer [typically 200 mM] and then dilute 20-fold into the running mobile phase [10 mM concentration on column]

  - *Example:* Prepare stock of 200 mM ammonium formate, pH 3. For a mobile phase containing 95% ACN and 5% water with 10 mM ammonium formate, pH 3, add 50 mL of stock buffer to 950 mL of ACN.

- For the best gradient performance and reproducibility, it is recommended that the additive or buffer be added to both aqueous and organic mobile phase bottles
### Before You Start: Column Equilibration and Wash Solvents

- **Instrument Wash Solvents**
  - Strong needle wash: 9:1 acetonitrile: water
  - Weak needle wash/purge solvent: initial mobile phase conditions [excluding salt, additive or buffer]

- **Brand new column**
  - Run 50 empty column volumes of 50:50 acetonitrile:water with 10 mM buffer or 0.2% additive solution

- **Column equilibration**
  - Equilibrate with 20 empty column volumes of initial mobile phase conditions

- **Gradient separations**
  - Re-equilibrate with 5 to 8 empty column volumes

*As with any column, insufficient equilibration can cause drifting retention times*
Sample diluent strongly influences solubility and peak shape (just like reversed-phase)

Sample diluent should be at least 75% acetonitrile or as close to initial mobile phase conditions as possible

However, polar analytes often have low solubilities in organic solvents

General purpose HILIC diluent
  — 75:25 acetonitrile:methanol works for most polar analytes
  — Offers a compromise between solubility and peak shape
  — Adjust according to your analytes (add 0.2% formic acid to increase solubility)
  — In some cases, 25% methanol may be too polar to use as an injection solvent
Influence of Sample Diluent

Water as the Polar Solvent

ACQUITY UPLC® BEH HILIC
2.1 x 100 mm, 1.7 µm

Analytes
1. Methacrylic acid
2. Cytosine
3. Nortriptyline
4. Nicotinic acid

Peak shape improves as % ACN in the diluent increases.

What about alternative polar organic solvents?
Influence of Sample Diluent
Methanol as the Polar Solvent

ACQUITY UPLC® BEH HILIC
2.1 x 100 mm, 1.7 µm

Analytes
1. Methacrylic acid
2. Cytosine
3. Nortriptyline
4. Nicotinic acid

Peak shape and solubility improve by replacing water with methanol
Peak shape improves as % ACN in the diluent increases.
Influence of Sample Diluent on Sensitivity

Sample Diluent: 95/5 ACN/H2O + 0.2% HCOOH

**gabapentin d10**
- MRM of 3 Channels ES+ 182.1 > 164
- 1.26e5

**gabapentin**
- MRM of 3 Channels ES+ 172 > 153.9
- 1.26e5

**pregabalin**
- MRM of 3 Channels ES+ 160 > 141.9
- 1.26e5

Sample Diluent: 75/25 ACN/MeOH + 0.2% HCOOH

**gabapentin d10**
- 0.94

**gabapentin**
- 0.94

**pregabalin**
- 0.89

ACQUITY UPLC BEH HILIC
Buffer: ammonium formate (pH 3)
Outline

- Overview of HILIC
- Retention mechanisms and characteristics
- Practical considerations
- HILIC method development strategy
  - Method development screening approach
  - Implementing the approach: practical example
  - Optimization steps
- Conclusions
Waters HILIC screening strategy

Where do I start?
- Initial scouting gradient from 95 to 50% acetonitrile over 2 minutes
- At least 5% should be a polar solvent (i.e., water or methanol)
Automated Screening Conditions

Instrument: ACQUITY UPLC system with ACQUITY PDA and Xevo TQ MS

Columns: ACQUITY UPLC BEH Amide, 2.1 x 50 mm, 1.7 µm
ACQUITY UPLC BEH HILIC, 2.1 x 50 mm, 1.7 µm
Atlantis HILIC Silica, 2.1 x 50 mm, 3 µm

MP A1: 20 mM HCOONH₄ and 0.125% HCOOH, pH 3.0*
MP B1: 95/5 ACN/H₂O with 20 mM HCOONH₄ and 0.125% HCOOH, pH 3.0*
MP A2: 20 mM CH₃COONH₄ and 0.04% NH₄OH, pH 9.0*
MP B2: 95/5 ACN/H₂O with 20 mM CH₃COONH₄ and 0.04% NH₄OH, pH 9.0*
Gradient: 99.5% B to 50% B in 2 min, reset (total run time = 3 min)

Flow rate: 0.6 mL/min
Inj. vol.: 5 µL

Sample Diluent: 75/25 ACN/MeOH
(0.2% HCOOH needed in some cases for solubility)

Column Temp.: 30°C
Strong and weak needle washes: 95/5 ACN/H₂O

*pH of stock buffer at 200 mM in water
As we go through the data for each separation, we should have some questions in mind to help select the best pH, organic solvent and column

- Adequate retention/separation from matrix components?
- Is a gradient or isocratic method desired?
- Sensitivity requirements?

Step 1: Review data and select pH
Step 2: Review data and select column
Step 3: Optimize/fine-tune separation as necessary
Implementing the Approach: Example 1: Fentanyl

- Fentanyl is a narcotic pain medicine nearly 100x more potent than morphine.
- Fentanyl is used to treat cancer pain that is not controlled by other medicines.

Fentanyl
m.w. 336.47
Stationary Phase Selectivity at Low pH: Fentanyl

**BEH Amide**

- Poor retention and peak shape on BEH Amide and HILIC
- MRM of 2 Channels ES+
  - 337.15 > 188.05
  - 7.20e6

**BEH HILIC**

- Good retention and peak shape on Atlantis HILIC Silica
- MRM of 2 Channels ES+
  - 337.15 > 188.05
  - 7.20e6

**Atlantis HILIC Silica**

- MRM of 2 Channels ES+
  - 337.15 > 188.05
  - 7.20e6

**Fentanyl**
- m.w. 336.47

**pH 3 Observations**

- Poor retention and peak shape on BEH Amide and HILIC
- Good retention and peak shape on Atlantis HILIC Silica
Mobile Phase Selectivity and Sensitivity: Fentanyl

Low pH

BEH Amide

MRM of 2 Channels ES+
337.15 > 188.05
5.34e6

BEH HILIC

High pH

BEH Amide

MRM of 2 Channels ES+
337.15 > 188.05
2.30e6

BEH HILIC
Final Chromatographic Method: Fentanyl

Chromatographic Method

Atlantis HILIC Silica
Low pH
95 – 50% ACN in 2 min
75/25 ACN/MeOH with 0.2% HCOOH

Fentanyl

Fentanyl d5

MRM of 2 Channels ES+
342.05 > 187.95
1.42e7

MRM of 2 Channels ES+
337.15 > 188.05
1.42e7

Fentanyl

m.w. 336.47

O
C

N
N
O
CH3

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Implementing the Approach:
Example 2, Organophosphonic Acids

**Cyclohexyl methylphosphonic acid (CMPA)**

**Isopropyl hydrogen methylphosphonate (IBMPA)**

**Pinacolyl methylphosphonic acid (PMPA)**

**Isopropyl methylphosphonic acid (IMPA)**

**Ethyl methylphosphonic acid (EMPA)**
Stationary Phase Selectivity at Low pH: Organophosphonic Acids

- **Compounds**
  1. PMPA
  2. CMPA
  3. MMPA
  4. IMPA
  5. EMPA

500 ng/mL each

**BEH Amide**

**BEH HILIC**

**Atlantis HILIC Silica**

**pH 3**

Atlantis HILIC Silica yields greatest retention

BEH Amide and Atlantis HILIC Silica yield similar selectivity
Mobile Phase pH Selectivity: Organophosphonic Acids

**Compounds**
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA

500 ng/mL each

**BEH Amide**
Higher sensitivity at pH 9
Similar selectivity independent of pH

**Compounds**
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA

500 ng/mL each
Stationary Phase Selectivity at High pH: Organophosphonic Acids

Compounds
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA

500 ng/mL each

pH 9
Greater Resolution for BEH Amide
No resolution between peaks 2 and 3
Further optimization needed

BEH Amide
1: SIR of 5 Channels ES-TIC
3.32e6

BEH HILIC
1: SIR of 5 Channels ES-TIC
3.32e6
Method Optimization Steps

1. Adjust gradient slope

2. Adjust column temperature

3. Adjust column length and flow rate

4. Isocratic mode instead of gradient
   - 95:5 ACN:H2O with 10 mM buffer or 0.2% additive

5. Replace a portion of the water in the mobile phase with a less polar solvent [MeOH, EtOH or IPA]

Evaluate result after each step. Stop after criteria for success has been met.
Consider injection solvent (sample diluent) if poor peak shape/resolution
Optimization Step 1: Adjust Gradient Slope

99.9% to 0.1% B in 5 min
SIR of 5 Channels ES-TIC
3.32e6

99.9% to 50% B in 5 min
SIR of 5 Channels ES-TIC
4.18e6

99.9% to 90% B in 5 min
SIR of 5 Channels ES-TIC
5.12e6

BEH Amide, pH 9
Shallower gradient slope results in improved resolution

Compounds
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA

500 ng/mL each
Optimization Step 2: Column Temperature

BEH Amide, pH 9
Shallow gradient
Increased temperature results in improved resolution

Compounds
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA
500 ng/mL each
Optimization Step 3: Column Length

2.1 x 50 mm

2.1 x 100 mm

BEH Amide, pH 9
Shallow gradient, 65 °C

100 mm column results in improved resolution
50 mm column results in shorter run time
Select result that meets method criteria

Compounds
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA
500 ng/mL each
Final Method: Organophosphonic Acids

Mass Spectrometer Conditions

Ionization Mode: ES-
Capillary: 2.5 KV
Cone: 30 V (EMPA, IMPA, PMPA); 40 V (CMPA); 35 V (MMPA)
Source Temperature: 120 °C
Desolvation Temperature: 400 °C
Desolvation Gas Flow: 800 L/HR
Cone: 5 L/HR
SIR m/z: 122.9 (EMPA); 136.95 (IMPA); 179.0 (PMPA); 177.0 (CMPA); 150.95 (MMPA)
Dwell Time: 0.1 s

Compounds
1. PMPA
2. CMPA
3. MMPA
4. IMPA
5. EMPA
500 ng/mL each

Chromatographic Conditions

Columns: ACQUITY UPLC® BEH Amide, 2.1 x 100 mm, 1.7 μm
Part Number: 186004801
Mobile Phase A: 50/50 MeCN/H₂O with 10 mM CH₃COONH₄ and 0.04% NH₄OH, pH 9.0
Mobile Phase B: 95/5 MeCN/H₂O with 10 mM CH₃COONH₄ and 0.04% NH₄OH, pH 9.0
Flow Rate: 0.5 mL/min
Gradient: Time Profile (min) %A %B
Initial 0.1 99.9
10.00 99.9 90.0
10.01 0.1 99.9
15.00 0.1 99.9
Injection Volume: 5.0 μL (PLNO)
Sample Concentration: 2 μg/mL each
Sample Diluent: 75/25 MeCN/MeOH
Column Temperature: 65 °C
Weak Needle Wash: 95/5 MeCN/H₂O
Instrument: Waters ACQUITY UPLC with ACQUITY SQD
## Screening approach

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Column conditioning*</td>
<td>30 minutes</td>
</tr>
<tr>
<td>2</td>
<td>3 Columns, 2 pH’s screening</td>
<td>30 minutes</td>
</tr>
</tbody>
</table>

## Optimization

<table>
<thead>
<tr>
<th>Step</th>
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<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Column conditioning [temp. equilibration]</td>
<td>30 minutes</td>
</tr>
<tr>
<td>2</td>
<td>Gradient slope and temperature</td>
<td>30 minutes</td>
</tr>
</tbody>
</table>

## Total method development time

2 Hours

*equilibration and 2 blank injections at each pH
Conclusions

- **For HILIC retention and selectivity:**
  - ACN is used the primary [weak] solvent in HILIC
  - Water, methanol, ethanol or isopropanol are strong [elution] solvents
  - Stationary phase charge and bonded phase can impact retention and selectivity
  - Analytes in their charged form exhibit greater retention [acids at high pH, bases at low pH]

- **Practical considerations:**
  - At least 10 mM buffer or 0.2% additive is recommended in mobile phase A and B
  - Sample diluent should contain at least 75% acetonitrile for solubility and peak shape
  - Weak needle wash must be in a high organic solution [90 – 95% ACN]

- **A systematic screening protocol was described to effectively and efficiently develop HILIC methods**
  - 3 column selectivities, 2 mobile phase pH’s
  - ACQUITY UPLC system used for rapid, automated method development
  - Total method development time of 2 hours