A New Approach to Retaining Hydrophilic Compounds in HPLC

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The presence of the hydride surface plus the bonded organic moiety make three modes of separation possible

1. Aqueous Normal Phase
2. Reversed-Phase
3. Organic Normal Phase
WHAT IS AQUEOUS NORMAL PHASE RETENTION?

A continuum of retention that provides a transition from the reversed-phase to the normal phase modes with water as a constituent in the mobile phase

Three distinct retention patterns are possible:

1. No overlap of reversed phase and normal phase retention for two or more compounds.
2. Overlap of reversed phase and normal phase retention for two or more compounds.
3. Individual compounds that can be retained by both reversed phase and normal phase modes.
WHAT IS HILIC RETENTION?

HILIC Retention

% Organic in Mobile Phase

Retention Time (min)

- Hydrophilic Compound
- Hydrophobic Compound
DIFFERENCES BETWEEN AQUEOUS NORMAL PHASE AND HILIC

Aqueous Normal Phase Silica Hydride-Based Column

- Retains nonpolar compounds by reversed phase mechanism
- Retains polar compounds by normal phase mechanism
- Both reversed phase and normal phase mechanisms can operate simultaneously
- Can separate samples with both polar and nonpolar compounds

Hydrophilic Interaction Chromatography (HILIC) uses ordinary Silica-Based Column

- Retains polar compounds by a normal phase mechanism
- Does not retain nonpolar compounds
- Cannot usually separate samples having both polar and nonpolar compounds
No overlap of reversed phase and normal phase retention for two or more compounds.

**ELUTION ORDER IN RP:** most polar, intermediate polarity, nonpolar

**ELUTION ORDER IN ANP:** nonpolar, intermediate polarity, most polar
Overlap of reversed phase and normal phase retention for two or more compounds.

Aqueous normal phase (1) and reversed-phase (2) compounds at three mobile phase compositions: A, 50:50 acetonitrile, DI water; B, 80:20 acetonitrile, DI water; and C, 85:15 acetonitrile, DI water.
Individual compounds that can be retained by both reversed phase and normal phase modes

ANP 3
Example of Separation Capabilities of Hydride Based Stationary Phase

METFORMIN
Aqueous Normal Phase

GLYBURIDE
Reversed Phase

Glyburide logP = 4.79

Metformin logP = -2.64
Comparison of Hydride-Based C18 and Ordinary Silica-Based C18 Columns in the Aqueous Normal Phase Mode
METFORMIN/GLYBURIDE ON BIDENTATE C18
FAST SEPARATION WITH UV DETECTION

Column dimensions: 2.1 x 20 mm

**Mobile Phase:**
A: 50:50 acetonitrile, DI water + 0.5% formic acid
B: 80:20 acetonitrile, DI water + 0.5% formic acid
C: 85:15 acetonitrile, DI water + 0.5% formic acid

**Flow rate:** 0.3 mL/minute

**Injection Volume:** 1 µL

**Samples:**
1. Metformin
2. Glyburide

100 µg/mL of each in the mobile phase

**Detection:** UV 254nm
Comparison of Choline and Acetylcholine Elution on Hydride-Based Cholesterol and BD C18 Columns under Aqueous Normal Phase Conditions
**SEPARATION OF ACETYLCHOLINE/CHOLINE ON STANDARD AND SHORT CHOLESTEROL COLUMNS**

On 2.1 x 20 mm Column in 92% ACN + 8% DI water with 0.5% formic acid

Challenging quaternary amine compounds

On standard 4.6x75 mm column using 90% ACN + 10% DI water with 0.5% formic acid

![Graph showing separation of Acetylcholine (m/z 146) and Choline (m/z 104) with time in minutes.]

5 min.
Aqueous Normal Phase Retention of the Basic Drug Tobramycin

Hydride Based Cholesterol, 4.6x75 mm Sample: TOBRAMYCIN

Retention time (min.)

% organic in DI water + 0.5% FA

methanol
acetonitrile
AQUEOUS NORMAL PHASE RETENTION OF CARBOHYDRATES ON A HYDRIDE-BASED COLUMN

Separation based on size

Detection by MS in the APCI+ mode
Method Conditions
Column: Cogent Bidentate C18, 4µm, 100A.
Catalog No.: 40018-75P
Dimensions: 4.6 x 75 mm
Mobile phase: A. 90:10 Acetonitrile/DI Water +0.5% formic acid
             B. 85:15 Acetonitrile/DI Water + 0.5 % formic acid
Flow rate: 0.5 mL/min.
Injection Volume: 10 µL
Samples:
1. Triacetylnormetanephrine (m/z 166.2)
2. Triacetylmetanephrine (m/z 180.2)
200 ng of each sample was dissolved in 1 mL of reverse osmosis water
Detection: Atmospheric Pressure Chemical Ionization in positive mode: APCI+
           Single Ion Monitoring
In detection single ion monitoring (SIM) was used. Mass transition of m/z 310.2 to m/z 166.2 (triacetylnormetanephrine) and m/z 324.2 to m/z 180.2 (triacetylmetanephrine) that correspond to the fragmentation of the (M+H⁺) ions were monitored.
The powerful anticancer drug, methotrexate (4-amino-N10-methylpteroyl glutamic acid) acts as an antimetabolite and is used for the treatment of many neoplastic diseases including acute leukemia, osteosarcoma, non-Hodgkins lymphoma, and breast cancer. There is a great interest in pharmacological studies and clinical monitoring of methotrexate.

A quadrupole mass spectrometer operating in the positive – ion mode and an atmospheric pressure ionization (API) source was used for selective detection and assured that no interfering peaks affect the quantitative results. A bidentate C18 column was the column of choice for the ANP gradient analysis of the drug. The retention of the methotrexate is more than sufficient. The LC-MS method developed assures both high specificity and sensitivity.

### Inverse Gradient Program

**A:** DI Water + 0.5% Formic Acid  
**B:** Acetonitrile

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>%A</th>
<th>%B</th>
</tr>
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<tbody>
<tr>
<td>0.00</td>
<td>10.0</td>
<td>90.0</td>
</tr>
<tr>
<td>1.00</td>
<td>10.0</td>
<td>90.0</td>
</tr>
<tr>
<td>5.00</td>
<td>80.0</td>
<td>20.0</td>
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<tr>
<td>10.00</td>
<td>80.0</td>
<td>20.0</td>
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<tr>
<td>10.01</td>
<td>10.0</td>
<td>90.0</td>
</tr>
<tr>
<td>12.00</td>
<td>10.0</td>
<td>90.0</td>
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</table>
ANP RETENTION CAN BE OBSERVED FOR HYDROPHILIC PEPTIDES

GENERAL PEPTIDE STRUCTURE: Ac-AXEXAHKAY-NH$_2$
SOME COMPOUNDS DISPLAY BOTH REVERSED PHASE AND ANP BEHAVIOR ON THE SAME COLUMN

Retention Map with Acetonitrile/Water (Formic Acid)

Retention Factor (k) vs. % Acetonitrile

- Cytidine-R1
- Cytidine-R2
- Cytidine-R3
USE OF TOF MS IN AQUEOUS NORMAL PHASE WITH A SILICA HYDRIDE COLUMN FOR DETECTION OF METABOLITES
ANP RETENTION ON DIAMOND HYDRIDE COLUMN
AMINO ACIDS - PHENYLALANINE

70:30 ACN/water + 0.1% FA
\( t_R = 2.96 \text{ min} \quad t_0 = 0.95 \text{ min} \)

80:20 ACN/water + 0.1% FA
\( t_R = 5.22 \text{ min} \)

90:10 ACN/water + 0.1% FA
\( t_R = 18.27 \text{ min} \)

Diamond Hydride: essentially an unmodified hydride surface

Detection: Agilent TOF-MS m/z = 166
Retention Time With Acetonitrile

Retention Time With Acetone
Extracted Ion Chromatogram Of Nineteen Amino Acid Separation

All of the critical amino acid pairs (those that are isobaric or have masses within one mass unit) are separated under these conditions except for the Leucine / Isoleucine pair. At present, the maximum separation is approximately 0.15 min with 0.30 minutes needed for resolution with the peak widths obtained for these two amino acids.
### GRADIENT REPRODUCIBILITY

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>G 1B 15 °C</th>
<th>G 1B inj 2 15 °C</th>
<th>Gr 1B inj 3 15 °C</th>
<th>Gr 1B inj 4 15 °C</th>
<th>%RSD</th>
<th>Gr 1B 30 °C</th>
<th>Gr 1B inj 2 30 °C</th>
<th>Gr 1B inj 3 30 °C</th>
<th>Gr 1B inj 4 30 °C</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamine</td>
<td>10.961</td>
<td>10.929</td>
<td>10.955</td>
<td>10.940</td>
<td>0.13</td>
<td>10.911</td>
<td>10.933</td>
<td>10.917</td>
<td>10.938</td>
<td>0.12</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>8.771</td>
<td>8.751</td>
<td>8.754</td>
<td>8.762</td>
<td>0.10</td>
<td>8.856</td>
<td>8.900</td>
<td>8.873</td>
<td>8.905</td>
<td>0.26</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>8.369</td>
<td>8.360</td>
<td>8.363</td>
<td>8.360</td>
<td>0.05</td>
<td>8.532</td>
<td>8.576</td>
<td>8.527</td>
<td>8.559</td>
<td>0.27</td>
</tr>
<tr>
<td>L-Proline</td>
<td>10.647</td>
<td>10.628</td>
<td>10.650</td>
<td>10.610</td>
<td>0.17</td>
<td>10.495</td>
<td>10.507</td>
<td>10.496</td>
<td>10.511</td>
<td>0.08</td>
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<tr>
<td>L-Tyrosine</td>
<td>8.491</td>
<td>8.483</td>
<td>8.495</td>
<td>8.498</td>
<td>0.08</td>
<td>8.641</td>
<td>8.686</td>
<td>8.641</td>
<td>8.679</td>
<td>0.28</td>
</tr>
</tbody>
</table>

The table shows retention time reproducibility for nine amino acids at two temperatures. Four replicates were performed at each temperature. The reproducibility was 0.28% or better for the amino acids. This is a significant improvement over what is usually observed for most HILIC analyses, especially considering this is gradient data with only a 5 minute re-equilibration time between runs.
ANP RETENTION OF SUCCINIC ACID ON DIAMOND HYDRIDE COLUMN

Mobile Phase: 95:5 ACN/water + 0.1% FA
Detection: TOF-MS @ m/z = 117
Retention increases to 5 min in 0.1% Ammonium Acetate
ANP RETENTION OF CARBOHYDRATES ON DIAMOND HYDRIDE COLUMNS

All have the same MW

Diamond Hydride
Dimensions: 4.6 x 75 mm
Flow rate: 0.5 mL/min
Mobile phase:
90% acetonitrile 0.1 formic acid /
10% DI water 0.1 % formic acid

Mannose 4.39 min
Glucose 4.68 min
impurity 4.99 min
(galactose)
FEATURES OF DIAMOND HYDRIDE FOR METABOLITE ANALYSIS

1. Retains amino acids, small organic acids and carbohydrates in the Aqueous Normal Phase Mode in acidic aqueous/organic mobile phases containing greater than 60% acetonitrile or acetone.

2. Higher temperature results in increasing retention time – opposite to what is typically observed in reversed phase retention.

3. Excellent reproducibility is obtained in either isocratic or gradient elution with retention time RSD better than 0.3%

4. Re-equilibration time is rapid (< 5 min) which is similar to or better than observed in most reversed phase applications and superior to re-equilibration times obtained in the HILIC mode.
USE OF HYDRIDE BASED STATIONARY PHASES FOR REVERSED PHASE SEPARATIONS
CAPILLARY LC SEPARATION OF STEROIDS IN REVERSED PHASE MODE

Column: Cogent Bidentate C18 capillary HPLC Column, 4 µm, 100 Å
Dimensions: 50 µm i.d. x 40 cm packed (50 cm total length),
Mobile phase: 70:30 acetonitrile/DI water + 0.1% formic acid
Flow rate: 0.010 mL/min.

SEPARATION OF CARBOHYDRATE STRUCTURAL ISOMERS

Mobile Phase: 100% water
All compounds have MW = 504
Each compound detected by MS in APCI+ mode with single ion monitoring (SIM) using a specific fragment ion.

- maltotriose α 1,4
- panose α 1,4 + α 1,6
- isomaltotriose α 1,6
- cellotriose β 1,4
Peptides: simple isocratic RP – HPLC analysis

An HPLC peptide standard mixture was resolved in under 5 minutes using a short (4.6 x 75 mm) Cogent BD C18 column and a simple isocratic RP-HPLC method. The separation was very reproducible. To achieve the separation presented on a conventional HPLC column, 4.6 x 250 mm, a gradient method is required.

If higher resolution is desired a longer column should be used. Columns from leading brands of manufacturers were evaluated using the same conditions and compounds 3 and 4 were never separated under RP-HPLC isocratic conditions.

**Mobile Phase:** 25% acetonitrile/75% DI water + 0.1% formic acid

**Flow rate:** 1 mL/minute

**Detection** UV 214 nm

4. Leu-enkephalin; 5. Angiotensin II

Chromatogram is a composite of 8 consecutive injections
# SEPARATION OF A MIXTURE OF POLAR AND NONPOLAR COMPUNDS

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<tbody>
<tr>
<td>1</td>
<td>Cytidine-R1</td>
<td>397</td>
<td>12.12</td>
<td>3.73</td>
<td>1.54</td>
</tr>
<tr>
<td>2</td>
<td>Cytidine-R2</td>
<td>454</td>
<td>12.07</td>
<td>3.73</td>
<td>3.66</td>
</tr>
<tr>
<td>3</td>
<td>Cytidine-R3</td>
<td>425</td>
<td>12.09</td>
<td>3.73</td>
<td>2.66</td>
</tr>
<tr>
<td>4</td>
<td>Quinolinedione-R1</td>
<td>536</td>
<td>8.65, 8.68</td>
<td>-</td>
<td>3.46</td>
</tr>
<tr>
<td>5</td>
<td>Tetramic acid</td>
<td>536</td>
<td>7.91, 10.98</td>
<td>5.36</td>
<td>2.96</td>
</tr>
<tr>
<td>6</td>
<td>Quinolinedione-R2</td>
<td>520</td>
<td>8.64</td>
<td>-</td>
<td>2.74</td>
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<tr>
<td>7</td>
<td>Benzopyran</td>
<td>396</td>
<td>-</td>
<td>-</td>
<td>2.80</td>
</tr>
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</table>

Mobile Phase: 60:40 acetonitrile/water
ANOTHER UNIQUE AND INTERESTING FEATURE OF HYDRIDE BASED STATIONARY PHASES

ORGANIC NORMAL PHASE RETENTION
NORMAL PHASE SEPARATION OF SUBSTITUTED PHENOLS

Atmospheric Pressure Chemical Ionization in positive mode - APCI+
Column: Bidentate C18, mobile phase: 95:5 Hexane/Ethyl Acetate
Flow rate: 1.0 mL/min.
Samples: 1 – phenol with aldehyde, 2 – parent phenol, 3 – phenol with ketone, 4 – phenol with acid
USE OF SILICA HYDRIDE COLUMN IN NORMAL PHASE MODE

Mobile phase: 10% Diethyl Ether in Hexane

A: HYDRIDE COLUMN

B: COMMERCIAL SILICA COLUMN

Stationary phase is unmodified silica hydride
NORMAL PHASE GRADIENT SEPARATIONS

A: 0-0.5 min 100% hexane; 0.5-7 min to 50:50 hexane/dichloromethane; 50:50 hexane/dichloromethane to 10 min.

B: 0.0 to 1.0 min 100% dichloromethane; 1.0 to 3.0 min to 100% ethyl acetate

A: Bidentate C18  B: Hydride Silica
IMPORTANT FEATURES OF HYDRIDE BASED STATIONARY PHASES

1. Hydride phases operate in the normal phase, aqueous normal phase and reversed phased modes

2. Hydride phases can be used in 100% aqueous mobile phases with no stationary phase collapse

3. Bases are retained at low pH so high pH mobile phases may not be necessary for many applications

4. Surface absorbs very little water so there is reproducible retention in the normal phase and rapid equilibration for gradient separations

5. Bidentate phases can be used over a broad range of pH and can be used at high temperatures
Funding Provided by:

National Science Foundation
National Institutes of Health
Camille and Henry Dreyfus Foundation

Special Thanks to
MicroSolv Technology
Supplier of Silica Hydride and Diamond Hydride Columns