Correlation of Retention and Selectivity in RP-HPLC with Molecular Structure of Analyte and Stationary Phase Chemistry

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Introduction

Chromatography has evolved as a powerful and rapid technique for the separation of compounds with close structural characteristics even from complex matrices. However, it is becoming more obvious that the selection of optimal separation conditions requires a profound knowledge of the effects of molecular parameters of solutes, stationary phases and mobile phase constituents and their mutual interactions on retention. The rational design of an optimal separation process requires the expert application of such knowledge.

Selectivity is the key parameter governing retention as well as resolution between two adjacent bands. In RP-HPLC, hydrophobic selectivity is a measure of the hydrophobic interactions between analytes and stationary phases. For solutes differing in functional groups, polar selectivity is an index of the polar interactions between that functionality with a stationary phase. Thus, selectivity in RP-HPLC is dependent upon the structure of the analyte, the nature and chemistry of the stationary phase, the composition of the mobile phase and temperature. Although a number of studies are documented in literature which report on the interaction of C\textsubscript{18} phase with a wide range of analytes, relative little information is available on stationary phases carrying polar functional groups. The current presentation compares Pursuit C\textsubscript{18} with polar-modified phases recently introduced by Varian. The effect of stationary phase structure on the retention of analytes is demonstrated. The results shed light on how to select a stationary phase that would furnish optimal separation for particular types of analytes.
Pursuit and Polaris Bonded Phases

Pursuit Phases

\[
\text{O-Si-R} \quad \text{O-Si-Me}_3
\]

Polaris Phases

\[
\text{O-Si-} \quad \text{O-Si-Me}_3
\]
Resolution Equation

\[ R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'}{1 + k'} \]

Resolution = Efficiency \cdot Selectivity \cdot Retention
Factors Influencing Selectivity in RP-HPLC

**Mobile Phase**
- Organic type
- Organic %
- pH
- Temperature
- Buffer
- Other additives (e.g., ion pair)

**Selectivity**

**Sample**
- Molecular size
- Shape/structure
- Functionality

**Stationary Phase**
- Bonded phase chemistry
- Particle chemistry
- Particle physical properties
**Hydrophobic or Methylene Selectivity** is generally taken as the relative retention of the adjacent members of a homologous series differing only in one methylene group. Its logarithm is proportional to the Gibbs free energy of transfer per methylene group from mobile phase to the stationary phase. It is calculated by dividing retention factors of neighboring members in a homologous series or from the slope of the regression line in the log $k$ vs $n_{CH_2}$ (number of methylene units) plot.

**Chemical or Polar Selectivity** comes about from strong interactions such as H-bonding, dipole and ionic interactions, complexation between the solute molecules and specific active sites (such as silanols or trace metal contaminants on the silica surface). In addition to the stationary phase polarity, polar selectivity also depends on the type of organic modifier and the mobile phase composition.
Columns for reversed-phase HPLC can be characterized by five retention-related parameters: \( H \) (hydrophobicity), \( S \) (steric selectivity), \( A \) (hydrogen-bond acidity), \( B \) (hydrogen-bond basicity) and \( C \) (cation-exchange behavior).

Linear free energy relationship for a solvent study under fixed solute and stationary phase conditions or for a stationary phase study under defined mobile phase conditions can be described as

\[
\log \left( \frac{k}{k_{\text{ref}}} \right) = \log \alpha = \eta' H + \sigma' S + \beta' A + \alpha' B + \kappa' C
\]

Here, \( k \) is the retention factor of any solute, \( k_{\text{ref}} \) is the value of \( k \) for a reference solute (ethylbenzene), and the remaining symbols denote selectivity-related properties of the solute (\( \eta' \), \( \sigma' \), \( \beta' \), \( \alpha' \), \( \kappa' \)).
κ’) or the column (H, S, A, B, C). Terms η’H, σ’S, β’A, α’B and κ’C describe various solute-column interactions which affect retention. Thus, the various column parameters (H, S, A, B, C) measure the following column properties: H, hydrophobicity; S, steric resistance to insertion of bulky solute molecules into the stationary phase (similar to, but not the same as, “shape selectivity”); A, column hydrogen-bond acidity, mainly attributable to non-ionized silanols; B, column hydrogen-bond basicity; C, column cation-exchange activity due to ionized silanols. The parameters η’, σ’, β’, α’ and κ’ are complementary properties of the solute. They can be obtained by multivariate regression analysis and are characteristic of the phase investigated.

Hydrophobic Selectivity

150 x 4.6 mm columns, 60:40 MeCN:water, 1 mL/min, ambient, UV 254 nm.
Polar Selectivity

150 x 4.6 mm columns, 60:40 MeCN:water, 1 mL/min, ambient, UV 254 nm.

Columns with polar activities
Selectivity Differences Between Pursuit and Polaris Columns

The Pursuit C\textsubscript{18} and Polaris Amide-C\textsubscript{18} columns yield differences in selectivity under neutral unbuffered mobile phase conditions. Figure A illustrates an analysis of parabens. With a Pursuit C\textsubscript{18} column, the relative retention ratios of ethyl, propyl and butyl parabens, as computed with methyl paraben as an internal marker, were 1.94, 4.41 and 10.73, respectively. With a Polaris Amide-C\textsubscript{18} column, the respective values were 1.80, 3.91 and 9.02. The methylene selectivity, calculated from the relative retention ratio between the butyl and propyl parabens, was 2.43 for Pursuit C\textsubscript{18} column and 2.31 for Polaris Amide-C\textsubscript{18} column.

We conducted a similar exercise with anticonvulsant drugs (Figure B), using clonazepam as an internal standard. Relative retention ratios for clorazepate and diazepam, respectively, were 2.83 and 3.34 using Pursuit C\textsubscript{18} column and 2.04 and 2.11 using Polaris Amide-C\textsubscript{18} column.

The parabens in Figure A are retained longer on the amide-functionalized bonded phase due to polar interactions; however, the relative retention ratios are higher for Pursuit C\textsubscript{18} columns because the homologous parabens differ from methyl paraben only with respect to the number of methylene groups, which can interact with the bonded phases by hydrophobic mechanism exclusively. The anticonvulsant drugs exhibit more striking differences in their retention times, as well as relative retention ratios. This trend is reversed as compared to the parabens. These drugs interact with the bonded phases predominantly through hydrophobic mechanisms, and since the Pursuit C\textsubscript{18} phase is more hydrophobic than the Polaris Amide-C\textsubscript{18}, these anticonvulsants are retained much longer on the former. Thus, selectivity differences between the two columns are attributable to the manner in which analytes interact with them, viz. hydrophobic or polar mechanisms.
Figure A. Parabens

Column: 150 x 4.6 mm columns, 5 micron particles
Mobile Phase: MeOH:H₂O, 40:60
Flow Rate: 1 mL/min
Detection: UV 254 nm

<table>
<thead>
<tr>
<th>Column</th>
<th>tₚMethylparaben(1)</th>
<th>tₚEthylparaben(2)</th>
<th>tₚPropylparaben(3)</th>
<th>tₚButylparaben(4)</th>
<th>tₚ(2)/tₚ(1)</th>
<th>tₚ(3)/tₚ(1)</th>
<th>tₚ(4)/tₚ(1)</th>
<th>tₚ(4)/tₚ(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18</td>
<td>6.797</td>
<td>13.184</td>
<td>29.984</td>
<td>72.904</td>
<td>1.94</td>
<td>4.41</td>
<td>10.73</td>
<td>2.43</td>
</tr>
<tr>
<td>Amide-C18</td>
<td>9.103</td>
<td>16.393</td>
<td>35.604</td>
<td>82.147</td>
<td>1.80</td>
<td>3.91</td>
<td>9.02</td>
<td>2.31</td>
</tr>
</tbody>
</table>
Figure B. Anticonvulsant Drugs

Column: 150 x 4.6 mm columns, 5 micron particles
Mobile Phase: MeOH:H₂O, 40:60
Flow Rate: 1 mL/min
Detection: UV 254 nm

<table>
<thead>
<tr>
<th>Column</th>
<th>tₐ Clonazepam (1)</th>
<th>tₐ Clorazepate (2)</th>
<th>tₐ Diazepam (3)</th>
<th>tₐ(2)/tₐ(1)</th>
<th>tₐ(3)/tₐ(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18</td>
<td>22.023</td>
<td>62.310</td>
<td>73.562</td>
<td>2.83</td>
<td>3.34</td>
</tr>
<tr>
<td>Amide-C18</td>
<td>17.817</td>
<td>36.416</td>
<td>37.540</td>
<td>2.04</td>
<td>2.11</td>
</tr>
</tbody>
</table>

Effect of Stationary Phase Structure on Retention/Selectivity of Anticonvulsant Drugs
Cephalosporins on Pursuit and Polaris Columns

Column: 150 x 4.6 mm ID columns, 5 micron particles
Mobile Phase: MeOH:25 mM KH2PO4, pH 3, 20:80
Flow Rate: 1.5 mL/min
Detection: UV 230 nm

Effect of Stationary Phase Structure on Retention/Selectivity of Cephalosporins

<table>
<thead>
<tr>
<th>Column</th>
<th>t_R Cefadroxil (1)</th>
<th>t_R Cefaclor (2)</th>
<th>t_R Cephalexin (3)</th>
<th>t_R(2)/t_R(1)</th>
<th>t_R(3)/t_R(1)</th>
<th>t_R(3)/t_R(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18</td>
<td>1.818</td>
<td>4.522</td>
<td>5.437</td>
<td>2.487</td>
<td>2.991</td>
<td>1.202</td>
</tr>
<tr>
<td>Amide-C18</td>
<td>1.519</td>
<td>2.794</td>
<td>2.573</td>
<td>1.839</td>
<td>1.694</td>
<td>0.921</td>
</tr>
</tbody>
</table>
Alkaloids on Pursuit and Polaris Columns

Column: 150 x 4.6 mm ID columns, 5 micron particles
Mobile Phase: MeOH:25 mM KH$_2$PO$_4$, pH 6.0, 48:52
Flow Rate: 1.5 mL/min
Detection: UV 254 nm
Temperature: Ambient
## Selectivity Comparison Between Pursuit and Polaris Columns: Alkaloids

<table>
<thead>
<tr>
<th></th>
<th>Codeine (1)</th>
<th>Strychnine (2)</th>
<th>Quinidine (3)</th>
<th>Quinine (4)</th>
<th>Papaverine (5)</th>
<th>Noscapine (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_R$</td>
<td>$k'$</td>
<td>$t_R$</td>
<td>$k'$</td>
<td>$t_R$</td>
<td>$k'$</td>
</tr>
<tr>
<td>C18</td>
<td>1.37</td>
<td>0.11</td>
<td>1.578</td>
<td>0.29</td>
<td>2.57</td>
<td>1.09</td>
</tr>
<tr>
<td>C18-B</td>
<td>1.30</td>
<td>0.06</td>
<td>1.52</td>
<td>0.24</td>
<td>2.44</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>$k'(2)/k'(1)$</td>
<td>$k'(3)/k'(1)$</td>
<td>$k'(4)/k'(1)$</td>
<td>$k'(5)/k'(1)$</td>
<td>$k'(6)/k'(1)$</td>
<td>$k'(4)/k'(3)$</td>
</tr>
<tr>
<td>C18</td>
<td>2.64</td>
<td>9.91</td>
<td>12.09</td>
<td>42.09</td>
<td>106.09</td>
<td>1.22</td>
</tr>
<tr>
<td>C18-B</td>
<td>4.00</td>
<td>16.33</td>
<td>19.50</td>
<td>51.67</td>
<td>109.83</td>
<td>1.19</td>
</tr>
</tbody>
</table>
Cold Remedy Ingredients on Pursuit and Polaris Columns

The separation of a mixture of acetaminophen and pseudoephedrine was studied on both Pursuit C_{18} and Polaris amide-C_{18} columns. The order of elution of the two drugs is reversed on the two columns, with acetaminophen retained longer on the Polaris Amide-C_{18} column and pseudoephedrine retained longer on the Pursuit C_{18} column. This difference can be attributed to the difference in the nature of interaction of each of these drug molecules with the stationary phases. Acetaminophen has a phenolic hydroxyl and an amide moiety in its structure and can exhibit strong polar interaction with the amide functionality on the Polaris Amide-C_{18} phase. On the other hand, pseudoephedrine carries a hydroxylated methylaminopropyl chain on a phenyl ring, which predominantly interacts through hydrophobic mechanisms. When the organic content in mobile phase is increased, the elution order of two drugs on the Pursuit C_{18} column is switched. However, the elution order of two drugs on the Polaris Amide-C_{18} column remains unchanged. This demonstrates clearly the difference in the polar nature of the stationary phases.

![Pseudoephedrine](image1)

![Acetaminophen](image2)
**Cold Remedy Ingredients on Pursuit and Polaris Columns**

Column: 150 x 4.6 mm columns, 5 micron particles  
Mobile Phase: MeCN:25 mM K₂HPO₄, pH 7.0  
Flow Rate: 1 mL/min  
Temperature: Ambient  
Detection: UV 214 nm

**15:85**

- 1. Pseudoephedrine  
- 2. Acetaminophen

**35:65**

- 1. Pseudoephedrine  
- 2. Acetaminophen
Antifungal Agents on Pursuit and Polaris Columns

Column: 150 x 4.6 mm ID columns, 5 micron particles
Mobile Phase: A: 0.1% HCOOH in water
            B: 0.1% HCOOH in MeCN
            A:B 80:20
Flow Rate: 0.7 mL/min
Detection: UV 254 nm
Sample: 1. 4-Aminobenzoic acid    2. Sorbic acid    3. Benzoic acid

![Graphs of Pursuit C₁₈, Polaris Amide-C₁₈, and Polaris C₁₈-B columns showing the separation of sample compounds over time.](image)
Antiulcers on Pursuit and Polaris Columns

Column: 150 x 4.6 mm ID columns, 5 micron particles
Mobile Phase: MeOH:10 mM K$_2$HPO$_4$ (pH 7), 20:80
Flow Rate: 1 mL/min
Detection: UV 214 nm
Temperature: Ambient
Conclusions

- The data presented herein make evident the excellent resolving power of the Pursuit and Polaris columns. The selectivity differences between Pursuit and Polaris columns are demonstrated by the separation of complicated drug mixtures.

- Stationary phases with different chemistries exhibit significant differences in hydrophobic selectivity for hydrophobic molecules. Polar interactions between analytes carrying polar groups and stationary phases of different structures are reflected through longer retention on polar functionalized bonded phases. Under reversed phase conditions, dispersive interactions are dominant, however, polar interactions lead to changes in resolution/selectivity on polar functionalized phases for analytes containing both hydrophobic and polar segments in their structures.

- Selectivity changes can be induced by varying stationary phase chemistry, solvent type, solvent strength and temperature. There are significant differences in selectivity between bonded phases comprised only of alkyl chains and phases with a polar functionality in the alkyl chain. With the Pursuit and Polaris columns, this is a powerful means of manipulating selectivity.

- Pursuit columns are more hydrophobic than Polaris columns. When selectivity is governed by hydrophobic interactions predominantly, Pursuit columns demonstrate higher $\alpha$ values. Polaris columns exhibit longer retention and greater selectivity for polar molecules, especially for acidic or basic moieties, compared to Pursuit columns.