Challenges of Peak Tracking in Information-Rich HPLC Experiments

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Lilly
Answers That Matter.
Method Development Strategy

Design space approach

• structured data generation across columns, aqueous buffers, and organic solvents leading to predictive retention models
• prospective design of analytical methods made possible using the retention models and an understanding of the specific separation requirements

Benefits

• identification of primary and orthogonal HPLC separation conditions
• efficient and consistent method development process
• high success rate for separations of <10-15 components
## Column Screening Experiments

<table>
<thead>
<tr>
<th>Column, Aqueous Phase</th>
<th>Organic Modifier</th>
<th>Gradient Range (%)</th>
<th>Gradient Times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zorbax SB-C8, 0.1% formic acid in water</td>
<td>ACN</td>
<td>4 – 77</td>
<td>9.5, 38.1</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>5 – 95</td>
<td>9.5, 38.1</td>
</tr>
<tr>
<td>Ace Phenyl, 0.1% formic acid in water</td>
<td>ACN</td>
<td>4 – 77</td>
<td>9.5, 38.1</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>5 – 95</td>
<td>9.5, 38.1</td>
</tr>
<tr>
<td>Zorbax Bonus RP, 0.1% formic acid in water</td>
<td>ACN</td>
<td>4 – 77</td>
<td>9.5, 38.1</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>5 – 95</td>
<td>9.5, 38.1</td>
</tr>
<tr>
<td>XBridge C18, 0.1% NH₄OH in water</td>
<td>ACN</td>
<td>4 – 77</td>
<td>9.5, 38.1</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>5 – 95</td>
<td>9.5, 38.1</td>
</tr>
</tbody>
</table>
Data Acquisition

Experimental

- 8 sets of chromatographic conditions
- 2 run times (17.8, 46.4 min) per condition
- X individual compound and analyte mixture solution injections per run time (median=13)
- 1 single channel UV detector

Cost/Benefit

- total instrument time (8 x 1.1hr x 13) = 111hr
- total injections = 208
- data manipulation can be time consuming
- peak tracking is relatively simple and reliable
Typical Results

API Starting Material and Potential Impurities

XBridge C18 NH₄OAc pH 10, ACN

Zorbax SB-C8, 0.1% formic, ACN
High Throughput Screening Protocol

Experimental
- 1 analyte mixture solution per project
- 8 sets of chromatographic conditions
- 1 gradient time (9.5min) per condition
- PDA and MS detection

Strategy
- detect and match peaks in MS signals
- detect and match peaks in UV signals
- reconcile component peak data across detector signals
- assign identity based on component spectra
- report retention time, peak width, and asymmetry of each component in each experiment
Method Development Suite for LC/MS

Signal Processing

- MS_MAP provides for very sensitive peak detection even when components are significantly overlapped with intelligent clustering of individual mass chromatograms and automated interpretation of component spectra
- UV_MAP reliably detects and matches components when they are partially separated

Additional Benefits

- cycle time for data analysis reduced from >1 week to <1 day
- software provides a platform for paperless flow of information from raw data files
Potential Failure Modes

Nature of component mixtures
- drug substance, starting materials, intermediates, synthetic impurities, and degradation products
- approximately 70% of projects require tracking structural isomers or stereo-isomers
- median number of components per project is 12

Sources of ambiguity
- detection sensitivity
- response changes with experimental conditions
- multiple components with similar spectra
- significant component overlap
Detection Sensitivity

MS Signal
• ionization efficiencies achieved in an electrospray source can vary dramatically among components
• about 20% of analytes provide insufficient response using ESI

UV Signal
• >95% of compounds of interest have adequate UV response
• authentic samples of most components are available for use in analyte mixture preparation
MS Response Changes

- response trends with pH are compound specific

Chromatography:
- Zorbax SB-C8 with 0.1% formic acid, ACN or MeOH with ESI+ detection
- XBridge C18 with 0.1% NH₄OH, ACN or MeOH with ESI+ detection
UV Response Changes

- spectral shifts with pH are compound specific

ACN or MeOH with 0.1% aqueous ammonium hydroxide pH 10
ACN or MeOH with 0.1% aqueous formic acid pH 3

Retinoic acid
## MS Spectral Similarity

### Table of Mass C.C.

<table>
<thead>
<tr>
<th>Number of maxima</th>
<th>m/z</th>
<th>Compon.</th>
<th>Notation</th>
<th>tR (min)</th>
<th>Areas (counts)</th>
<th>Height (counts)</th>
<th>Color</th>
<th>Carbon</th>
<th>A+2</th>
<th>12C/3C</th>
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<td>12C</td>
<td>Br 0 Cl 0 S 0</td>
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</tr>
</tbody>
</table>

### API process impurity mix

- **Monoisotopic Mass** = 339.184587 Da

### Chromatogram

- XBridge C18, 3.5 micron aqueous NH\(_4\)OH, ACN gradient
- API process impurity mix
- **Monoisotopic Mass** = 339.184587 Da

<table>
<thead>
<tr>
<th>Isomer</th>
<th>Relative Amount</th>
<th>Relative Response</th>
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<td>0.4</td>
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<td>P.I. #5C</td>
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<tr>
<td>API</td>
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<td>1.0</td>
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</tbody>
</table>

**API**

**P.I. #5C**

**P.I. #6C**
UV Spectral Similarity

Ace Phenyl, 3.5 micron aqueous formic acid, ACN gradient API starting material and impurities
Peak Overlap in MS Signal

Zorbax SB-C8, 3.5 micron
0.1% formic acid, ACN gradient
response ratio = 200:1
critical pair $R_s = 0.39$

Terfenadine
Monoisotopic Mass = 471.31373 Da

Warfarin
Monoisotopic Mass = 308.104859 Da

<table>
<thead>
<tr>
<th>m/z</th>
<th>Component</th>
<th>Notation</th>
<th>tR</th>
<th>Color</th>
<th>Carbon</th>
<th>12C/13C</th>
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</table>
Peak Overlap in MS Signal

Zorbax SB-C8, 3.5 micron
0.1% formic acid, MeOH gradient
response ratio = 130:1
critical pair $R_s = 0.07$

Propranolol
Monoisotopic Mass = 259.157229 Da

Indoprofen
Monoisotopic Mass = 281.105193 Da

<table>
<thead>
<tr>
<th>m/z</th>
<th>Component</th>
<th>Notation</th>
<th>tR (min)</th>
<th>Color</th>
<th>Carbon</th>
<th>12C/13C</th>
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<tbody>
<tr>
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<tr>
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<td>283.006</td>
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<td></td>
<td>6.039</td>
<td>13C</td>
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</tr>
</tbody>
</table>
Peak Overlap in UV Signal

Atlantis dC18, 3 micron
0.1% formic acid, ACN gradient
relative amount = 1:1
critical pair $R_s = 0.7$
Peak Overlap in UV Signal

Zorbax SB-C8, 3.5 micron
0.1% formic acid, ACN gradient
relative amount: 1.8 : 1 : 2.1
critical pair $R_s$:
- $C_{09}/C_{17}=0.4$
- $C_{17}/C_{10}=0.5$
- $C_{09}/C_{10}=1.0$
Ensuring Component Identity

Lack of adequate MS detection sensitivity
- rely on UV signal for tracking components across experiments
- need additional data to identify components based on UV spectra

Multiple components with similar spectra
- MS and UV signals will not provide the required specificity
- control relative amounts of components in analyte mixture solution

Mitigation Plan
- collect MS and UV spectra at low and high pH for each component of interest individually
- use information to define relative amounts of components in analyte mixture solution
- cost: typically adds about 8 hours of instrument time and 2 hours of data processing time to screening protocol
Conclusions

• A high throughput approach to screening columns, aqueous buffers, and organic solvents can dramatically reduce cycle time

• ACD/Labs Method Development Suite for LC/MS is well-suited for processing MS and UV signals and managing project information

• Components of interest in pharmaceutical separations can have similar structures which lead to identical UV spectra and in the case of isomers, identical MS spectra

• Collecting MS and UV spectral and response data at low and high pH for each component of interest provides additional information needed to resolve ambiguities in assigning identity of components in complex mixtures
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