Applications of 2D-LC in Pharmaceutical Analysis

C. J. Venkatramani, Genentech, USA

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Agenda

• Business driver – why 2D-LC?

• Flavors of Two-Dimensional Chromatography

• Two-Dimensional Liquid Chromatography

• Application of 2D-LC in pharmaceutical analysis

• Acknowledgements
Pharmaceutical Industry

Highly regulated industry

Deliver safe and efficacious medications patients

**DS Specifications:** Appearance, Identity, *Assay and Related Substances*, Residual Solvents, Water Content, Heavy Metals and Specified Metals …. 

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ICH Q3A:

Reporting threshold $> 0.05\%$

Identification threshold $\geq 0.10\%$ or max 1.0 mg/day

Qualification threshold $\geq 0.15\%$ or max 1.0 mg/day

Exceptions: Impurities with tox coverage, metabolites

Genotoxic impurities (M7):

Limited by max daily dose & duration of exposure

Usually low parts per million
Drug substance synthetic scheme (Hypothetical)

Regulatory Starting Material (RSM1)

5-chloro-2,4-difluorobenzoic acid
RSM 1

Intermediate

Drug Substance
Isomers are likely to react and produce associated API impurities which will be difficult to purge, analyze.
Strategy for impurity characterization

Screen columns of different selectivity, pH’s, peak tracking

Demonstrate specificity - SM’s, intermediates, potential imps

Demonstrate method is stability indicating with stressed samples - acid, base, peroxide, heat, humidity, light

Relies on DAD and MS for detection - limiting factor

Peaks eluting around main component have similar UV spectra - Limits DAD
Isomers - Limits MS

**Potential Solution:** Two-dimensional Chromatography
Flavors of 2D-LC
Heart-Cutting 2D-LC
Part of primary column eluent sampled into secondary column
Easy to operate, adequate for most applications – *camera*

Comprehensive 2D-LC
Entire primary column eluent sampled into secondary column
Challenging, detailed analysis– *camcorder*

Pseudo-comprehensive 2D-LC
Comprehensive separation of select region of primary column eluent (*Targeted analysis*)
Moderate level of difficulty, adequate for most applications – *smart phone*
Heart-cutting 2D-LC


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Primary Chromatogram

Secondary Chromatograms

2D Contour Plots

Primary Ret. (min)

Sec. Ret. (sec)
### Primary Chromatogram

<table>
<thead>
<tr>
<th>Retention (minutes)</th>
<th>Detector Response (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>-200</td>
</tr>
<tr>
<td>5.50</td>
<td>0</td>
</tr>
<tr>
<td>6.00</td>
<td>200</td>
</tr>
<tr>
<td>6.50</td>
<td>400</td>
</tr>
<tr>
<td>7.00</td>
<td>600</td>
</tr>
<tr>
<td>7.50</td>
<td>800</td>
</tr>
<tr>
<td>8.00</td>
<td>1000</td>
</tr>
</tbody>
</table>

### Secondary Chromatograms

2D Contour Plots

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Case study: 2D-LC-MS method development strategy for complex, high molecular weight compound
Analytical challenges

Extremely complex synthesis involving multiple steps

Lot to lot variability requiring iterative method development

Some of the intermediates are extremely reactive and toxic

Molecular weights is usually very high

Qualitative and quantitative analysis of potential impurities is critical as it could have significant bearing on the downstream process and long term stability
## Chromatographic conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Ace, Excel 2 PFP C18, 150 mm x 3.0 mm, 2.0um</td>
</tr>
<tr>
<td>Wavelength</td>
<td>205 nm</td>
</tr>
<tr>
<td>Oven Temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.5 mL/min.</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>5 µL</td>
</tr>
<tr>
<td>Mobile Phase A</td>
<td>0.05% phosphoric acid in water</td>
</tr>
<tr>
<td>Mobile Phase B</td>
<td>0.05% phosphoric acid in ACN</td>
</tr>
<tr>
<td>Gradient program</td>
<td>Time (min)   A%  B%</td>
</tr>
<tr>
<td></td>
<td>0.0          95   5</td>
</tr>
<tr>
<td></td>
<td>8.0          60   40</td>
</tr>
<tr>
<td></td>
<td>23.0         47   53</td>
</tr>
<tr>
<td></td>
<td>28.0         5    95</td>
</tr>
<tr>
<td></td>
<td>30.0         5    95</td>
</tr>
<tr>
<td></td>
<td>30.1         95   5</td>
</tr>
<tr>
<td></td>
<td>37.0         95   5</td>
</tr>
<tr>
<td>Run Time</td>
<td>37 min</td>
</tr>
<tr>
<td>Diluent</td>
<td>50/50 (v/v) Acetonitrile / Water</td>
</tr>
</tbody>
</table>
Chromatographic profile of sample
Modify primary column gradient to elute component of interest within 5 to 15 minutes

Evaluate complementary phases in the secondary dimension
  SB-CN, Primesep-B, diamond hydride phase, SB-Phenyl, SB-Aq…

Re-assess original method using most complementary phase in the secondary dimension
## Modified expt. conditions – primary column

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Ace, Excel 2 PFP C18, 150 mm x 3.0 mm, 2.0 um</td>
</tr>
<tr>
<td>Wavelength</td>
<td>260 nm &amp; 300 nm</td>
</tr>
<tr>
<td>Oven Temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.05 mL/min.</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>5 μL</td>
</tr>
<tr>
<td>Mobile Phase A</td>
<td>0.05% formic acid in water</td>
</tr>
<tr>
<td>Mobile Phase B</td>
<td>0.05% formic acid in ACN</td>
</tr>
<tr>
<td>Gradient program</td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>A%</td>
</tr>
<tr>
<td>0.0</td>
<td>60</td>
</tr>
<tr>
<td>22.0</td>
<td>50</td>
</tr>
<tr>
<td>25.0</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>30.1</td>
<td>60</td>
</tr>
<tr>
<td>Run Time</td>
<td>Varies depending upon secondary column run time</td>
</tr>
<tr>
<td>Diluent</td>
<td>50/50 (v/v) Acetonitrile / Water</td>
</tr>
</tbody>
</table>
Trace in red: Primary column gradient
Trace in blue: Secondary column gradient

Fractions transferred to sec. column

1st gradient

Last gradient
Experimental conditions – secondary column

**2D-LC Mode**
- Comprehensive
- Heart-Cutting
- HiRes sampling

**2D Gradient**
- Time [min]:
  - 0.00: 45.00%
  - 2.00: 60.00%

**Solvents**
- **A:** 55 %
  - A1: 100.0 % Water V.03
- **B:** 45 %
  - B1: 100.0 % Acetonitrile V.03

**Sampling table**

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Mode</th>
<th>Sampling time [s]</th>
<th>Cuts</th>
<th>Loop filling [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.15</td>
<td>Time based</td>
<td>4.50</td>
<td>10</td>
<td>94</td>
</tr>
</tbody>
</table>

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*Fractions 1 to 5 are parked in Deck A, analyzed in reverse order (5, 4, 3,...)
*Fractions 6 to 10 parked in Deck B
*Fractions 6 through 10 in Deck B is analyzed in reverse order (10, 9, 8...) following Deck A analysis
*Trapping and analysis can repeated throughout the chromatogram
High-resolution sampling 2D-LC
Composite picture of 2D-LC separation (main comp)

Cut 2

Cut 3

Cut 4

Cut 5

Cut 6

Cut 7

Cut 8

Cut 9

Sec Column Separation

Primary Column Separation

Sec Column Separation

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Stack plot of 2nd dimension separation

Lot #1

Final intermediate

Imp A

Imp B

Imp C

Main component

Stack plot of sec column separation showing potential co-elution in the primary dimension

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2D-LC detection: UV v/s MS

*Co-eluting impurities from the primary column are resolved in the secondary, complementary SB-CN column

*Impurities were not detected by MS

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*Co-eluting impurities from the primary column are resolved in the secondary, complementary SB-CN column
*Impurities were not detected by MS
Zones of co-elution in the 1st dimension

Impurities A and B observed in fractions 2 to 5 across the main peak
Zones of co-elution in the 1\textsuperscript{st} dimension

Impurity C observed in fractions 5 to 9 across the main peak
Assessment of 1st dimension for potential co-elusion

Sec column separation (UV)
8.31 min
8.85 min

Primary column separation
Selective assessment of 1\textsuperscript{st} dimension (co-elusion)

Sec column separation (UV)
10.95 min
11.03 min
11.11 min

Primary column separation
Selective assessment of 1\textsuperscript{st} dimension (co-elusion)

**Sec column separation (UV)**

11.45 min
Main Comp

**Primary column separation**
Selective assessment of 1st dimension (co-elution)

Sec column separation
13.49 min
13.69 min
13.89 min
Selective assessment of 1st dimension (co-elusion)

(1) 8.31 min 8.85 min

(2) 10.95 min 11.03 min 11.11 min

(3) 11.45 min Main Comp

(4) 13.49 min 13.69 min 13.89 min
Based on 2D-LC-MS analysis of sample, multiple components co-elute in the primary dimension, several of these components are partially resolved in the secondary SB-CN column.
Sec. column separation of lot 2 (main component)

Stack plot of secondary column separation showing potential co-elution in primary dimension

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Isocratic separation in secondary SB-CN column
Lot 1, HPLC UV at 260 nm

Secondary column separation - Isocratic

Final intermediate

Secondary column separation
isocratic separation (Water:ACN:FA 48:52:0.05)

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Sec. column separation - Isocratic

Lot 2, HPLC UV at 260 nm

Secondary column separation
isocratic separation (Water:ACN:FA 48:52:0.05)

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Quantitation by 2D-LC
Peak ID & quantification in 2\textsuperscript{nd} dimension

Secondary column peak integration

Secondary column separation (min)
Real component will shows up in multiple, sequential chromatograms confirming its presence in sample

<table>
<thead>
<tr>
<th>Cut #</th>
<th>2D Retention time [min]</th>
<th>Area</th>
<th>Height</th>
<th>Width</th>
<th>Symmetry</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.41</td>
<td>37.46</td>
<td>13.87</td>
<td>0.04</td>
<td>0.77</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>1.41</td>
<td>730.71</td>
<td>271.44</td>
<td>0.04</td>
<td>0.75</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>1.42</td>
<td>3436.59</td>
<td>1246.35</td>
<td>0.04</td>
<td>0.75</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>1.42</td>
<td>4684.61</td>
<td>1627.74</td>
<td>0.05</td>
<td>0.73</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>1.42</td>
<td>2204.79</td>
<td>767.27</td>
<td>0.04</td>
<td>0.73</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>1.42</td>
<td>485.61</td>
<td>164.22</td>
<td>0.04</td>
<td>0.72</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>1.42</td>
<td>187.23</td>
<td>62.20</td>
<td>0.05</td>
<td>0.72</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>1.42</td>
<td>84.97</td>
<td>27.66</td>
<td>0.05</td>
<td>0.75</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>1.42</td>
<td>48.26</td>
<td>16.15</td>
<td>0.04</td>
<td>0.79</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>1.41</td>
<td>30.45</td>
<td>10.13</td>
<td>0.05</td>
<td>0.75</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>1.42</td>
<td>11930.68</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>
Relative PA’s of the impurities are comparable between runs low %RSD (< 10%) for peaks in the LOQ range

Lot#1 has an additional impurity compared to lot 1

%PA is the level of impurities co-eluting in the main component

### Lot 1

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>Run#1</th>
<th>Run#2</th>
<th>Run#3</th>
<th>Average</th>
<th>Std Dev</th>
<th>%RSD</th>
<th>%Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>7.3</td>
<td>7.9</td>
<td>7.5</td>
<td>7.6</td>
<td>0.30</td>
<td>3.98</td>
<td>0.06</td>
</tr>
<tr>
<td>0.99</td>
<td>12.4</td>
<td>14.0</td>
<td>13.9</td>
<td>13.4</td>
<td>0.92</td>
<td>6.83</td>
<td>0.11</td>
</tr>
<tr>
<td>1.21</td>
<td>32.045</td>
<td>30.935</td>
<td>30.998</td>
<td>31.3</td>
<td>0.62</td>
<td>1.99</td>
<td>0.26</td>
</tr>
<tr>
<td>1.42</td>
<td>11922.2</td>
<td>11875.9</td>
<td>11907.3</td>
<td>11901.8</td>
<td>23.65</td>
<td>0.20</td>
<td>99.56</td>
</tr>
</tbody>
</table>

### Lot 2

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>Run#1</th>
<th>Run#2</th>
<th>Run#3</th>
<th>Average</th>
<th>Std Dev</th>
<th>%RSD</th>
<th>%Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>6.4</td>
<td>6.8</td>
<td>7.6</td>
<td>6.9</td>
<td>0.61</td>
<td>8.73</td>
<td>0.06</td>
</tr>
<tr>
<td>0.99</td>
<td>12.9</td>
<td>13.9</td>
<td>13.6</td>
<td>13.5</td>
<td>0.52</td>
<td>3.84</td>
<td>0.11</td>
</tr>
<tr>
<td>1.42</td>
<td>11930.7</td>
<td>11903.2</td>
<td>11895.3</td>
<td>11909.8</td>
<td>18.55</td>
<td>0.16</td>
<td>99.83</td>
</tr>
</tbody>
</table>

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Comparison of blank and sample at 260 and 300 nm
Sec. column separation - Isocratic

Lot 2, HPLC UV at 260 nm

Effective retention space

Secondary column separation
isocratic separation (Water:ACN:FA 48:52:0.05)

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Hi-speed separation in secondary column
Secondary Column Separation on RRHD SB-CN column
Assessment of potential co-elution in original method
Sec. column separation – SB-CN

Lot 1, 260 nm
Cut 3
A B
Final Int

Lot 1, 260 nm
Cut 6
C?
Final Int

Lot 1, 260 nm
Original method
Phosphoric acid system
Sec. column separation – SB-CN

Lot 1, 260 nm
Lot 1, 300 nm

Deg 1

Residual level of new degradation product observed in aged sample
Imp C resolved from main component in the release method

Lot 1, 260 nm
Original method
Phosphoric acid system
Analysis of sample using Primesep-B column in secondary dimension
Sec. column separation – PS-B

Primary column separation

LC-UV @ 260 nm

Secondary column separation

Secondary column separation on a 2 cm Primesep-B guard column
Analysis of sample using diamond hydride column in secondary dimension
Secondary column separation – DH

Primary column separation

LC-UV @ 260 nm

Secondary column separation

HPLC-UV

Final Intermediate

Secondary column separation on a Diamond Hydride column (5 cm x 4mm x 4 micron)
Analysis of sample using SB-Aq column in secondary dimension
Sec. column separation – SB-Aq

Sec column separation on a SB-AQ column, 2.1 mm x 30 mm, 1.8 uM

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Conclusions

Successfully demonstrated the applications of 2D-LC in resolving residual, co-eluting impurities in the midst of main component.

Successfully demonstrated the capability of hi-resolution 2D-LC in quantitative analysis of co-eluting impurities.

SB-CN offered most complementary separation in the secondary dimension.

Commercial systems have extended the capabilities of 2D-LC from being a research tool in select laboratories to real world applications.

Multiple heart cutting, flexibility …..
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