Integrated approach to HPLC method development:
Using all the tools in the chromatographer’s toolbox

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Overall Strategy

- Determine aim of analysis
- Look at structure (estimate $pK_a$) or use ACD (Advanced Chemistry Development)
- Try to use shorter columns for scouting experiments (5cm x 4.6 mm) - 3 um or use Acquity system with 10 cm x 2.1 mm, 1.7 um particles
- Use 35 - 45C as starting temperature.
- Run probe linear gradient with hold at high organic on short column, 5cm column
- Run pH studies isocratically to determine optimal pH (5 cm column)
  - Acquity system very effective for doing so
- Run linear gradient with hold at high organic with optimized pH on 5 cm x 4.6 mm column
- If sufficient resolution Finished
- If need more resolution then go to 15 cm x 3 mm id column
- If resolution obtained- Finished
- If desired resolution not obtained use AMDS system (need to optimize gradient and selectivity)
- Use AMDS system for gradient optimization on 15 cm x 3 mm id column. (2 organics, steep/shallow gradient)- we will discuss today.

Sample Prep

- Use methanol as sample prep solvent if using second dilution. (check for sample reactivity)
- If using methanol for only dilution look at impact on peak shape (diluent/mobile phase mismatch for components with $k'<2$)
- Sample prep constitutes apx. 70% of solvent usage, try to use methanol if possible.
First Step: pKₐ Estimation Using ACD Software

• Product T is a diprotic base with two pKₐ's 3.3 and 5.3 estimated by ACD

• It has two pKₐ values and at mobile phase pH values between 3 and 5, multiple species exist.

• Two equilibria could be written for this amphoteric species

• Since pKₐ's are close to one another (<4 pKₐ units apart) the inflection points overlap making titration and or chromatographic pKₐ prediction difficult.

• At pH=4.3 basic site (A) will be predominately neutral (90%) and the other basic site (B) will be predominately ionized (90%).
Initially a steep gradient was run to estimate the isocratic elution conditions in order to study the effect of pH on analyte retention.
Retention Dependence on Mobile Phase pH

Chromatographic conditions:
Column: Phenomenex Luna 3u C8(2) [150x4.6mm, 3µm]
MP: 10 mM K₂HPO₄:ACN (71:29,v/v)
  pH adj. w/ H₃PO₄
Flow rate: 1.0 mL/min
Injection Vol.: 10 µL
Wavelength: 247 nm
Column Temp.: 35 C

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<td>1.6</td>
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- Initial isocratic conditions predicted from probe gradient run correlates very well with actual retention obtained at isocratic conditions.
- pH 6 is the optimal pH to run for the analysis.
- Check which wavelength gives the best sensitivity.

- Try 5 cm x 4.6 mm column on conv. HPLC system
- Try 10 cm x 2.1 mm column on Acquity system
What should the starting pH be in order to analyze this molecule in its neutral form?

- The downward $pK_a$ shift for basic analytes must be accounted for.
- The working pH should be at least 2 pH units above the basic analyte $pK_a$ to be fully neutral.
- The upward pH shift of the aqueous acidic buffer upon addition of the organic must be accounted for.

**Example:** The higher $pK_a$ of Product T is 5.3 and initial eluent conditions are: 30% MeCN and 70% Buffer.

What should the pH of the buffer be in order to obtain the basic analyte in its neutral form?

\[
5.3 - (3 \times 0.2) = 4.7 \quad \text{Downward analyte } pK_a \text{ shift.}
\]
\[
4.7 + 2 = 6.7 \quad \text{pH at which basic analyte would be neutral}
\]
\[
3 \times 0.2 = 0.6 \quad \text{Upward pH shift of aqueous acidic buffer upon addition of organic}
\]
\[
6.7 - 0.6 = 6.1 \quad \text{Max pH of buffer in order to have analyte in fully neutral form.}
\]

This prediction agrees well with the experimental!!
Chaotropic Effect: Increasing retention in low pH region

Chromatographic Conditions
Column: Luna C8(2) 150x4.6 mm
MP: Aqueous :ACN (71:29, v/v)
Wavelength: 247 nm
Col. Temp.: 35 C
Inj. vol.: 10 µL

- Chaotropic anions, TFA and HClO₄ interact with positively charged analyte and lead to an increase in the analyte retention.

- Retention decreases from pH 1.90 to 1.86 for MP with H₃PO₄. Indicates analyte not completely ionized in this pH region.

- TFA is MS compatible however is not the best MS mobile phase additive to employ (ion suppression in gas phase) so went to high pH analysis.
Chromatographic overlays of Labetalol analyzed at different analyte concentrations using increasing mobile phase concentration of perchlorate anion.

Chromatographic conditions: Analyte load: 3.3, 6.5, 31.2 mg, (a) 75%: 0.1 v/v% H3PO4: 25% acetonitrile, (b) 75%: 0.05 v/v% HClO4: 25% acetonitrile, (c) 75%: 0.2 v/v% HClO4: 25% acetonitrile, (d) 75%: 0.4 v/v% HClO4: 25% acetonitrile, (e) 75%: 0.5 v/v% HClO4: 25% acetonitrile.

Increase of analyte efficiency and reduction of peak tailing.
Fig. 5. Chromatographic overlays of Dorzolamide HCl analyzed at different analyte concentrations using increasing mobile phase concentration of perchlorate anion
Chromatographic conditions: Analyte load: 1.4, 5.2, 9.2, 48 mg, (a) 90%: 0.1 v/v% H3PO4: 10% acetonitrile, (b) 90%: 0.05 v/v% HClO4: 10% acetonitrile, (c) 90%: 0.2 v/v% HClO4: 10% acetonitrile, (d) 90%: 0.4 v/v% HClO4: 10% acetonitrile, (e) 90%: 0.5 v/v% HClO4: 10% acetonitrile
Effect of analyte load and perchlorate counteranion conc. on labetalol apparent efficiency and tailing factor.

Chromatographic conditions:
Analyte load: 0.06 - 31.2 mg, 75% water: 25% acetonitrile. Water adjusted with 0.025 - 0.5 v/v% HClO4. (A) N (h/2) vs. perchlorate concentration. (B) Tailing factor vs. perchlorate concentration.
Mobile phase: 0.1 v/v% phosphoric acid + xBF4 [1 mM – 50 mM]: acetonitrile, Ophthalmic compounds (10% acetonitrile), phenols (25% acetonitrile), (A) N(h/2) vs. tetrafluoroborate concentration. (B) Tailing factor vs. tetrafluoroborate concentration.
Mobile phases (A) Dorzolamide HCl: 90% water: 10% acetonitrile. Water adjusted with either 0.1 v/v% phosphoric acid or 0.5 v/v% HClO4. Phenol: 75% water: 25% acetonitrile. Water adjusted with either 0.1 v/v% phosphoric acid or 0.5 v/v% HClO4. (B) Benzyamine: 95% water: 5% acetonitrile. Water adjusted with either 0.1 v/v% phosphoric acid or 0.5 v/v% HClO4. Toluene: 50% water: 50% acetonitrile. Water adjusted with either 0.1 v/v% phosphoric acid or 0.5 v/v% HClO4.
Option 2- Analysis of Analyte (Free Base) as Neutral Species

Chromatographic Conditions:
- Column: Luna C8(2) 150x4.6 mm
- MP: Aqueous : Acetonitrile
- Wavelength: 247 nm
- Col. Temp.: 35 C
- Flow: 1 mL/min
- Inj. Vol.: 10 µL

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<td>3</td>
<td>23</td>
<td>5</td>
<td>95</td>
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- Analyte is in neutral form at the mobile phase pH of the aqueous phases used.
- Ammonium Carbonate and ammonium acetate have a UV cutoff < 220 nm.
- Both buffers are compatible with LC-MS.
- Ammonium carbonate buffer may be undesired because it can form CO₂ and alter the mobile phase pH.
• Diode array spectra show that this main peak is not spectrally homogenous.
• Need to perform method optimization experiments to resolve the impurity from active.
• LC-MS studies were also done in parallel.

Chromatographic Conditions:
Column: Luna C8(2) 150x4.6 mm
MP: 10 mM NH₄OAc:Acetonitrile
Col. Temp.: 35 C
Flow: 1.0 mL/min
Inj. Vol.: 10 µL

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</tr>
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<td>23</td>
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%A: 10 mM NH₄OAc, pH 5.8
%B: ACN
Spectral Purity: MS

Product T free base

Chromatographic Conditions:
Column: Luna C8(2) 150x4.6 mm
MP: Aqueous : Acetonitrile
Wavelength: 247 nm
Col. Temp.: 35°C
Flow: 1 mL/min, split 10:1
Inj. Vol.: 10 µL

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<tr>
<td>3</td>
<td>23</td>
<td>1</td>
<td>5</td>
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</tbody>
</table>

%A: 10 mM Amm. Acetate, pH 5.8
%B: ACN

ESI: + ion mode
Single Quadrupole, Open access
Capillary: + 3.5 kV
Cone: 25 V
Source Temp: 150°C
Cone Temp: 20°C
Desolvation Temp: 400°C
Cone gas flow: 113 L/hr
Desolvation gas flow: 419 L/hr
Indicates peak is not spectrally homogenous.
• [M+H] ion of active = 469
• [M+H] ion of impurity = 438

Indicates impurity has odd number of nitrogens.

We will revisit the mass spectra once we separate!!!
Waters Automated Method Development System (AMDS)

The Waters AMDS was used for method optimization by varying several chromatographic parameters in order to satisfy a user's set criteria.

**Analytical goals for the Analysis of Product T on the AMDS:**

- Organic solvents: Methanol, Acetonitrile
- Aqueous: 10 mM NH₄OAc, pH 5.8
- Columns: Luna C18 (2) 3µm(150x4.6 mm), Sunfire C18 3.5µm (150x4.6 mm)
- Resolution more important than run time
- Runtime < 10 min.
- Minimum Resolution > 3.0 (between identified peaks)
- Pressure < 3000 psi
AMDS for Method Optimization

Luna C18(2), 3µm, 150x4.6 mm

1 – (Steep gradient) 5% - 90% Methanol in 6 min
2 – (Shallow gradient) 5% - 90% Methanol in 17 min
3 – (Steep gradient) 5% - 90% Methanol in 6 min
4 – (Shallow gradient) 5% - 90% Methanol in 17 min

Sunfire C18, 3.5 µm, 150x4.6 mm

Flow rate = 0.8 mL/min

Future suggestion: Gradients used should be 15 – 95% Methanol
AMDS for Method Optimization

Luna C18(2), 3µm, 150x4.6 mm

1 – (Steep gradient) 5% - 85% Acetonitrile in 6 min
2 – (Shallow gradient) 5% - 85% Acetonitrile in 17 min
3 – (Steep gradient) 5% - 85% Acetonitrile in 6 min
4 – (Shallow gradient) 5% - 85% Acetonitrile in 17 min

Sunfire C18, 3.5 µm, 150x4.6 mm

Flow rate=1.5 mL/min
• Acetonitrile is the preferred organic solvent for this separation.
• Greater resolution between the critical pair is observed on the Sunfire column.
• Further optimization is necessary to shorten run time.
Predicting Method Conditions Using DryLab

- Entered chromatographic results into DryLab to predict minimum resolution between active and impurity.
- Used the resolution map to determine optimal gradient run time and temperature condition for analysis.

Gradient Conditions

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<tr>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>12</td>
<td>75</td>
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</tbody>
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Col. Temp. = 35° C  
Flow Rate = 1.5 mL/min  
Sunfire C18, 3.5 µm, 150x4.6 mm  
10 mM NH₄OAc, pH 5.8, :ACN
**DryLab Prediction vs. Actual Chromatogram**

DryLab Predicted Chromatogram  
\[ R_s = 2.5 \]

Actual chromatogram  
\[ R_s = 3.1 \]

Chromatographic Conditions:
- Column: Sunfire C18, 3.5um, 150x4.6 mm
- MP: Aqueous:Acetonitrile
- Wavelength: 247 nm
- Col. Temp.: 35° C
- Flow: 1.5 mL/min
- Inj. Vol.: 10 µL

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%A: 10 mM NH₄OAc, pH 5.8  
%B: ACN

**DryLab predicted chromatogram and actual chromatogram are similar.**  
**Note that Drylab gives accurate predictions only if the analyte ionization state is not changing with increasing organic**  
**The run time was decreased without compromising the resolution between the active and the impurity.**
Predict/Confirm possible deg. products of your analyte
Forced Deg studies

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<td>1.5</td>
<td>25</td>
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%A = 10 mM NH₄OAc, pH 5.8
%B = ACN

Rs=3.0

50°C / 1 week, pH 1

• Sample was stressed at 50°C for 1 week in pH 1 diluent.
• Decreased the initial organic composition of the gradient from 35% to 25%.
• Carboxylic acid impurity has enhanced retention at lower organic.
• Resolution between active and impurity eluting after main component was not compromised.
MS analysis of Product T

RRT = 1.00

RRT = 1.04

%A = 10 mM NH₄OAc, pH 5.8
%B = ACN
Sunfire C18, 4.6 x 150 mm, Flow 1.5 ml/min, split 10:1
Temp: 35°C
Cone voltage: 25 V, Single quad.
MS analysis confirmed the separation of the active from the MW 437 impurity.

RRT=1.00, active

\[ [\text{M+H}]^+ \]

RRT:1.04, Imp.

\[ [\text{M+H}]^+ \]

\[ [2\text{M+H}] \]

\%
A = 10 mM NH₄OAc, pH 5.8
\%
B = ACN
Sunfire, 4.6 x 150 mm, Flow 1.5 ml/min, split 10:1
Cone voltage: 25 V, Single quad.

Product T
Monoisotopic mass: 468.2

Impurity at RRT 1.04
Monoisotopic mass: 437.2
• DS loses methyl amine (31 dalton) to form stable ion (m/z 438).
• Note if too high a fragmentor voltage used fragment could not be deconvoluted from RRT: 1.04 impurity [M+H]= 438.
• The proposed fragmentation pattern was confirmed by MS/MS analysis and deuterated experiments.
Conclusion

• Use ACD to estimate $pK_a$ of molecule and predict optimal pH for analysis
• Run steep gradient to predict isocratic conditions for further pH optimization
• Determine retention behavior of active as function of pH (isocratic)- use Acquity if possible
• Determine best mobile phase pH for further experiments
• Co-elution of impurities
  - Determine spectral purity with PDA spectra
  - LC-MS to elucidate spectral homogeneity (watch out for isomers)
    - Run all intermediates, precursors, forced deg. samples
• Method optimization
  - Use AMDS/Dry Lab for method optimization
    - Use MS to confirm the separation of active from possible co-eluting species
• MS/MS analysis can be performed for structural elucidation of impurities
• Deuterated experiments can be performed to support structural assignments
Acknowledgements

A. Jones
Frances Liu
Y. Kazakevich
Rich Vivilecchia
James Mullins
Background Slides
Deuterated experiments

• Deuterium-exchange experiments can also be used to further elucidate and define structural detail.

• The fine structural details of analytes could be further defined by a deuterium-exchange experiment that measures the number of exchangeable protons in each molecule.

• The number of exchangeable protons in a molecule can be determined based on the mass shift.

• This technique allows an understanding of which protons are susceptible to exchange, but also can be used to differentiate compounds of the same molecular weight that have a different number of exchangeable protons.
Deuterated exchange provides strong evidence to support degradation product and synthetic by-product elucidation.
Deuterated exchange provides strong evidence to support degradation product and synthetic by-product elucidation.
Case Study 1
Structural elucidation of compounds with the same mass

Mass spectra of these different analytes are similar and the same \([\text{M+H}]^+\) ion obtained. Application of the “nitrogen rule” does not help (odd number of nitrogens in both).

Deuterium exchange might help in structural elucidation since 5-aminoindazole has 3 exchangeable protons and 1-Aminoindan has only two exchangeable protons.

80:20, 0.1% HAc in water:MeCN
HPLC analysis using on-line H/D exchange

Conditions:
70% D2O:
30% MeCN,
Flow: 0.5 ml/min.
Symmetry Shield
50 x 4.6 mm, 5 um

• The mass of the first component on the chromatogram increases by 5 therefore 3 exchangeable protons are present, which suggests that this analyte is 5-aminoindazole.
• The mass of the second compound increases by 4 therefore only 2 exchangeable protons are present.
• Knowledge of the number of labile H atoms in a molecule is useful for comparing proposed impurity structures with that of the parent drug to determine the presence or absence of these functional groups.
HPLC References

• LoBrutto, R., Kazakevich, Y.V. Retention of Ionizable Components in Reversed Phase HPLC (Book Chapter), Practical Problem Solving in HPLC, Wiley-VCH, 122-158 (2000).