ACD/AutoChrom—Assisted Method Development for Challenging Separations

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Resolution of isomeric species is one of the most challenging areas of separation science due to the inherent similarity of the molecules and their respective energies. A number of pharmaceutical active ingredients and intermediates contain moieties that may exist as isomers that could potentially contaminate the material. The accepted approach for finding optimal separation conditions initiates with the screening of multiple columns/conditions followed by gradient optimization. Traditional trial-and-error studies to optimize gradient slope for a given separation may require several days. In comparison, computer simulation can deliver the optimized conditions within minutes.
Typical Reversed Phase HPLC Method Development Workflow for Isomeric Separations

1. Search literature/available databases for existing methods
   - Method available?
     - Yes: Prepare mixture of markers
     - No: Screen multiple columns/generic gradient at multiple pHs/choose the best column

2. Prepare mixture of markers
   - Screen multiple column at best pH/choose the best column
   - Peak co-elution check (UV and MS)
   - Method criteria met?
     - Yes: Finalize method
     - No: Gradient and/or Temperature optimization through modeling

3. Finalize method
   - Method criteria met?
     - Yes: Further screening of organic modifiers/fine tuning pH
     - No: Specialized method development

   1. Specialty columns;
   2. Chiral columns
   3. Chiral GC
   4. CE
   5. Normal Phase, etc
Typical Instrumentation/Software Use with Generic Approach to Method Development

- pH screen
- Initial condition: Generic method Empower
- Column Screening
- Meduza/Shimadzu Class VP

Manual evaluation of results
- Drylab or manual twicking
- Manual transfer of data from Empower or file conversion

Optimization

LC-MS ChemStation
- LC/MS/UV Peak Purity
Automated Method Development
What is ACD/AutoChrom?

A multiple software tools in one convenient and fully automated package.

- Offers calculations of Phys-Chem properties for analytes with known structures prior to MD (pKa, LogP).
- Controls HPLC, UHPLC, and LC/MS instruments - fully automated, needs minimal programming.
- Spectrus: LC-GC-UV-LC/MS-GC/MS-SPECTROSOOPY Processor
- Intellextact – automated peak mapping capabilities based on MS/UV spectra, identifies and matches relevant peaks based on spectral information (MS; UV) thus allowing to see peak distribution and choose the best separation based on user defined criteria and the number of peaks found.
- ACD/LC & GC Simulator
- More than 10,000 chromatograms in Chromatography Application Database—offer search for starting conditions for known structures.

Guides workflows that facilitate the entire method development process: runs screening/maps peaks based on UV and MS data/provides optimization/modeling/suggests best results.
ACD/AutoChrom
Method Development Cycle

Method Optimization: Release
Suitability criteria has been changed to allow quantitation of p-TsOH (k > 1); max run time: 25 min

1. Acquire data
2. Track peaks between injections
3. Generate peak table

Gradient
Temperature

End
Generic Approach vs AutoChrom

- HPLC (Agilent or Waters)
- Column screening system (Shimadzu)
- LC-MS
- Drylab
- ACD/AutoChrom
Method Development for Cis/Trans isomers and Closely Eluting Impurities Separation: Case Study 1
Control of trans-isomer is critical since this step is a quality gate-keeper for API
Best Results by Traditional Column Screening
ACD/AutoChrom: Strategy for Method Development

Strategy:

2 pHs, 11 columns

<table>
<thead>
<tr>
<th>Weak Phase</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.05 mM TFA)</td>
<td>pH 2</td>
</tr>
<tr>
<td>Water/MeCN (95:5) (+ 10 mM AcONH4)</td>
<td>pH 7</td>
</tr>
</tbody>
</table>

Experiment | Rs | Score | Total |
---|---|---|---|
Zorbax ECLIPSE PLUS & pH 2 & pH 2 | - | 0/26 |
ZORBAX SB AQ C18 & pH 2 | 0.44 | 13/26 |
ABZ PLUS SUPELCO, ser# 123990-05 & pH 2 | - | 0/26 |
AQUA SEP ES & pH 2 | - | 0/26 |
ACE C18-AR ser#A79091 & pH 2 | - | 0/26 |
BONUS RP & pH 2 | - | 0/26 |
ACSENTIS EXPRESS C18 & pH 2 | - | 0/26 |
FORTIS DIPHENYL, ser# A05111301 & pH 2 | - | 0/26 |
Phenomenex Aqua C18 & pH 2 | - | 0/26 |
ATLANTIS T3 & pH 2 | - | 0/26 |
Poroshell 120 SB-C18 & pH 2 | - | 0/26 |
Zorbax ECLIPSE PLUS & pH 6.99 | - | 0/26 |
ZORBAX SB AQ C18 & pH 6.99 | - | 0/26 |
ABZ PLUS SUPELCO, ser# 123990-05 & pH 6.99 | - | 0/26 |
Phenomenex Aqua C18 & pH 6.99 | - | 0/26 |
Poroshell 120 SB-C18 & pH 6.99 | - | 0/26 |
ACSENTIS EXPRESS C18 & pH 6.99 & BONUS RP & pH 6.99 | 0.519 | 20/26 |
FORTIS DIPHENYL, ser# A05111301 & pH 6.99 | - | 0/26 |
Phenomenex Aqua C18 & pH 6.99 | - | 0/26 |
ATLANTIS T3 & pH 6.99 | 0.56 | 15/26 |
Poroshell 120 SB-C18 & pH 6.99 | - | 0/26 |
Three Best Columns: Choice for Optimization

Mobile Phase A: Water/ACN (95:5 v/v); 10mM Ammonium Acetate
Mobile Phase B: Water/ACN (5:95 v/v); 10mM Ammonium Acetate
10-90B (30 min)
Column Name: BONUS RP
Sample Name: BMT-082676_ML

Retention Time (min) Response
0 8 16 24 32 40 48
8
16
24
32
40
48

Mobile Phase A: Water/ACN (95:5 v/v); 10mM Ammonium Acetate
Mobile Phase B: Water/ACN (5:95 v/v); 10mM Ammonium Acetate
10-90B (30 min)
Column Name: ATLANTIS T3
Sample Name: BMT-082676_ML

Retention Time (min) Response
0 8 16 24 32 40 48
8
16
24
32
40
48

Mobile Phase A: Water; 0.05mM TFA
Mobile Phase B: ACN/Water (95:5 v/v); 0.05mM TFA
10-90B (30 min)
Column Name: ZORBAX SB AQ C18
Sample Name: BMT-082676_ML

Retention Time (min) Response
0 8 16 24 32 40 48
8
16
24
32
40
48

* Critical pair
Based on the modeling data, Atlantis T3 was abandoned since Bonus RP showed better separation for the isomeric pair.
Baseline separation of trans-isomer and other impurities from the main peak were achieved. Accurate correlation between modeled and experimental data.
Method Development for Multiple Diastereomers Mixture Case Study 2
Develop a method suitable for IPC rxn completion and release of an intermediate (diastereomers separation) preferably in one run;

Challenge:
multiple chiral centers, no pure markers available, time constrain.

Strategy:

Negotiate critical pairs

pH Evaluation:
Run generic method at different pHs

Further screening
No separation
Baseline separation
Transfer method
Initial screening:
pH Evaluation Using Generic Method
Day 1

<table>
<thead>
<tr>
<th>pH</th>
<th>Status</th>
<th>Rs Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Complete</td>
<td>0.164</td>
</tr>
<tr>
<td>6.99</td>
<td>Complete</td>
<td>0.167</td>
</tr>
</tbody>
</table>

Mobile Phase A: Water/ACN (95:5 v/v); 10mM Ammonium Acetate
Mobile Phase B: Water/ACN (5:95 v/v); 10mM Ammonium Acetate
10-90B (25 min)
Column Name: Zorbax ECLIPSE PLUS
Sample Name: 98395-040-F2 BMT-129167

Continue screening since starting material and Compound 1 co-elute.

Compound 1 (Product) mixture of all isomers
SM
diastereomer marker
Strategy of Method Development
Day 1

**Strategy for overnight screening**

**Since generic column screening scored better at neutral pH, further column screening was conducted only with NH4Ac.**

**Strategy for Automated Processing**

Process only promising separations, choose best scored chromatogram for further optimization

**Column Screening Results**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Status</th>
<th>Rs Score</th>
<th>Min Rs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zorbx ECLIPSE PLUS</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>ZORBAX SB AQ C18</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>ABZ PLUS SUPELCO</td>
<td>Complete</td>
<td>0.680</td>
<td>1.23</td>
</tr>
<tr>
<td>AQUA SEP ES</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>ACE C18-AR</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>BONUS RP</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>ASCENTIS EXPRESS C18</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>FORTIS DIPHENYL</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Phenomenex Aqua C18</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>ATLANTIS T3</td>
<td>Complete</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Poroshell 120 SB-C18</td>
<td>Complete</td>
<td>0.613</td>
<td>0.165</td>
</tr>
</tbody>
</table>
Building an Accurate model – MD
Day 2

\[
\ln K = a + b X
\]

\[
K' = a + b X
\]

\[
\ln K' = a + b X
\]

\[
\ln K = a + b X + c X^2
\]

\[
\ln K = a + b X + c X^{1/3}
\]

\[
\ln K' = a + b X^{-1}
\]

\[
k'^{-1} = a + b X
\]
Software Optimized Final Method – MD

Day 2

Baseline separation of all impurities of interest were achieved within 2 days.

*) Separation of diastereomers 2 and 3 of Compound 1 is not critical for the process
The Importance of Software-Assisted Peak Co-elution Check in Addressing Root Cause of Reaction Stalling
Case Study 3.
During the course of development for a BMS compound, an improvement to the yield of one of the early intermediates was desired due to an expensive starting material (SM 1). This improvement would not only reduce the cost of the entire synthesis but also increase the throughput. Systematic analysis of in-process reaction samples showed that the reaction “stalled” at ~80% yield, regardless of process conditions, as both starting materials were not completely consumed and an acid of SM 2 was observed as a major side product.
Initial Screening results

In-process monitoring of the reaction completion was challenging due to the lack of chromophore and highly polar nature of SM1. A generic HPLC method was assessed initially with a combination of Evaporative Light Scattering Detector (ELSD) and UV detection since SM1 could not be detected by UV while SM 2 was not visible by ELSD.

Chromatogram of Markers Overlay

Hypothesis: the apparent “reaction stalling” effect is due to an unknown impurity co-eluting with the SM1.
Peak Purity Check with ACD/AutoChrom Software

ACD/AutoChrom General Method Development cycle

1. Acquire data
2. Track peaks between injections based on MS data or UV spectra similarity
3. Generate peak table, identify peak co-elution, suggests best separation based on the resolution score
4. Acquire data to model separation
5. Suggest Next Experiments

Initial Screening Results

Gradient used

Results of LC/MS column screening

Zorbax Eclipse Plus column was assigned the best overall resolution score by the software.

Peak purity was checked based on the results of LC/MS column screening.
While the majority of impurities are baseline resolved, 2 unknowns were identified under the SM1 peak with the difference in [M+H]/retention time 14 Daltons/0.14 min (major peak) and 29 Daltons/0.9 min respectively, indicating peak co-elution in all the columns tested. Composite chromatogram simulated by the software (on the right) shows 3 components identified under the peak at 5.2 min detected by MSD (on the lower left).

Data were also intended to be used for the release method development, so “the worst case scenario” reaction mixture with multiple impurities was used for the screening of 10 columns under acidic conditions.
Further software-assisted modeling/optimization resulted in partial separation of piperidines on ACE PFP column:
Ratio of piperidines in the marker mixture: ~ 20/40/40
Ratio of piperidines in the reaction mixture: ~ 7/83/10

The retention times of the peaks in the marker mixture match with those of the peaks identified in the reaction mixture. NMR analysis also confirmed the proposed structures. A reliable IPC method was needed with baseline resolution of all starting material-related species and the product.
Further Method Development: Mixed Mode Chromatography

Mixed-mode chromatography adds another dimension to the separation of polar ionizable analytes allowing resolution of compounds based on their differences in both hydrophobicity and pKa/pKb.

All compounds of interest were baseline resolved under isocratic conditions on a Primesep A column, enabling further process optimization. The reaction yield was vastly improved by altering the original starting material SM 2 to an ethyl ester, thus eliminating the undesired alkylation. The initial 80% yield was improved to 94% with a mechanistic understanding of the reaction enabled by the analytical investigation.
Summary

- Peaks that overlap with the starting material can lead to false results during reactions monitoring, which can hinder process optimization. Software assisted peak co-elution check that includes automated processing of LC/MS data can significantly expedite process development and provide knowledge of generated impurities, as described in this work.

- Findings obtained during AutoChrom-assisted method development allowed to increase the yield and provided better understanding of the reaction mechanism.

- Isocratic HPLC method that employed mixed mode chromatography and CAD was developed and qualified enabling successful process development and DOE study followed by the transfer to an outside vendor.
Conclusions

Using AutoChrom software in tandem with Agilent LC/MS:

1. Significantly simplifies Method Screening - runs column, buffer, and solvent screening experiments, automatically finds and tracks all peaks in your samples, and selects the best result.

2. Assists in Method Optimization - guides through data processing and method optimization, helps to avoid the experiments that are unlikely to work, and develops high quality separation methods.

3. Tracks peaks from run-to-run based on UV or MS spectral similarity, finds all trace components to avoid missed peaks.

4. Build a model based on peak tracking. Offer a choice of mathematical equations for more accurate model.

5. Manages projects – allows to see a clear overview of your experiments, and dig down into the original data when necessary. Experiments are summarized in a peak table as you work.

6. Provides knowledge about process impurities.

7. Assists in peak co-elution check.
Scheme of Automated Method Development for Difficult Separations

Start

Prepare Analyte Mixture → Ran buffer screening using generic column/gradient → Process MSD/DAD Signals → Evaluate against design criteria, select experiment with best Rs

Process signals for promising data select best combinations for optimization → Screen 11 columns/1 or 2 MP using best pH conditions

Separation Achieved

Yes → Create Report → End

No → Run 2 additional gradients requested by the software for optimization → Process MSD/DAD signals, verify elution model, optimize conditions against design criteria