Care, Maintenance, and Troubleshooting of HPLC Columns

Columns and Consumables

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Applications Engineer
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Goals for this presentation:

1. Introduce the most commonly observed column related problems in HPLC.
2. Explore the reasons for these column problems.
3. Propose preventative maintenance and method development/optimization approaches to minimize HPLC column problems and increase column lifetimes.
Troubleshooting in HPLC
Major Areas of Column Problems - Dramatic Changes in 3 Key Areas:

1. HPLC System Pressure
2. Chromatogram - Peak Shape
3. Chromatogram - Peak Retention/Selectivity
## 1. Pressure Issues

<table>
<thead>
<tr>
<th>Column Observations</th>
<th>Potential Problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large pressure change</td>
<td>Plugged inlet frit</td>
</tr>
<tr>
<td></td>
<td>Column contamination</td>
</tr>
<tr>
<td></td>
<td>Plugged packing</td>
</tr>
</tbody>
</table>
Determining the Cause and Correcting High Back Pressure

• Check pressure with/without column - many pressure problems are due to blockages elsewhere in the system.

If Column pressure remains high:

• Rinse column *(remove detector from flow path!)*
  – Eliminate column contamination and plugged packing
  – high molecular weight/adsorbed compounds
  – precipitate from sample or buffer

• Back flush column – may clear plugged column inlet frit

• Change column inlet frit (… or discard column)

Eliminate pressure issues – add a disposable 0.5 or 2 um in-line filter to system.
Pressure Problem I

Pressure Too High

- Column inlet frit contaminated
- Frit in purge valve contaminated
- Column contaminated
- Blockage in a capillary, particularly needle seat capillary
- Rotor in injection valve plugged
- Injection needle or needle seat plugged

Use this valve to divide the system

Pressure Measurement
Pressure Problem II

Pressure Too Low

- Solvent inlet frit plugged
- Leak in a capillary connection or other part (pump seals)
- Wrong solvent or flow rate
- AIV (Active inlet valve) defective
- Multichannel Gradient valve incorrectly proportioning
- Ball valve defective
- Column defective (stationary phase)
1100 and 1200 Pumps
Exploded View

- Piston Support Rings
- Plunger Housing
- Pistons
- Seals
- Holding Screw
- Outlet Valve
- Purge Valve
- Pump Housing
- Active Inlet Valve

Agilent Technologies
Pump Check Valves

Active Inlet Valve (common to all)

New style
G1312-60010

Cartridge
5062-8562

Old style
5062-8568

Outlet Ball Valve
Iso/Quat Pump G1311-60012
Binary Pump G1312-60012

1. Gold Washer  5001-3707
2. Plastic cap  01018-21207
3. Gold Seal  5001-3707
4. Cap(4pk)  5062-2485

Purge Valve
G1312-60009

5. Gold Seal  5001-3707
6. Cap(4pk)  5062-2485
7. PTFE (5pk)  01018-22707
Column Cleaning

Flush with stronger solvents than your mobile phase. Make sure detector is taken out of flow path.

Reversed-Phase Solvent Choices in Order of Increasing Strength

Use at least 10 x $V_m$ of each solvent for analytical columns

1. Mobile phase without buffer salts (water/organic)
2. 100% Organic (MeOH or ACN)
3. Is pressure back in normal range?
4. If not, discard column or consider more drastic conditions:
   75% Acetonitrile:25% Isopropanol, then
5. 100% Isopropanol
6. 100% Methylene Chloride*
7. 100% Hexane*

When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.
Column Cleaning

Normal Phase Solvent Choices
in Order of Increasing Strength

- Use at least 50 mL of each solvent
- 50% Methanol : 50% Chloroform
- 100% Ethyl Acetate
Preventing Back Pressure Problems: In-Line Devices

Filter and Guard Column Act on Sample
Pre-Column Acts on Mobile Phase

To Detector
Preventing Column Back Pressure Problems

1. Filter mobile phase:
   - filter non-HPLC grade solvents
   - filter buffer solutions
   - Install an in-line filter between auto-sampler and column (removes pump seal debris, ALS rotor debris, and sample particulates). Use 2 um frit for 3.5 um columns, use 0.5 um frit for 1.8 um columns.

2. Filter all samples and standards

3. Perform sample clean-up (i.e. SPE, LLE) on dirty samples.

4. Appropriate column flushing – flush buffers from entire system at end of day with water/organic mobile phase.
2. Peak Shape Issues in HPLC

- Split peaks
- Peak tailing
- Broad peaks
- Poor efficiency (low N)
- Inconsistent response

- Many peak shape issues are also combinations - i.e. broad and tailing or tailing with increased retention
Split Peaks

Can be caused by:

- Column contamination
- Partially plugged frit
- Column void (gap in packing bed)
- Injection solvent effects
Split Peaks
Column Contamination

Column: StableBond SB-C8, 4.6 x 150 mm, 5 μm
Mobile Phase: 60% 25 mM Na₂HPO₄, pH 3.0 : 40% MeOH
Flow Rate: 1.0 mL/min
Temperature: 35°C
Detection: UV 254 nm

Injection 1
Injection 30
Injection 1 After Column Wash with 100% ACN

- Column washing eliminates the peak splitting, which resulted from a contaminant on the column.
Split Peaks

Injection Solvent Effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 μm  Mobile Phase: 82% H₂O : 18% ACN
Injection Volume: 30 μL  Sample: 1. Caffeine  2. Salicylamide

A. Injection Solvent
100% Acetonitrile

B. Injection Solvent
Mobile Phase

- Injecting in a solvent stronger than the mobile phase can cause peak shape problems, such as peak splitting or broadening.
- Note: earlier peaks (low k) most affected
Peak Shape Problems - Doublets

- Void Volume in Column
- Partially Blocked Frit
- Only One-Peak a Doublet- Coeluting Components
- Early (low k) peaks most affected
Determining the Cause of Split Peaks

1. Complex sample matrix or many samples analyzed - likely column contamination or partially plugged column frit.

2. Mobile phase pH > 7 - likely column void due to silica dissolution (unless specialty column used, Zorbax Extend-C18 stable to pH 11)

3. Injection solvent stronger than mobile phase - likely split and broad peaks, shape dependent on injection volume and k value.
Peak Tailing, Broadening and Loss of Efficiency (N, plates)

May be caused by:

1. Column “secondary interactions”
2. Column packing voids
3. Column contamination
4. Column aging
5. Column loading
6. Extra-column effects
Peak Tailing

Column “Secondary Interactions”

Column: Alkyl-C8, 4.6 x 150 mm, 5μm  
Mobile Phase: 85% 25 mM Na₂HPO₄ pH 7.0 : 15% ACN  
Flow Rate: 1.0 mL/min  
Temperature: 35°C  

- Peak tailing of amine analytes eliminated with mobile phase modifier (TEA, triethylamine) at pH 7
Peak Tailing
Column “Secondary Interactions”

Column: Alkyl-C8, 4.6 x 150 mm, 5 μm  Mobile Phase: 85% 25 mM Na₂HPO₄ : 15% ACN  Flow Rate: 1.0 mL/min  Temperature: 35°C  Sample: 1. Phenylpropanolamine  2. Ephedrine  3. Amphetamine  4. Methamphetamine  5. Phenteramine

- Reducing the mobile phase pH reduces interactions with silanols that cause peak tailing. No TEA modifier required.
Peak Tailing
Column Contamination

Column: StableBond SB-C8, 4.6 x 250 mm, 5μm
Mobile Phase: 20% H₂O : 80% MeOH
Flow Rate: 1.0 mL/min
Temperature: R.T.
Detection: UV 254 nm
Sample: 1. Uracil  2. Phenol  3. 4-Chloronitrobenzene  4. Toluene

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<tr>
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<th>TF</th>
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<td>2.</td>
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<td>3.</td>
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<td>4.</td>
<td>13355</td>
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QC test forward direction

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QC test reverse direction

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<td>4.</td>
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QC test after cleaning 100% IPA, 35°C
Analytical vs. Preparative Scale HPLC. Non-linear Adsorption Isotherms, or Overload Conditions:
Peak Tailing/Broadening
Sample Load Effects

Columns: 4.6 x 150 mm, 5μm
Mobile Phase: 40% 25 mM Na₂HPO₄ pH 7.0 : 60% ACN
Flow Rate: 1.5 mL/min
Temperature: 40°C

Tailing
Eclipse XDB-C8
USP TF (5%)

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<tr>
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<th>A</th>
<th>B</th>
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<tr>
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<tr>
<td>6</td>
<td>1.25</td>
<td>1.25</td>
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High Load x10

B. Low Load

C. Broadening
Competitive C8 Plates

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<th>D</th>
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<tr>
<td>6</td>
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</table>
Peak Broadening, Splitting
Column Void

- Multiple peak shape changes can be caused by the same column problem. In this case a void resulted from silica dissolved at high pH.

Mobile Phase: 50% ACN: 50% Water : 0.2% TEA (~ pH 11)

Initial

After 30 injections
Peak Tailing
Injector Seal Failure

Column: Bonus-RP, 4.6 x 75 mm, 3.5 μm  
Mobile Phase: 30% H₂O : 70% MeOH  
Flow Rate: 1.0 mL/min  
Temperature: R.T.  
Detection: UV 254 nm  

Before

<table>
<thead>
<tr>
<th>Plates</th>
<th>USP TF (5%)</th>
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<td>1. 2235</td>
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<tr>
<td>2. 3491</td>
<td>1.48</td>
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<tr>
<td>3. 5432</td>
<td>1.15</td>
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</tbody>
</table>

After replacing rotor seal and isolation seal

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<th>USP TF (5%)</th>
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<tr>
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<td>2. 10457</td>
<td>1.09</td>
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<tr>
<td>3. 10085</td>
<td>1.00</td>
</tr>
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</table>

• Overdue instrument maintenance can sometimes cause peak shape problems.
Peak Tailing
Extra-Column Volume

Column: StableBond SB-C18, 4.6 x 30 mm, 3.5 μm
Mobile Phase: 85% H₂O with 0.1% TFA : 15% ACN
Flow Rate: 1.0 mL/min
Temperature: 35°C
Sample: 1. Phenylalanine  2. 5-benzyl-3,6-dioxo-2-piperazine acetic acid  3. Asp-phe  4. Aspartame

10 μL extra-column volume

50 μL extra-column volume (tubing)
Determining the Cause of Peak Tailing

- Evaluate mobile phase effects - alter mobile phase pH and additives to eliminate secondary interactions
- Evaluate column choice - try column with high purity silica or different bonding technology
- Reduce sample load – volume injection and concentration
- Eliminate extra-column effects – tubing, fittings, Uv cell
- Flush column and check for aging/void
Reproducibility

Typically,

• Area and Peak Height problems together point to the autosampler system

• Area and Retention Time problems together point to the pump

Peak retention time precision:
⇒ with oven: _________ < 0.3%
⇒ without oven: ________ < 0.7%
Peak area precision:  ≤ 1.5%
Problems with Reproducibility – Peak Areas

Peak Areas not Reproducible

With peak height
- Rotor seal cross-port leak or injection valve not tight
- Piston seal of metering unit leaking
- Needle partially blocked

With retention time
- Variable pump flow rate

Other
- Capillary from injector to detector not tight
- Detector equilibration problems
3. Retention Issues

- Retention time changes ($t_r$)
- Retention factor changes ($k'$)
- Selectivity changes ($a$)
Retention time $t_R$, Retention factor $k'$, and Selectivity factor $\alpha$

The Chromatogram

$t_0$ - elution time of unretained peak
$t_R$ - retention time - determines sample identity

Retention factor $k' = (t_R - t_0)/t_0$

Selectivity factor $\alpha = k_2/k_1$
Changes in Retention (k) - Same Column, Over Time

May be caused by:

1. Column aging
2. Column contamination
3. Insufficient column equilibration
4. Poor column/mobile phase combination
5. Change in mobile phase
6. Change in flow rate
7. Change in column temperature
8. Other instrument issues
Mobile Phase Change Causes Change in Retention

- Volatile TFA evaporated/degassed from mobile phase. Replacing it solved problem.
- Chromatography is from a protein binding study and peak shape as expected.
Separation Conditions That Cause Changes in Retention*

Flow Rate  ± 1%  ± 1% $t_r$
Temp  ± 1° C  ± 1 to 2% $t_r$
%Organic  ± 1%  ± 5 to 10% $t_r$
pH  ± 0.01%  ± 0 to 1% $t_r$

Determining the Cause of Retention Changes

Same Column

1. Determine $k'$, $a$, and $t_r$ for suspect peaks
2. Wash column
3. Test new column - note lot number
4. Review column equilibration procedures
5. Make up fresh mobile phase and test
6. Check instrument performance
Change in Retention/Selectivity
Column-to-Column

1. Different column histories (aging)
2. Insufficient/inconsistent equilibration
3. Poor column/mobile phase combination
4. Change in mobile phase
5. Change in flow rate
6. Other instrument issues
7. Slight changes in column bed volume ($t_r$ only)
Example Change in Retention/Selectivity

Column-to-Column

Mobile Phase Variation

“I have experimented with our mobile phase, opening new bottles of all mobile phase components. When I use all fresh ingredients, the problem ceases to exist, and I have narrowed the problem to either a bad bottle of TEA or phosphoric acid. Our problem has been solved.”
Minimize Change in Retention/Selectivity
Lot-to-Lot

Evaluate:

1. All causes of column-to-column change*
2. Method ruggedness (buffers/ionic strength)
3. pH sensitivity (sample/column interactions)

*All causes of column-to-column change should be considered first, especially when only one column from a lot has been tested.
Lot-to-Lot Selectivity Change - pH

- pH 4.5 shows selectivity change from lot-to-lot for basic compounds
- pH 3.0 shows no selectivity change from lot-to-lot, indicating silanol sensitivity at pH 4.5
- Evaluate several pH levels to establish most robust choice of pH
Problems with Reproducibility – Peak Areas

Peak Areas not Reproducible

With peak height
- Rotor seal cross-port leak or injection valve not tight
- Piston seal of metering unit leaking
- Needle partially blocked

With retention time
- Variable pump flow rate

Other
- Capillary from injector to detector not tight
- Detector equilibration problems
Problems with Reproducibility – Retention Time

Retention Times not Reproducible

• Pump Problems
  – Mobile phase composition problems
  – Valves AIV, ball valve defective
  – Flow rate problems

• Column Oven Problems
  – Temperature fluctuations

• Other
  – Column equilibration
  – Column deterioration
**Autosampler**

**Principle of Operation**

- **Standard loop volume**: 300µl
- **Total delay volume**: 300µl + Vinj
- **Minimal (bypass) delay volume**: 6.2µl

**Diagram**

- **Vial gripper**
- **Sampling unit**
- **Metering device**
- **Rheodyne 7750**
- **4-port rotor seal**
- **To waste**
- **From pump**
- **To column**

**Widest dynamic injection range:**
0.1 µl-1.5 ml (w/add'l hardware)
Evaluate Retention Changes
Lot-to-Lot

1. Eliminate causes of column-to-column selectivity change
2. Re-evaluate method ruggedness - modify method
3. Determine pH sensitivity - modify method
4. Classify selectivity changes
5. Contact manufacturer for assistance*

Agilent Column Support: 800-227-9770, option 4, option 2 (LC columns)
Conclusions

HPLC column problems are evident as:

1. High pressure
2. Undesirable peak shape
3. Changes in retention/selectivity

These problems are not always associated with the column and may be caused by instrument and experimental condition issues.
The End – Thank You!

Agilent LC Column Tech Support: 800-227-9770 #4, #2  Email: Edward_kim@agilent.com
Agilent LC Columns and Agilent J&W GC Columns Scientific Technical Support

800-227-9770 (phone: US & Canada)*

302-993-5304 (phone)
For LC columns

Select option 4, then option 2
For GC Columns

* Select option 4, then option 1.

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January 22, 2008 – 1:00 pm EST

A Look at Column Choices- Series 3
February 13, 2008 – 1:00 pm EST

Method Development – Series 4
March 18, 2008 – 2:00 pm EST