Tips and Tricks of HPLC System Troubleshooting

Agilent Technologies, Inc.
LC Tips And Tricks Seminar Series

Trouble Shooting Steps

You Have Recognized There is a Problem!

How Do You Fix It?

• 1st Did System Suitability or Sample Fail?
• 2nd Review Method for Compliance
  – Is The Procedure Being Followed Properly?
  – Are Instrument Settings Correct?
• 3rd Ask More Questions!
  – When Did the System Last Function Properly?
  – Has Anything Been Changed?
• 4th Review ALL parameters!
  – The Obvious Is Not Always the Cause
  – Was There More Than One Change?
HPLC System Components

Pump
Injector/Autosampler
Column
Detector
Data System/Integrator

Problems Can Be Related to All Components in the System

Categories of Column and System Problems

A. Pressure
B. Peak shape
C. Retention
Pressure Issues

<table>
<thead>
<tr>
<th>Column Observations</th>
<th>Potential Problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>High pressure</td>
<td>· Plugged frit</td>
</tr>
<tr>
<td></td>
<td>· Column contamination</td>
</tr>
<tr>
<td></td>
<td>· Plugged packing</td>
</tr>
<tr>
<td>Low Pressure</td>
<td>· Leak</td>
</tr>
<tr>
<td></td>
<td>· Flow Incorrect</td>
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</tbody>
</table>

Determining the Cause and Correcting High Back Pressure

- Check pressure with/without column - many pressure problems are due to blockages in the system or guard col.
  - Remove Column - Pressure Still High?
  - Remove Guard – Pressure Still High?

- **If Column pressure is high:**
  - Back flush column – Clear “dirty” frit surface
  - Wash column – Eliminate column contamination and plugged packing
    - high molecular weight/adsorbed compounds
    - precipitate from sample or buffer
  - Change frit – Clear plugged frit **PREVENT THIS!**
Column Cleaning

*Flush with stronger solvents than your mobile phase.*

Reversed-Phase Solvent Choices
in Order of Increasing Strength

Use at least 25 mL of each solvent for analytical columns

- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile
- 75% Acetonitrile:25% Isopropanol
- 100% Isopropanol
- 100% Methylene Chloride*
- 100% Hexane*

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

Must Reverse to Re-Equilbrate

Changing a Frit May Not Be a Good Idea

May not be possible with new generation columns
May damage high performance columns

Tip: Prevention is a Much Better Idea!
The Trick: Prevention Techniques - A Better Choice!

- Use column protection
  - In-line filters
  - Guard columns
- Filter samples
- Filter buffered mobile phases

Easy

• Sample clean-up (i.e. SPE)
• Appropriate column flushing

Not As Easy

Inexpensive Filters Prevent Column Frit Plugging

Regenerated Cellulose (RC) Recommended
• Universal hydrophilic membrane, compatible with most solvents - aqueous and organic
• High purity, extremely low extractables and binding
• More Uniform Surface
• Different than Other Cellulose Filters!!

In-line Filters Easy to Use and replace
Frits Available in 0.2, 0.5 and 2.0µ Porosity
Much Less expensive than a Column
Easier and Faster to Replace than a Column Frit
What Are Common Peak Shape Issues?

1. Split peaks
2. Peak tailing
3. Broad peaks

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

• Symptoms do not necessarily affect all peaks in the chromatogram

• Each of these problems can have multiple causes

Peak Splitting Caused By Disrupted Sample Path

• Flow Path Disrupted by Void
• Sample Allowed to Follow Different Paths Through Column
• Poorly Packed Bed Settles in Use
• High pH Dissolves Silica

Normal Peaks — Split or Double Peaks

Tip: Similar Effect Can be Caused by Partially Plugged Frit
### Split Peaks from 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  
**Sample:** Filtered OTC Cold Medication: 1. Pseudoephedrine  2. APAP  3. Unknown  4. Chlorpheniramine  

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

**Tip:** Column washing eliminates the peak splitting, which resulted from a contaminant on the column. How could this be prevented? (Guard Column, SPE clean up of samples, Periodic column wash)

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### Split Peaks from 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**

**Tip:** Injecting in a solvent stronger than the mobile phase can cause peak shape problems such as peak splitting or broadening.  
**Trick:** Keep Organic Concentration in Sample Solvent < Mobile Phase
Peak Tailing, Broadening and Loss of Efficiency

May be caused by:

- Column “secondary interactions”
- Column contamination
- Column aging
- Column loading
- Extra-column effects

Peak Shape: Tailing Peaks

Causes

Some Peaks Tail:
- Secondary - Retention Effects.
- Residual Silanol Interactions.
- Small Peak Eluting on Tail of Larger Peak.

All Peaks Tail:
- Extra-Column Effects.
- Build up of Contamination on Column Inlet.
- Heavy Metals.
- Bad Column.
Peak Tailing
Identifying Column “Secondary Interactions”

Tip: Mobile phase modifier (TEA) competes with Sample for surface ion exchange sites at mid-range pH values.

Peak Tailing
Low pH Minimizes “Secondary Interactions” for Amines

Tip: Reducing mobile phase pH reduces interactions with silanols and peak tailing.
Peak Tailing
High pH Eliminates “Secondary Interactions” for Amines

Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 μm
Mobile Phase: See Below
Flow Rate: 1.0 mL/min
Temperature: RT
Detection: UV 254 nm

Sample:
1. Maleate
2. Scopolamine
3. Pseudoephedrine
4. Doxylamine
5. Chlorpheniramine
6. Triprolidine
7. Diphenhydramine

pH 7
30% 20 mM Na₂HPO₄
70% MeOH

pH 11
30% 20 mM TEA
70% MeOH

$t_R = 8.5$
$t_R = 11.4$

Peak Shape and Retention of this sample of basic compounds improves at high pH where column has high IEX activity. Why?

Peak Tailing - Column Contamination

Tip: Quick Test to Determine if Column is Dirty or Damaged

Trick: Reverse Column and Run Sample – If Improved, Possible Cleaning Will Help - No improvement - Column Damaged and Needs to be Replaced
Peak Shape: Fronting Peaks

- Columns Overload
- Normal Fronting
- Symmetry < 0.9

Causes:
- Column Overload

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%) i

A. High Load x10
B. Low Load

Broadening
Competitive C8 Plates

C. D
1. 850  5841
2. 815  7842
3. 2776  6231
4. 2539  8359
5. 2735  10922
6. 5189  10725

Tip: Evaluate Both Volume and Mass Loading
**Peak Shape: Broad Peaks**

All Peaks Broadened:
- Loss of Column Efficiency.
- Column Void.
- Large Injection Volume.

Some Peaks Broadened:
- Late Elution from Previous Sample (Ghost Peak).
  - High Molecular Weight.
  - Sample - Protein or Polymer.

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**Unknown “Phantom” Peaks**

Column: Extend-C18, 4.6 x 150 mm, 5 μm  
Mobile Phase: 40% 10 mM TEA, pH 11 : 60% MeOH  
Flow Rate: 1.0 mL/min  
Temperature: R.T.  
Detection: UV 254  

Tip: The extremely low plates for moderately retained peaks are an indication of a very late eluting peak from a preceding run.
Extra-Column Dispersion

- Use short, small internal diameter tubing between the injector and the column and between the column and the detector.
- Make certain all tubing connections are made with matched fittings.
- Use a low-volume detector cell.
- Inject small sample volumes.

Peak Broadening
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 mL extra-column volume

50 mL extra-column volume (tubing)
Tip: Poorly Made HPLC System Connections Can Cause Peak Broadening

The System Has Been Optimized and:

- All Tubing Lengths Are Minimum
- Smallest Diameter Tubing Used
- Proper Flow Cell Volume

Symptom Still Seems to Have Too Much Extra-Column Volume

What Is Wrong?

Have You Made the Connections Properly?

Column Connectors Used in HPLC


Swagelok

Waters

Parker

Rheodyne

Valco

Uptight

0.090 in.

0.130 in.

0.090 in.

0.170 in.

0.080 in.

0.090 in.
What Happens If the Connections Poorly Made?

Wrong ... too long

Ferrule cannot seat properly

Wrong ... too short

If Dimension X is too long, leaks will occur

If Dimension X is too short, a dead-volume, or mixing chamber, will occur

Stainless Steel and Polymer Fittings

Which type is used and when?

Stainless Steel (SS) fittings are the best choice for reliable high pressure sealing

- Agilent uses Swagelok type fittings with front and back ferrules – which give best sealing performance – throughout all our LC systems

PEEK (<400b bar System Pressure) fittings are ideal where:

- Connections are changed frequently, i.e. connecting columns
- Pressure is less critical

PolyKetone

- Easy, hand tighten column connection
- **600 bar Pressure Rating** PN: 5042-8957 (10/pk)
- Fits to SS Tubing
Changes in Retention Can Be Chemical or Physical

May be caused by:

- Column aging
- Column contamination
- Insufficient equilibration
- Poor column/mobile phase combination
- Change in mobile phase
- Change in flow rate
- Different Gradient Delay Volumes

Column Aging/Equilibration Causes Retention/Selectivity Changes

- The primary analyte was sensitive to mobile phase aging/conditioning of the column
- The peak shape was a secondary issue (metal chelating compound) resolved by "de-activating" the active metal contamination
Metal Sensitive Compounds Can Chelate

Hint: Look for Lone Pair of Electrons on :O: or N Which Can Form 5 or 6 Membered Ring with Metal

![Chemical structures](image)

- Salicylaldehyde
- 6-membered ring complex
- 8-hydroxyquinoline
- 5-membered ring complex
- a-benzoinoxomine
- 5-membered ring complex

Acid Wash Can Improve Peak Shape

Before Acid Wash

After Acid Wash

- Columns: ZORBAX SB-Phenyl
  - 4.6 x 150 mm
- Mobile Phase: 75% ammonium phosphate buffer 25% ACN
- Flow Rate: 1.0 mL/min.
- Temperature: RT
- Sample Size: 5 mL

<table>
<thead>
<tr>
<th>Before Acid Wash</th>
<th>After Acid Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 – 100 mLs 1% H₃PO₄</td>
<td>50 – 100 mLs 1% H₃PO₄</td>
</tr>
<tr>
<td>Tf: 3.7</td>
<td>Tf: 1.2</td>
</tr>
</tbody>
</table>

- A 1% H₃PO₄ solution is used on SB columns, 0.5% can be used on endcapped columns.
Example: Change in Retention/Selectivity

Unintended Mobile Phase Variation

Tip: The Source of the Problem is Often Not the Obvious Change

“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.”

Tip: Dwell Volume Differences Between Instruments Can Cause Changes in Retention and Resolution

- Column: ZORBAX Rapid Resolution Eclipse XDB-C8
  - 4.6 x 75 mm, 3.5 µm
- Mobile Phase: Gradient, 0 - 100 %B in 52.5 min.
  - A: 5/95 methanol/25 mM phosphate pH 2.50
  - B: 80/20 methanol/25 mM phosphate pH 2.50
- Flow Rate: 0.5 mL/min
- Temperature: 25°C
- Injection: 5 µL
- Detection: 250 nm
- Sample: Mixture of antibiotics and antidepressants
  - Upper trace simulates actual run data entered into DryLab® 3.0 software
  - Lower trace is simulated chromatogram for larger VD
Trick: Measure and Correct for Dwell Volume ($V_D$)

If $V_{D1} > V_{D2}$
Compensate for longer $V_{D1}$ by adding an isocratic hold to $V_{D2}$, such that
Hold + $V_{D2} = V_{D1}$

If $V_{D1} < V_{D2}$
Delay injection, such that $V_{D2} - \text{delay} = V_{D1}$

Mobile Phase pH and pH Buffers
Why Are These So Important in HPLC?

• pH Effects Ionization
  – Silica Surface of Column
  – Sample Components of Interest

• Buffers
  – Resist Changes in pH and Maintain Retention
  – Improve Peak Shape for Ionizable Compounds

• Effects Column Life
  – Low pH strips Bonded Phase
  – High pH Dissolves Silica
Minimize Change in Retention/Selectivity
Lot-to-Lot

Evaluate:

• All causes of column-to-column change*
• Method ruggedness (buffers/ionic strength)
• 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 Related to pH Choice

• pH 4.5 shows selectivity change from lot-to-lot for basic compounds
• pH 3.0 shows no selectivity change from lot-to-lot
• Indication of poorly controlled ionization
Why Worry About pH?

**pH, pKa and Weak Acids**

\[
\text{RCOOH} \rightleftharpoons \text{RCOO}^- + H^+ \quad K_a = \frac{[\text{RCOO}^-][H^+]}{[\text{RCOOH}]} \\
K_a = 6.4 \times 10^{-5} \\
pK_a = 4.2
\]

At pH 4.2 – the sample exists as benzoic acid and the benzoate ion in a ratio of 1:1. Peak shape can be poor.

At pH 5.2 – 91% of the sample exists as the benzoate ion. RP retention decreases.

At pH 3.2 – 91% of the sample exists as benzoic acid. RP retention increases.

Effect of pH on Peak Shape at or Near the Sample pK_a

- Column: ZORBAX SB-C8 4.6 x 150 mm, 5 mm
- Mobile Phase: 40% 5 mM KH_2PO_4: 60% ACN
- Flow Rate: 1.0 mL/min.
- Temperature: RT

**Ibuprofen**

- pK_a = 4.4

Inconsistent and tailing peaks may occur when operating close to an analyte pKa and should be avoided.
Why Worry About pH? pH, pKa and Weak Bases

\[ R_3\text{NH}^+ \rightleftharpoons R_3\text{N} + H^+ \]

\[ K_a = \frac{[R_3\text{N}][H^+]}{[R_3\text{NH}^+]} \]

\[ K_a = 1 \times 10^{-9} \]

\[ pK_a = 9 \]

At pH 9 – the sample exists as protonated and unprotonated diphenhydramine in a ratio of 1:1. Peak shape can be poor.
At pH 10 – 91% of the sample exists as unprotonated diphenhydramine.
At pH 8 – 91% of the sample exists as protonated diphenhydramine.

pH vs. Selectivity for Acids and Bases

- Retention and selectivity can change dramatically when pH is changed.
Importance of pH and Buffers
A Practical Example

• Why the Sample Dictates Use
• What Happens When Buffer Used Effectively
• What Happens When Buffer Ignored or Used Improperly

Optimized Isocratic Conditions for Cardiac Drugs

Column: StableBond SB-C18, 4.6 x 150 mm, 5 mm
Mobile Phase: 45% 25 mM NaH₂PO₄, pH 3.0, 55% MeOH
Flow Rate: 2.0 mL/min.
Temperature: 35°C
Detection: UV 254 nm
Sample: Cardiac Drugs
1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. Lidoflazine
5. Flunarizine
I Don’t Have Time to Make Buffers or Adjust pH …

Column: StableBond SB-C18
4.6 x 150 mm, 5 mm
Mobile Phase: A: 20% H2O
B: 80% MeOH
Flow Rate: 1.0 mL/min.
Temperature: 35°C
UV Detection: 254 nm
Sample: Cardiac Drugs

Even at very high % MeOH Most Components Strongly Retained with Poor peak Shape Due to IEX at Surface

* Buffers are critical to good retention and peak shape in many separations.

What If You Work Outside the Buffer Range?

Columns: StableBond SB-C18
4.6 x 150 mm, 5 mm
Mobile Phase: A: 30% 25 mM NaH2PO4, pH 4.8 unbuffered
4.8 unbuffered
B: 70% MeOH
Flow Rate: 1.0 mL/min.
Temperature: 35°C
UV Detection: 254 nm
Sample: Cardiac Drugs
1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. Lidoflazine
5. Flunarizine

Unsuitable Peak Shape
Don’t Forget - Match Column to pH of Mobile Phase for Maximum Column Lifetime

low pH and high temperature (pH 0.8, 90°C)

Purge Solvent: 50% methanol/water with 1.0% TFA
Solute: Toluene


Don’t Forget - Match Column to pH of Mobile Phase for Maximum Column Lifetime

High pH and Room Temperature (pH 11 RT)

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

Initial

After 30 injections

Tip: Use Columns Designed for chosen pH
Detection Issues

Recognize Where the Problem Originates
• Is it a consequence of technique?
• Is it expected due to use of certain mobile phase components?
• Can it be corrected by adjusting detector parameters?
• Answers Will Help Find a Solution!

Let’s Explore Some Problems and Solutions

Causes:
- Absorbance of sample is less than the mobile phase.
- Equilibrium disturbance when sample solvent passes through the column.
- Normal with Refractive Index Detectors.

Peak Shape: Negative Peaks

Causes:
- Absorbance of sample is less than the mobile phase.
- Equilibrium disturbance when sample solvent passes through the column.
- Normal with Refractive Index Detectors.
Ghost Peaks

- Peaks which appear even when no sample is injected.
- Problem: Dirty Mobile Phase

Noisy Baselines

Possible Causes:
- Dirty Flow Cell
- Detector Lamp Failing
- Pulses from Pump if Periodic
- Temperature Effects on Detector
- Air Bubbles passing through Detector
Drifting Baselines

- Gradient Elution
- Temperature Unstable (Refractive Index Detector)
- Contamination in Mobile Phase
- Mobile Phase Not in Equilibrium with Column
- Contamination Bleed in System

Chromatographic Results with “Wrong” Lamp at 214 nm Wavelength

Tip: Could also be a symptom of aging lamp
**Expanded View of Chromatographic Results**

Generic Source Lamp at 214 nm Wavelength

<table>
<thead>
<tr>
<th>Peak</th>
<th>S/N</th>
</tr>
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<tbody>
<tr>
<td>Peak 1</td>
<td>150</td>
</tr>
<tr>
<td>Peak 2</td>
<td>400</td>
</tr>
<tr>
<td>Peak 3</td>
<td>300</td>
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</table>

<table>
<thead>
<tr>
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<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>15</td>
</tr>
<tr>
<td>Peak 2</td>
<td>50</td>
</tr>
<tr>
<td>Peak 3</td>
<td>50</td>
</tr>
</tbody>
</table>

Tip: Poor S/N makes it difficult to detect low level impurities

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**Effect of Detector Response Time**

The System is operating well—the settings were poorly made!

Slow Data Rates Can Hinder Impurity Detection and Reduce Sensitivity

<table>
<thead>
<tr>
<th>Response Time</th>
<th>0.1 sec</th>
<th>0.2 sec</th>
<th>0.5 sec</th>
<th>1.0 sec</th>
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<tbody>
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<td>1.2 sec</td>
<td>1.2 sec</td>
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<td>1.2 sec</td>
</tr>
<tr>
<td>1st peak</td>
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<td>1.2 sec</td>
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Tip: Adjust the response rate of your detector for best peak detection.
Conclusions

HPLC column problems are evident as
• High pressure (prevention better than the cure)
• Undesirable peak shape
• Changes in retention/selectivity

Often these problems are not associated with the column and may be caused by instrument and chemistry issues.
• pH of mobile Phase
• Instrument Connections
• Detector Settings
• Metal Contamination

Start With the Correct Questions
• Find the Answers
• The Answers will Lead to Solutions