HPLC

TROUBLE SHOOTING GUIDE

GL Sciences
Separation Sciences for All

High Performance Liquid Chromatography

HPLC Troubleshooting Guide

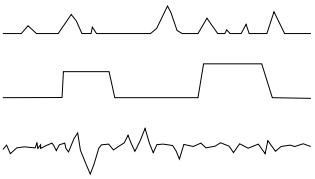
HPLC method development has benefited from substantial advancements in column technology and instrumentation, but problems during applications are inevitable. A systematic approach must be applied to effectively identify problems and troubleshoot issues with the HPLC system. This guide is organized into 13 major categories of troubleshooting to quickly identify the causes of potential problem(s).

Troubleshooting Menu

Periodic noise

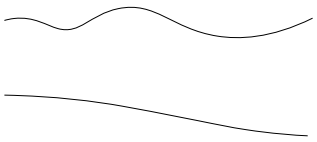
Main cause	Solution
Problems caused by the detector	
Weak detector lamp.	Check the lamp energy and the running time. Change the lamp if required.
Contamination in the detector cell.	Clean or replace the cell.
Air in the mobile phase.	Check the degassing unit.
Air in the detector cell.	Flush the system to remove excess air from the detector cell or pump. Backing pressure must be applied on the detector outlet line. (CAUTION: The flow cell may crack under excess pressure.)
Air in the pump.	Purge the pump or draw air out with a syringe.
Other electronic equipment on the same line.	Isolate the LC, detector, and recorder to determine if the source of the problem is external, and take necessary measures to resolve the problem.
Problems caused by the HPLC column	
Contamination in the HPLC column.	Clean the HPLC column.

Random noise



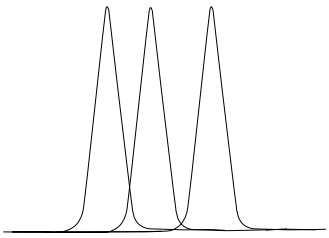
Main cause	Solution
Problems caused by the detector	
Weak detector lamp.	Check the lamp energy and running time. Change the lamp if necessary.
Contamination in the detector cell.	Clean the cell.
Air in the mobile phase.	Check the degassing unit.
Air in the detector cell.	Flush the system to remove any excess air from the detector cell or pump. Backing pressure must be applied on the detector outlet line. (CAUTION: The flow cell may crack under excess pressure.)
Air in the pump.	Purge the pump or remove the air using a syringe.
External electrical interference.	Isolate the LC, detector, and recorder to determine if the source of the problem is external. Correct the issue as necessary.
Problems caused by the pump	
Leak	Replace the plunger or the plunger seal.
One or more check valves have failed.	Clean the check valve, and replace it if it is defective.
Problems caused by the HPLC column	
Contamination in the column.	Clean the HPLC column.
Column leaking silica or packing material.	Replace the column, and clean the system.
Problems caused by the mobile phase	
Mobile phase is contaminated, deteriorated, or prepared from low-quality materials.	Check the composition of the mobile phase.
Wavelength is set at an absorbance higher than that of the mobile phase.	Lower the wavelength according to the absorbance of the mobile phase.

Drift is large



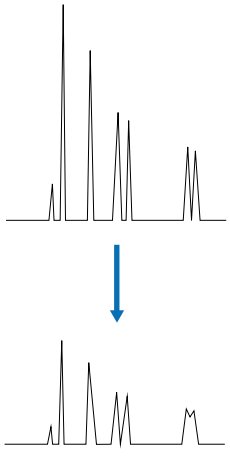
Main cause	Solution
Problems caused by the detector	
Weak detector lamp.	Check the lamp energy and running time. Change the lamp if necessary.
Contamination in the detector cell.	Clean the cell.
The warm-up period is very short.	Run the instrument for an hour after powering it on and let it warm up.
Wavelength is set at an absorbance higher than that of the mobile phase.	Lower the wavelength according to the absorbance of the mobile phase.
Instability in Detector Temperature.	Ensure that the ambient temperature where the detector is installed conforms to the its specifications. In particular, verify that the airflow from the air conditioning is not directly hitting the detector.
Problems caused by the HPLC column	
Contamination in the column.	Clean the HPLC column.
Column leaking silica or packing material.	Replace the column, and clean the system.
Fluctuating column temperature.	Control the temperatures of the column and the mobile phase.
Strongly retained materials (with high k') exist in the sample and may elute as very broad peaks, leading to a rising baseline (gradient analyses can aggravate the problem).	Use a guard column. If necessary, flush the column with a strong solvent between injections or flush periodically during analysis.
Problems caused by the mobile phase	
Mobile phase is contaminated, deteriorated, or prepared from low-quality materials.	Check the composition of the mobile phase.
Low-quality solvent, especially in the case of drift in the gradient system.	Ensure that the solvent used is of HPLC grade.
Problematic mixing of the mobile phase or change in flow rate.	Correct the composition or the flow rate. Adjust the mixing volumes.
Buffer concentration is too high.	Decrease the buffer concentration.

Retention time reproducibility is poor



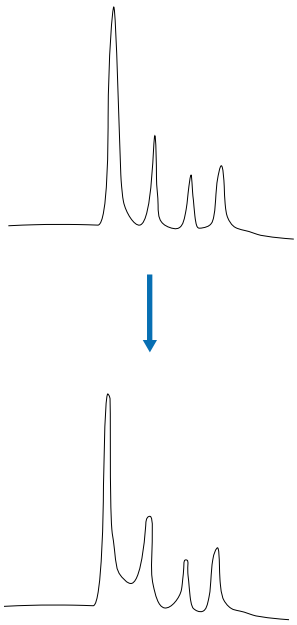
Main cause	Solution
Problems caused by the HPLC system	
Leak	Check the system for loose fittings. Check the pump for leaks, salt buildup, and unusual noises. Change pump seals if necessary.
Air trapped in the pump.	Purge the pump or draw air out with a syringe.
One or more check valves have failed.	Clean the check valves, and replace the defective ones.
Clogged solvent filter.	Clean the solvent filter or replace it if necessary.
Decreased flow rate.	Check and reset the pump flow rate; check the pump itself for defects.
Problems caused by the HPLC column	
Overloaded column.	Inject a smaller volume (e.g., 1 µL vs. 10 µL) of the sample or dilutions, such as 1:10 and 1:100.
The solvent in the sample is incompatible with the mobile phase.	Adjust the solvent. Whenever possible, inject the sample directly into the mobile phase.
Void at the column inlet.	Replace the column or open its inlet, and fill the void.
The guard column is contaminated/worn out.	Replace the guard column.
Column was contaminated.	Replace the column with a new one of the same type.
Problematic column.	Substitute a new column with another one of the same type to confirm that the original column is causing a problem.
Problems caused by the mobile phase	
Change in mobile phase composition. (small changes can lead to large changes in retention times.)	Check the composition of the mobile phase. If the mobile phase is machine-mixed using proportioning values, hand mix it, and supply it into the instrument from a single reservoir.
Increase or decrease the solvent ionic strength, pH, or additive concentrations (these parameters strongly affect the ionic solutes).	Measure the pH of the mobile phase and sample compounds. (pH is almost the same as pKa; pH range of column is not compatible with mobile phase, etc.)

Loss of resolution



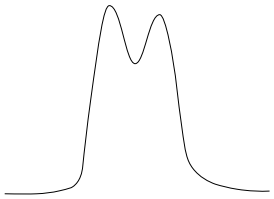
Main cause	Solution
Problems caused by the HPLC system	
The mixing volumes are insufficient.	Increase the mixing volumes (ex. 50 µL → 150 µL). Use a dynamic mixer.
Problems caused by the column	
Obstructed guard or analytical column.	Wash the guard and analytical columns. Replace the guard or analytical column with a new one of the same type, if necessary.
Problems caused by the mobile phase	
The mobile phase is contaminated/deteriorated (causing retention times and/or selectivity to change).	Prepare a fresh mobile phase.

Broad peak(s)



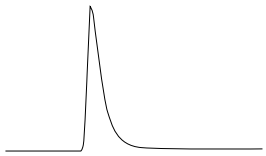
Main cause	Solution
Problems caused by the detector	
Detector settings are incorrect.	Adjust settings.
Detector response time is long or the cell volume is very large.	Reduce the response time or use a smaller cell.
The response time is very high.	Reduce the response time.
Problems caused by the HPLC column	
Column overloaded.	Inject a smaller volume (e.g., 1 µL vs. 10 µL) or dilutions of the sample, e.g., 1:10 and 1:100.
Tubing between the column and detector or the inner diameter (I.D.) of the tube is very large.	Narrow down and shorten the tubing to 0.007–0.010 mm I.D. to avoid extra column volume.
Void at the column inlet.	Replace the column or open the inlet end and fill the void.
The guard column is contaminated/worn out.	Replace the guard column.
The column is contaminated/ worn out.	Replace the column with a new one of the same type.
Column temperature is low.	Increase the temperature, but it should not exceed 80°C unless higher temperatures are indicated as acceptable by the column manufacturer.
Problems caused by the mobile phase	
Mobile phase composition has changed.	Prepare a new mobile phase.
The flow rate of the mobile phase is very low.	Adjust the flow rate.
Buffer concentration is too low.	Increase the concentration.
Increase or decrease the solvent ionic strength, pH, or additive concentration (these parameters strongly affect the ionic solutes).	Check the pH of the mobile phase and the pKa of the sample.
The solvent viscosity of the mobile phase is very high.	Increase the column temperature. Switch to a solvent of lower viscosity.

Split peak(s)



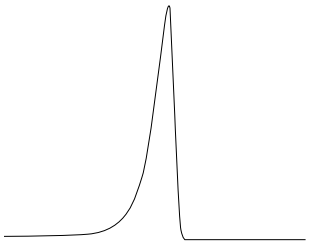
Main cause	Solution
Problems caused by the HPLC column	
Column is overloaded.	Inject a smaller volume (e.g., 1 µL vs. 10 µL) or dilutions of the sample, e.g., 1:10 and 1:100.
The solvent in the sample is incompatible with the mobile phase.	Adjust the solvent. Whenever possible, inject samples directly into the mobile phase.
Void at the column inlet.	Replace the column or open the inlet, and fill the void.
The guard column is contaminated/worn out.	Replace the guard column.
The column is contaminated/ worn out.	Wash the column. Replace the column with a new one of the same type.
Problematic column.	Substitute a new column of the same type to confirm that the original column is the cause of the issue.
Problems caused by the mobile phase	
Change in mobile phase composition.	Check the composition of the mobile phase. If the mobile phase is machine-mixed using proportioning values, hand mix and supply from one reservoir.

Tailing peak(s)



Main cause	Solution
Problems caused by the HPLC column	
Wrong column type.	Try another column type.
The sample solvent is incompatible with the mobile phase.	Adjust the solvent. Whenever possible, inject the samples into the mobile phase (peaks exhibit tailing when the sample is injected in a solvent with a stronger eluting effect than that in the mobile phase.)
Void at the column inlet.	Replace the column or open the inlet, and fill the void
The guard column is contaminated/worn out.	Replace the guard column.
The column is contaminated/ worn out.	Wash the column. Replace the column with a new one of the same type.
Interfering components exist in the sample.	Check the column performance with standard solutions.
Problems caused by the mobile phase	
Wrong mobile phase pH.	Check the pKa of the sample and adjust the pH. For basic compounds, a lower pH usually delivers more symmetric peaks.
The mobile phase is contaminated/ deteriorated.	Check the composition of the mobile phase.

Fronting peak(s)

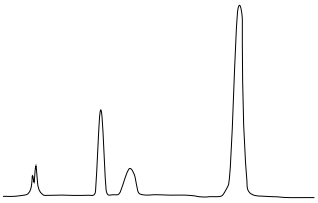


Main cause	Solution
Problems caused by the HPLC column	
Wrong column type.	Try another column.
Column is overloaded.	Inject a smaller volume of the sample than that in the previous injection (e.g., 1 µL vs. 10 µL) or its dilutions (e.g., 1:10 and 1:100).
The solvent in the sample is incompatible with the mobile phase.	Adjust the mixing and ratio and/or type of solvent. Whenever possible, inject the samples directly into the mobile phase (Peaks exhibit tailing when the sample is injected in a stronger solvent than the mobile phase).
Void at the column inlet.	Replace the column or open the inlet, and fill the void.
The guard column is contaminated/worn out.	Replace the guard column.
The column is contaminated/ worn out.	Wash the column. Replace the column with a new one of the same type.
Interfering components exist in the sample.	Check the column performance with standard solutions.
An undesirable sample component exists.	Increase the efficiency or selectivity of the system to improve peak-to-peak resolution. Test another column type if necessary.
Problems caused by the mobile phase	
Wrong pH of the mobile phase.	Check the pKa of the sample and adjust its pH. For basic compounds, a lower pH usually provides more symmetric peaks.
The mobile phase is contaminated/ deteriorated.	Check the composition of the mobile phase.

Negative peak(s)

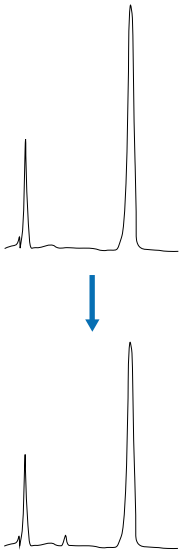
Main cause	Solution
Problems caused by the HPLC system	
Recorder leads are reversed.	Check the polarity.
Problems caused by the mobile phase	
The mobile phase is more absorptive of ultraviolet wavelengths than the sample components.	Change the UV wavelength or use a mobile phase that does not adsorb the chosen wavelength.
Sample solvent and mobile phase differ greatly in composition.	Adjust or change the sample solvent. Dilute the sample using the mobile phase whenever possible.

Ghost peak(s)



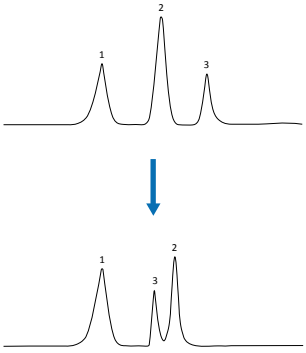
Main cause	Solution
Problems caused by the HPLC system	
Contamination in the auto-sampler.	Flush the auto-sampler between analyses.
Problems caused by the HPLC column	
Contamination in the column.	If necessary, run a strong solvent through the column to remove late-eluting compounds. Include a final washing step in gradient analyses to remove compounds that are strongly retained on the column.
Elution of analytes retained from the previous injection.	Check the steps of sample preparation. Include (step) gradient to quickly elute all components.
Problems caused by the mobile phase	
Upset equilibrium (Ion-pair chromatography).	Prepare the sample in the mobile phase; reduce the injection volume.
The mobile phase is contaminated/ deteriorated.	Check the composition of the mobile phase.
Oxidation of the mobile phase (TFA, THF, etc.)	Change the mobile phase.

Change in peak height



Main cause	Solution
Problems caused by the sample	
One or more sample components have deteriorated.	Use a fresh sample or standard compound to confirm the sample as the source of the problem. If some or all analyte peaks are still smaller than expected, replace the column. If some specific peaks are smaller than expected, cool the sample vial.
Problems caused by the HPLC system	
Inconsistent sample volume.	Ensure that the sample volumes injected are consistent. For a fixed volume sample loop, inject 2–3 times the loop volume to ensure the loop is filled. Ensure that the automatic sampler vials contain sufficient samples with no air bubbles.
Weak detector lamp.	Replace the lamp.
Contamination in the detector cell.	Clean the cell.
Leak, particularly between the injection port and column inlet (this will also change the retention).	Check the system for loose fittings. Check the pump for leaks, salt buildup, and unusual noises. Change the pump seals if necessary.

Change in selectivity



Main cause	Solution
Problems caused by the HPLC column	
The column was changed, and the new column has a different selectivity from that of the old column.	Confirm the composition of the new column packing. For reproducible analyses, use the same column type. Establish whether a change in the analytical records occurred gradually. If so, the bonded phase may have been stripped. Column activity may have changed, or the column may have become contaminated.
Problems caused by the mobile phase	
An increase or decrease in the ionic strength, pH, or additive concentration of the solvent (these parameters strongly affect ionic solutes).	Check the composition of the mobile phase.
Problems caused by the sample	
The sample is injected in the incorrect solvent.	Adjust the solvent. Whenever possible, inject the sample directly into the mobile phase.

What is a buffer?

The importance of controlling the pH of the mobile phase, when analyzing ionizable compounds using reverse-phase (RP) HPLC, is recognized and understood well. However, it is often equally important to control the pH, when working with samples comprising nonionizable compounds, due to the presence of ionizable impurities.

A partial list of common buffers used for RP-HPLC and their corresponding pH values are presented in Table 1. Generally, the most commonly used HPLC buffer is a form of acetic acid or phosphoric acid. A definition of buffer strength is given in Figs. 1 and 2 by demonstrating the change in the formation of acetic and phosphoric acid conjugates with pH. Note that the buffer capacity (i.e., the ability of a solvent to resist pH change when a sample of different pH is introduced) is 100% only at the pK value of the acid or base.

As a rule, one should work within ±1 pH unit of the pKa of the buffer to effectively control the pH of the mobile phase.

When the pH must be controlled at lower values (2–3), phosphate or stronger organic acids, such as TFA, are used, while acetic acid may be used when volatility is a concern. If the pH is to be controlled at 4–5 is desired, organic acid buffers, such as acetate or citrate, should be considered in place of phosphate acids.

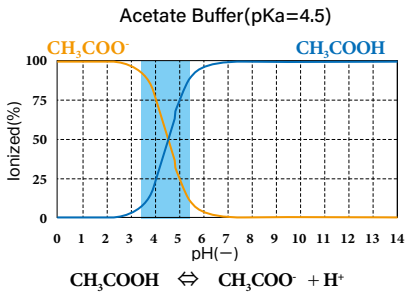


Figure 1 **Buffer capacity of acetate**

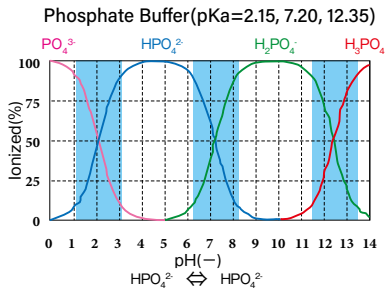


Figure 2 **Buffer capacity of phosphate**

Table 1 **Properties of Common Buffers**

	pKa		
Acetate	4.56		
Fomate	3.55		
Phosphate	2.15	7.20	12.35
Citrate	2.90	4.34	5.66
Tris	8.30		
Ammonia	9.25		

RP methods are developed with buffers that have little or no buffering capacity at the pH specified for the mobile phase. Methods that instruct using a phosphate buffer at a pH range of 4–6 or an acetate buffer in the range of 6–7 are, unfortunately, common. These buffers are not just unusable in these pH ranges; they unnecessarily complicate the preparation of the mobile phase, giving the analyst a false sense of controlling the reproducibility of the separation.

Examples of good and poor buffers are shown in Fig. 3. At pH 4.5, acetate is a good buffer, but phosphate is a poor buffer; consequently, it would change rapidly toward one of the used buffer's pKa values if a more acidic or basic sample were introduced.

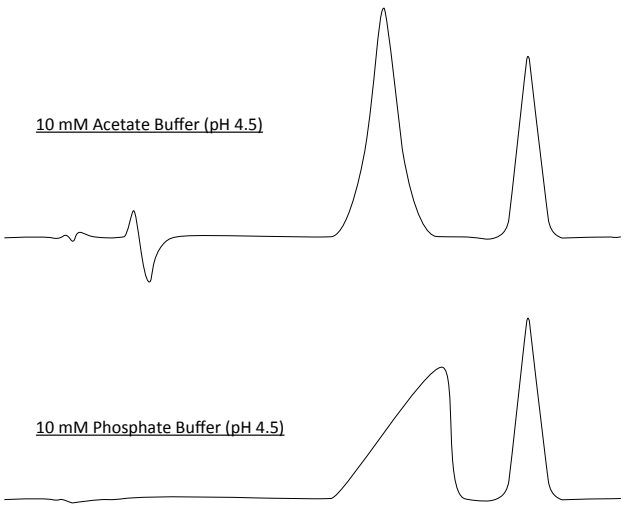
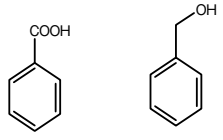


Figure 3



1. Benzoic acid
2. Benzyl alcohl

Column	: (4 µm, 150 x 3.0 mm I.D.)
Mobile phase	: CH_3CN / 10 mM Buffer (pH 4.5) = 15 / 85
Flow rate	: 0.4 mL/min
Column Temp.	: 40 ℃
Detection	: UV 215 nm
Injection	: 5 µL

Contact us or your local GL Sciences representative.
https://www.glsciences.com/contactus/

Authorized distributor:
https://www.glsciences.com/company/distributor.html

For analytical use only. Information subject to change without prior notice.

GL Sciences
https://www.glsciences.com
E-Mail world@gls.co.jp

Published in Japan, December 23, 2023
LCTG0401EN