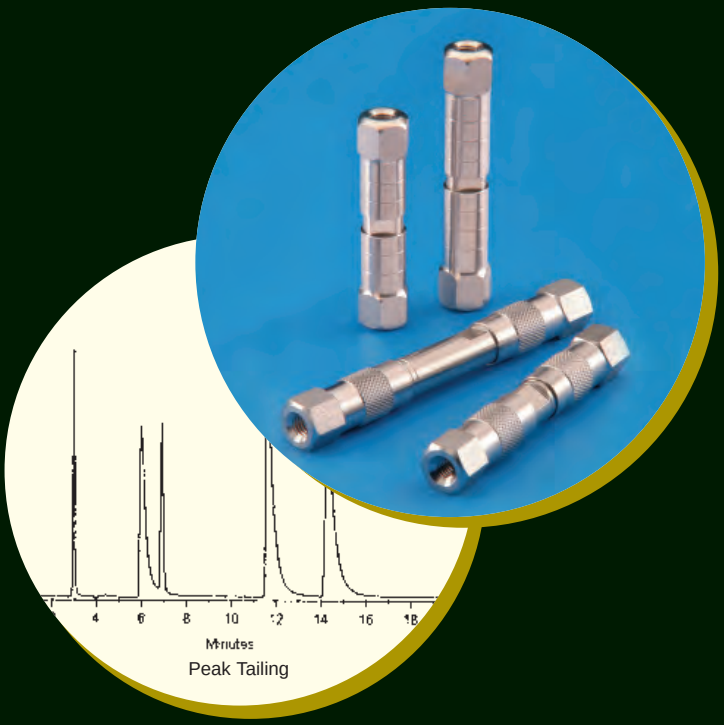
HPLC Troubleshooting Guide



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John Dolan

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HPLC Troubleshooting Guide

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Quick Reference Tables

If you have a problem you need to solve now, use these Troubleshooting Tables. Locate the table for the type of problem you have, find the possible cause and use the short description of the solution or the cross-reference to the main body of the guide to help you identify and solve your problem quickly.

Problems with Peak Shape

Possible Cause	Prevention/Solution
Peak Tailing	
Interaction with active silanols	1. Use ultra-high purity silica based stationary phase (see 1.1) 2. Add basic mobile phase additive (eg. TEA) – not needed with ultra-high purity phases
Chelation with metal ions in stationary phase	As above
Wrong mobile phase pH	1. Decrease mobile phase pH to suppress silanol ionisation (see 1.2) 2. Increase buffer concentration (see 1.2)
Blocked frit	1. Reverse flush the column (see 5.2) 2. Use in-line filter (see 1.3, 4.2)
Column void	1. Reverse flush the column (see 5.2) 2. Replace the column
Unswept dead volume	1. Minimise number of connections 2. Use shorter connection tubing 3. Check all fittings are tight
Split Peaks	
Contamination on guard or analytical column inlet	1. Remove guard cartridge and carry out analysis – replace guard if necessary 2. Reverse flush analytical column (see 5.2) 3. For strongly retained contaminants, try regeneration procedure (see 5.2) 4. Replace column
Blocked frit	1. Reverse flush the column (see 5.2) 2. Use in-line filter (see 1.3, 4.2)
Sample solvent incompatible with mobile phase	1. Inject sample in mobile phase
Simultaneous elution of second component	1. Use sample clean-up prior to injection 2. Change selectivity by changing mobile phase or column phase
Column overloaded	1. Use higher capacity stationary phase 2. Increase column diameter 3. Decrease sample amount

Problems with Peak Shape (continued)

Peak Fronting	
Formation of channels in column	1. Replace the column 2. Operate within recommended pH limits of column (see 1.5)
Column overloaded	1. Inject smaller volume or more dilute sample solution 2. Use higher capacity stationary phase
Sample solvent incompatible with mobile phase	1. Inject sample in mobile phase
Low temperature	1. Increase column temperature

Problems with Retention Variation

Possible Cause	Prevention/Solution
Decreasing Retention Times	
Loss of bonded stationary phase	1. Replace column 2. Operate at pH 2-8 for silica based RP columns (see 2.2)
Active groups on stationary phase	1. Use organic modifier in mobile phase 2. Increase buffer strength
Increasing flow rate	1. Check and adjust pump flow rate (see 2.4)
Column overloaded	1. Reduce amount of sample injected 2. Use column with larger i.d.
Increasing Retention Times	
Changing mobile phase composition	1. Cover solvent reservoirs (see 2.1) 2. Prepare fresh mobile phase (see 2.1)
Loss of bonded stationary phase	1. Replace column
Decreasing flow rate	1. Check and adjust pump flow rate (see 2.4) 2. Check for leaks in system, including pump seals (see 2.4)
Bubbles in mobile phase	1. Check flow rate and pressure (see 2.4) 2. Degas mobile phase (see 2.4)
Fluctuating Retention Times	
Insufficient column equilibration	1. Equilibrate column longer between runs 2. Condition the column with concentrated sample
Change in mobile phase composition	1. Check make-up of mobile phase and make up new if necessary (see 2.1) 2. Check proportioning-valve accuracy (see 2.5)
Insufficient buffer capacity	1. Use buffer concentrations >20mM
Fluctuating column temperature	1. Stabilise ambient temperature (see 2.3) 2. Thermostat the column (see 2.3)

Problems with Ghost Peaks

Possible Cause	Prevention/Solution
Ghost Peaks	
Contamination in column or injector	1. Use only HPLC grade solvents 2. Flush column to remove impurities (see 5.2) 3. Flush injector between analyses
Late eluting peak from previous injection	1. Extend run time (see 3.1) 2. Flush column with strong mobile phase at end of each run (see 3.1) 3. For gradient runs, end at higher concentration (see 3.1)
Contaminated water in RP HPLC	1. Use HPLC grade water (see 3.2)
Unknown interferences in sample	1. Use sample clean-up (e.g. SPE)
Negative Peaks	
Refractive index of solute lower than that of mobile phase (RI detector)	1. Use mobile phase with lower refractive index 2. Reverse detector polarity to obtain positive peaks
Absorption of solute lower than absorption of mobile phase (UV detector)	1. Change UV wavelength 2. Use mobile phase with lower UV absorption (see 3.3)
Sample solvent and mobile phase differ in composition	1. Change sample solvent and dissolve sample in mobile phase if possible
Spikes	
Air bubbles in mobile phase	1. Degas mobile phase 2. Install back pressure restrictor at detector outlet 3. Ensure all fittings are tight
Column stored without endcaps	1. Store columns with endcaps (see 5.3) 2. Flush RP column with degassed methanol

Problems with Column Backpressure

Possible Cause	Prevention/Solution
High Backpressure	
Wrong pump setting	1.Check and correct setting
Normal for system	Increased backpressure normal if: 1. Switched to longer column 2. Changed to smaller particles 3. Changed to smaller diameter 4. Increased flow rate *if* no other changes made
Pressure higher during middle of gradient	1. Normal
Temperature too low	1. Adjust column oven temperature
Column ageing	1. Gradual increase in pressure normal over column lifetime
Blocked column frit	1. Reverse flush the column (see 5.2) 2. Use in-line filter (see 1.3, 4.2) 3. Centrifuge or filter samples 4. Use guard cartridges (see 4.3)
Blocked in-line filter	1. Replace in-line filter frit (see 4.2) 2. Centrifuge or filter samples 3. Pre-filter mobile phase
Blocked guard cartridge	1. Replace guard cartridge more frequently (see 4.3)
System blockage	1. Systematically investigate system to find blockage (see 4.1)
Buffer precipitation	1. Reverse flush the column with water(see 4.4) 2. Review evaluation conditions (see 4.4)
Low Backpressure	
Leak in system	1. Locate leak and correct
Column temperature too high	1. Lower temperature
Flow too low	1. Increase flow rate

Introduction

This troubleshooting guide contains examples of some of the most common problems observed in reversed-phase HPLC (RP-HPLC) separations. Four major problem areas are covered: peak shape, retention time changes, ghost peaks and problems related to column backpressure. In addition, a section on column care is included – procedures that will help you get maximum lifetimes from your columns. Within each section, several examples are given to illustrate various problems. A set of troubleshooting tables corresponding to each section will help you quickly identify problem causes and solutions. If you are in a hurry, you can go directly to the tables to help you solve an existing problem. Otherwise, we suggest that you read the entire guide so as to pick up some ideas that will help you avoid problems in the future. We hope that you find this guide useful to diagnose problems and to gain an understanding of the underlying causes so that you can prevent, or at least minimize, their future occurrence.

1. Peak Shape

1.1. Peak tailing has been the most common peak shape problem since the early days of RP-HPLC. Most peak tailing is due to interaction with acidic or ionized silanol groups on the surface of the silica particles within the column. The low-purity silica (often called "Type-A" or acidic silica) has a high content of acidic silanol (-Si-OH) groups and the presence of metal impurities (especially iron and aluminum) further increases the ionization of these groups to $–Si-O^-$, which provides cation exchange sites. The pK_a of these materials is in the pH 4-5 region, meaning that at pH>6 most of the silanol groups are ionized. Efforts to improve the purity and lower the acidity of silica led to higher purity silica particles ("Type-B") and since their initial introduction, the purity of these silica packings has improved. High-purity silica has a pK_a of >8, so there is minimal silanol ionization in the pH-stable range of 2<pH<8 for most columns.

Basic compounds are the most susceptible to silanol tailing and because a high proportion of sample molecules contain basic nitrogen functional groups, few compounds are completely immune to silanol interactions. Silanol tailing is illustrated in Figure 1. Toluene, a neutral compound, is not subject to silanol tailing, but the remaining analytes of Figure 1 are strongly basic drugs. Further challenging the separation is a mobile phase pH of 6, which ionizes the silanols of the less-pure-silica columns. The lowest-purity silica (Fig. 1a), representative of Type-A silica, has such strong interactions with the bases that the analytes are not eluted from the column. The early Type-B materials (Fig. 1b) significantly improved peak shape, so that all peaks in the sample are visible, even though they tail badly. Further improvements in silica purity (Fig. 1c) have reduced peak

tailing to acceptable levels. It is unlikely that peak tailing will be eliminated as long as silanol-containing silica is used in RP-HPLC.

Silanol tailing is best reduced by using a high-purity silica column, but there are other techniques to reduce tailing. For many years, triethylamine (TEA), a small-molecular-weight base, was added to the mobile phase (e.g. at 25 mM) for this purpose. TEA is a very effective competitor for acidic silanol groups, but with today's high-purity silica, TEA is not needed and is rarely used.

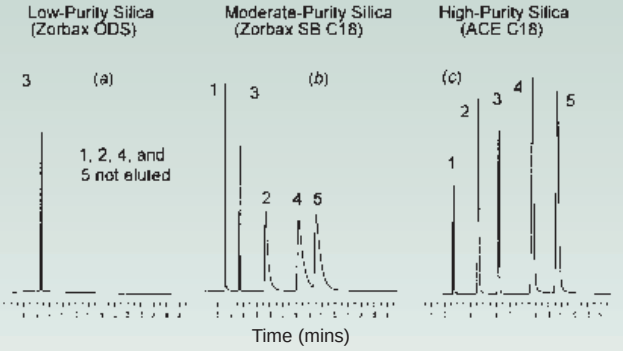


Figure 1. Silica purity and peak tailing. (a) low-, (b) moderate-, and (c) high-purity silica. Columns: 250x4.6mm, 5µm; Mobile phase: 80:20 MeOH/25mM KH_2PO_4 (pH 6.0); Flow: 1.0 mL/min; Components: 1, norephedrine; 2, nortriptyline; 3, toluene; 4, imipramine; 5, amitriptyline.

1.2. Insufficient buffer or mobile phase additive also can result in peak tailing. A primary function of a buffer is to keep the sample in a constant ionization state, so as to stabilize retention and to minimize peak tailing due to ionic interactions. The buffer also suppresses ionization of silanol groups on the silica surface. This, of course, is more of a challenge for less-pure silica materials, where ionized silanols are much more likely. The effect of the additive concentration on peak shape is illustrated in Figure 2. In this case, trifluoroacetic acid (TFA) acts as an ion-pairing reagent for the protein sample components and also creates a low-pH mobile phase to suppress silanol ionization. Traditionally, 0.1% TFA has been used as an additive for protein and peptide separations. As can be seen in Figure 2a and b, this concentration is sufficient to minimize tailing on both the high-purity and moderate-purity silica columns. However, when the TFA concentration is dropped tenfold (Fig. 2c, d), tailing increases with both phases. The high-purity silica still maintains acceptable peak shape, but the peak tailing on the moderate-purity column is now unacceptable.

A common characteristic of buffers and other mobile phase additives is that their effect (e.g. reduction of peak tailing, stabilization of retention times) begins at low concentrations and continues as the concentration is increased, but gradually levels off into a plateau. Select an additive concentration on the plateau for stable operation; excessive concentrations can cause solubility problems. Additives in the 10-25 mM region usually are sufficient for most applications, but it is a good idea to determine this on a case-by-case basis.

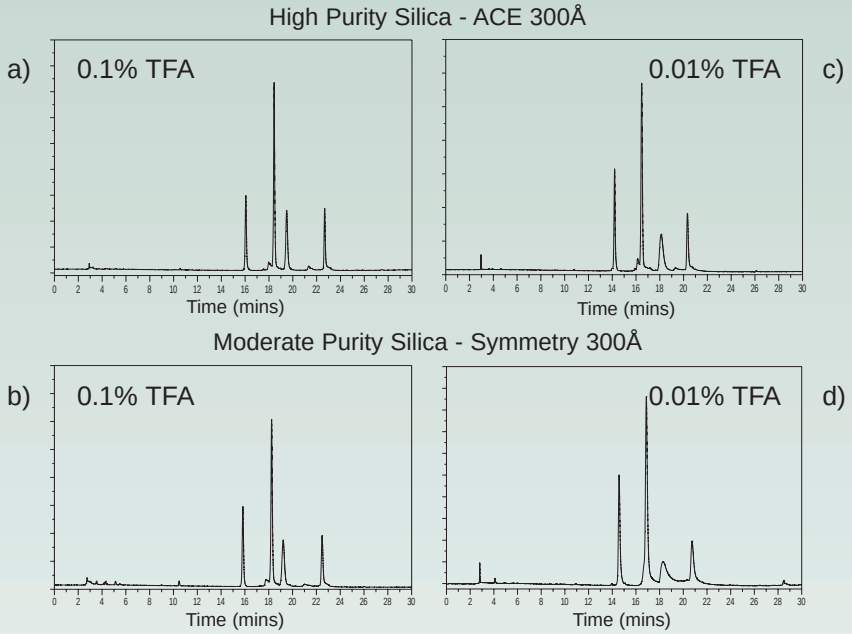


Figure 2. The effect of buffer concentration on peak tailing. 0.1% TFA with (a) high- and (b) moderate-purity silica column; 0.01% TFA with (c) high- and (d) moderate-purity silica column. Columns: 250x4.6mm, 5µm, C18 300Å; Mobile phase: A: 0.1% or 0.01% TFA in H_2O; B: 0.1% or 0.01% TFA in ACN; 5-70% B in 30 min; Flow: 1.0 mL/min, 280nm. Components (in retention order): ribonuclease A, cytochrome C, holo-transferrin, apomyoglobin.

1.3. Peak tailing or distortion for all peaks in the chromatogram usually is the result of a physical, rather than a chemical problem. When all peaks in the chromatogram show the same type of distortion (Fig. 3), the initial problem occurred before the analytes began migrating through the column. The most common cause of this type of peak distortion is a partially blocked frit or a void

at the head of the column. Today's columns are not very susceptible to voids unless they are operated outside of their recommended pH range. However, frit blockage remains a common problem. The frit at the column inlet typically is 2.0 µm porosity for 5 µm particle columns, and 0.5 µm porosity for columns ≤3 µm. If particulate matter from the sample, worn pump seals, or the mobile phase reaches the column, it usually collects on the frit. This material can distort the distribution of the sample at the column inlet, such that part of the sample reaches the column via a different flow path and thus later than another portion of the sample. Since no separation has taken place at this point, all analytes are distorted in the same manner and the chromatogram shows similar peak tailing or distortion for all peaks. To prevent this problem, filter the mobile phase if it has potential to contain particles (e.g. buffer precipitate or dust); replace the pump seals before they wear enough to shed particles. If the sample contains particulate debris, either filter it (e.g. 0.5 µm porosity filter) or centrifuge it briefly (e.g. 5 min, >1500 x g) to remove particles. We strongly recommend installing a 0.5 µm porosity in-line filter just downstream from the autosampler to catch any particulate matter that inadvertently enters the HPLC system. The system backpressure will rise when this in-line filter frit becomes blocked; replacement of the frit is a simple, fast, and inexpensive task. Use of an in-line filter is one of the least expensive ways to prolong the column lifetime.

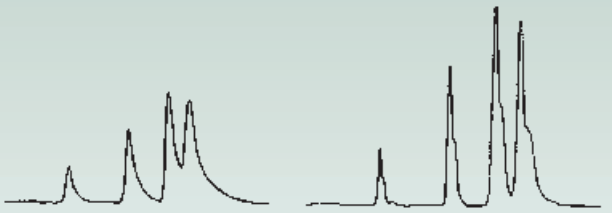


Figure 3. Peak distortion of all peaks in the chromatogram due to a partially blocked frit or column void.

1.4. Poorly resolved peaks can masquerade as a column or a buffer problem. If two peaks are only partially resolved, peak splitting or doubling can occur. This is seen in Figure 4. In Figure 4a, peak distortion, much like that of Figure 3, was observed, suggesting a blocked frit. Changing the column did not correct the problem, so a blocked frit was unlikely. When the mass of sample on column was reduced (Fig. 4b), the peak looked more like two peaks than a shoulder. This led to further investigation and it was determined that a second peak was present. The method conditions were modified to fully separate the two peaks.

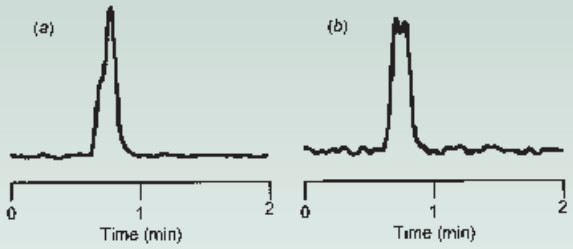


Figure 4. Split peaks due to the presence of a second component. (a) 25 ng/mL, and (b) 10 ng/mL of drug (second peak) in plasma. Adapted from [1].

1.5. Fronting peaks are a fairly rare phenomenon to encounter with today's well-packed columns. One source of peak fronting is channelling in the column or collapse of the column structure. This is rare with today's columns if they are operated under conditions recommended by the manufacturer.

Most silica columns are stable in the pH range 2-8. Below pH 2, the bonded phase hydrolyzes; above pH 8, the silica can dissolve. If you operate the HPLC system outside this pH range, be sure to select a column that is designed to be stable under the selected conditions. Peak fronting due to channelling within the column is illustrated in Figure 5. A normal peak is shown in Figure 5a; after ≈500 injections, peak fronting (Fig. 5b) was observed. This change was characteristic of the method and often appeared suddenly, from one run to the next. Once fronting peaks appeared, the only effective fix was to replace the column. The column was a 100x2.1 mm i.d. column packed with 5 µm C18 particles. Mobile phase A was 10 mM ammonium bicarbonate (pH 9.0); B was methanol (MeOH). The method comprised an isocratic elution segment at 5% B followed by an 80% B flush. This particular column was designed to work with up to 100% water mobile phases and was not endcapped. Endcapping helps to protect the silica from dissolution as the mobile phase pH is increased. In this case, the column was used with a mobile phase pH well above its recommended range and lacked protective endcapping. The silica gradually dissolved at pH 9 until the column bed structure was no longer stable and the bed shifted, causing a void or channel, which in turn resulted in the fronting peaks of Figure 5b. This problem could have been avoided by using a lower mobile phase pH or selecting a column that was stable at higher pH.

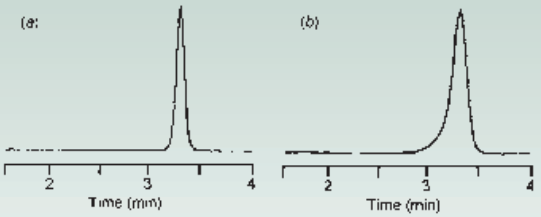


Figure 5. Peak fronting due to channelling in column. (a) normal peak shape; (b) fronting peak. Adapted from [2].

2. Retention Variation

When all peaks change retention times, most likely it is due to a change in mobile phase composition, column chemistry, column temperature or flow rate. Errors in on-line mixing of isocratic or gradient mobile phases also can cause retention time problems. Each of these sources is examined briefly below.

2.1. Mobile phase composition changes usually occur abruptly when a change is made by the operator – either by improperly setting the mobile phase mixture with an on-line mixing system or replacing the mobile phase with a new batch that was not prepared properly. In rare cases, selective loss (e.g. evaporation) of one mobile phase component may occur. When a mobile phase change is made, peaks usually move in the same direction – to shorter or longer retention times – and relative retention (the selectivity factor, a) often changes. The best way to check for mobile phase composition errors is to double-check the system settings and if necessary, make up a new batch of mobile phase. Method documentation may contain information about the effect of specific mobile phase changes. For example, a small change in the %-organic solvent or pH may have a characteristic effect on the chromatogram, such as a change in resolution or a shift in retention. If you suspect the equipment is at fault, move the column and mobile phase to another HPLC system and try another run. If the problem persists, it is due to the mobile phase or column, but if it goes away, it can be correlated with other system components or parameters.

2.2. Column chemistry changes will occur over the lifetime of the column and generally are gradual over several weeks or months. Column ageing usually is accompanied by rising column backpressure, gradually shifting retention times (longer or shorter) and more peak tailing. Exchange the column for a new one to confirm a column-ageing problem. Column lifetimes of 500-2000 injections should be considered satisfactory; at this point, the cost contribution of the column to the overall analysis is small, so replacement is justified easily. If the column lifetime seems unacceptably short, carefully examine the operating conditions to be sure that they are reasonable for the column. Figure 5 shows an example of short column lifetime due to extreme operating conditions.

2.3. Column temperature changes can cause retention time changes of 1-3% per 1°C change in temperature. When a column oven is not used (i.e. "ambient" conditions), the temperature often cycles over the course of the day (and night) due to laboratory temperature changes. Although the laboratory temperature may seem to be constant, as measured at the room's thermostat, the micro-environment at the HPLC system may change significantly, especially if a heating or air conditioning vent blows directly at the system. Column temperature problems can be eliminated with the use of a column oven and location of the HPLC system away from such vents.

2.4. Flow rate problems can be due to bubbles, leaks or pump problems. Bubble problems will correlate with low or fluctuating pressure and increased retention times. With two-headed pumps, the flow and pressure may pulse if a bubble is present in only one pump head. Degas the mobile phase, then purge the pump by opening the purge valve and pumping 5-10 mL of mobile phase through the pump at several times the normal flow rate to displace bubbles. In some cases, it may be necessary to use a low-viscosity, degassed solvent, such as methanol (MeOH) or acetonitrile (ACN) to purge stubborn bubbles from the pump.

Leaks also will increase retention times. Look for dripping fittings or crystalline deposits on fittings as evidence of leaks. Pay special attention to fittings upstream from the column. Fittings and seals inside the autosampler may be hard to inspect – a flashlight and small mirror can be helpful. If stainless steel fittings are in use, usually a ¼ turn of the fitting nut will stop a leak. With PEEK fittings, it is best to stop the pump, loosen the fitting, push the tubing to the bottom of the fitting port and then tighten the fitting prior to restarting the pump. Tightening a PEEK fitting with the flow on may cause the tubing to slip in the fitting, creating extra-column volume, which can degrade the separation.

Faulty check-valves or worn pump seals can result in low or fluctuating flow rates. Check-valve problems will be accompanied with pressure fluctuations. If purging bubbles from the pump does not correct pressure fluctuation problems, check-valves are the next most likely source. Check-valves can be replaced with new ones, but effective cleaning can be accomplished by sonicating the check-valves in a beaker of MeOH for a few minutes. If you cannot easily distinguish between inlet and outlet check-valves, mark them with a scribe or label the beakers clearly. Place each check-valve in a separate beaker for cleaning as they can come apart during cleaning. If the parts do come out, carefully reassemble them so as to avoid contamination (dust-free gloves, avoid scratching the ball, etc.). Pump seals wear with use and lifetimes tend to be shorter with buffered mobile phases or high-salt conditions, such as ion exchange methods. By keeping good records of seal replacement intervals, you will be able to set up a preventive maintenance program that involves seal replacement prior to failure. In absence of other indicators, replace the seals at least once a year.

2.5. Proportioning-valve failures and on-line mixing problems will degrade gradient elution methods. The example of Figure 6 shows two consecutive injections of a peptide sample analyzed by gradient elution. In this case, the system suitability test allowed for 0.1 min variation in retention between runs – the first peak just fails this criterion and the last peak barely passes, but the middle two peaks are obviously out of specification.

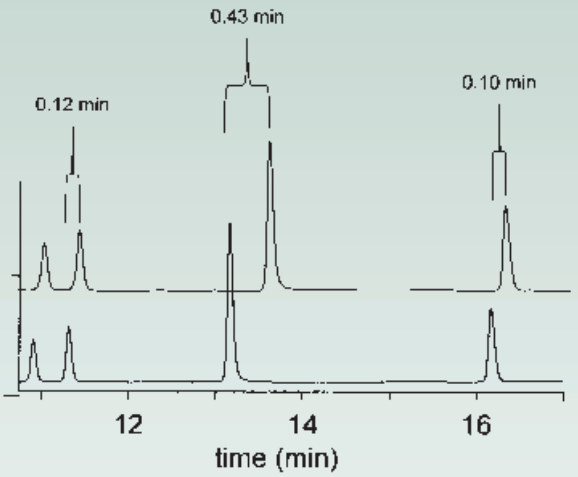


Figure 6. Chromatograms from two consecutive gradient runs showing larger errors for peaks near the gradient midpoint (13 min). Adapted from [3].

A simple way to check mobile-phase proportioning accuracy is as follows. Replace the column with ≈1 m of 0.005 in. i.d. (0.12 mm) tubing, place water in the A-reservoir and water containing 0.1% acetone in the B-reservoir, set the detector wavelength to 265 nm and use a flow rate high enough that the check-valves will work reliably (e.g. 2 mL/min). Run a series of steps at 10% increments (10%, 20%, 30%... 90%, 100% B). Since problems often occur near 50% B, add an extra step at 45% B and 55% B. The result should be a smooth, stair-step plot (see Fig. 8a). For the sample of Figure 6, the plot of Figure 7 was observed for the 40% - 60% B steps. The steps are distorted, and the step from 45% to 50% B is 8.4% rather than 5%. The dashed lines in Figure 7 approximate the gradient – there is an offset somewhere between 45% and 50% B. Unfortunately, this is where the peaks with large retention variations were eluted. The HPLC system had a procedure for proportioning-valve adjustment and when this was performed, the steps became smooth and even and the retention times fell within specifications.

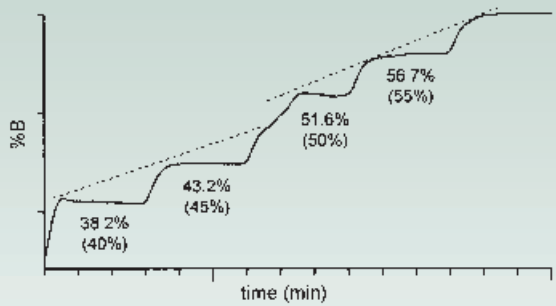


Figure 7. Results of proportioning step test performed near the midpoint of the gradient used for Figure 6. Theoretical values shown in parentheses. Adapted from [3].

The step-test for a well-behaved system should look similar to that of Figure 8a, with even steps throughout the plot. A companion test that should be run is a 0-100% B gradient with no injection. This should appear as a linear baseline, a linear gradient segment, and a linear post-gradient hold, with a smooth curve transition between each segment. The example of Figure 9 shows a blank-gradient run with regular deviations from linearity (arrows) at ≈25%, 50% and 75% B.

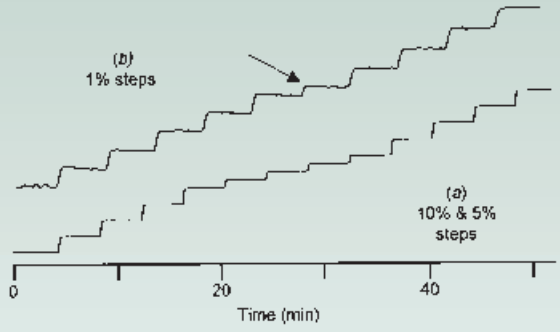


Figure 8. Gradient step test results for HPLC system of Figure 9. (a) Steps of 0, 10, 20, 30, 40, 45, 50, 55, 60, 70, 80, 90, and 100% B; (b) upper trace is 45 – 55% in 1% steps. Arrow showing "short" step between 50 and 51% B. Adapted from [4].

The step-test corresponding to these conditions is shown in Figure 8a, and on this scale looks quite good. To more closely examine the problem region, a step-test was run in 1% increments over 45-55% B, as shown in Figure 8b. This expanded plot clearly shows an irregularity in the step between 50% and 51% B. Errors at regular intervals in the linearity plot (Fig. 9) suggest problems with the algorithm controlling the proportioning valves, or the proportioning valves themselves. In the present case, adjustment of the controlling software did not correct the problem, so the proportioning valves were replaced and the problem was corrected.

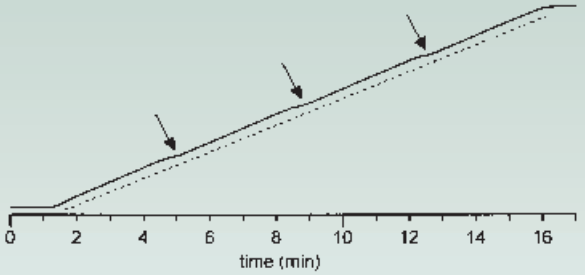


Figure 9. Plot of linear gradient with faulty proportioning valves. Arrows show deviations from linearity; dashed line drawn below plot for reference. Gradient 0 – 100% B in 15 min at 1 mL/min; A = water, B = 0.1% acetone in water; detection UV 265 nm. Adapted from [4].

Although it is a good assumption that a single source is responsible for a particular HPLC problem, this is not always the case. Figure 10a shows three consecutive injections of a peptide sample run with a very shallow gradient (19-24% ACN in 30 min). Flow rate problems were suspected, so all 8 check valves and 4 pump seals were replaced for this dual-piston, two-pump system. This improved the retention variation considerably, from a 2.1 min retention range to 1.0 min (Fig. 10b), but this still was unacceptable variation. To investigate the problem further the solvents were premixed to 15% ACN in the A-reservoir and 25% ACN in the B-reservoir. When the instrument settings were adjusted to generate the same gradient as in Figure 10a and b, the results shown in Figure 10c were obtained. Although the proportioning accuracy of the instrument was within the ±0.1% specification, it was not sufficient for a very shallow gradient. Premixing the solvents changed the effective accuracy from 0.1% to 0.01%, which was necessary for satisfactory retention time reproducibility for this sample. Premixing can improve system performance for demanding separations.

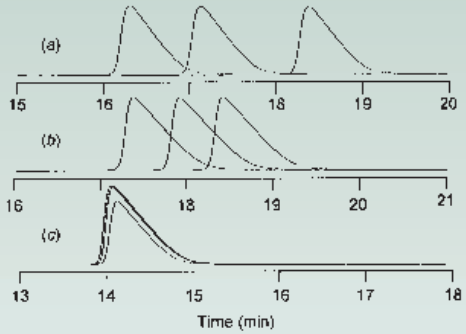


Figure 10. Expanded chromatograms from three consecutive injections of a peptide standard. Chromatograms generated (a) using the original system configuration, (b) after replacing all check-valves and pump seals, and (c) using premixed mobile phase. Column: 250x4.6 mm, 5 µm C18 operated at 1.5 mL/min and 35°C with detection at 215 nm. Gradient: 19-24% ACN/0.1% TFA in water over 30 min. Adapted from [5].

3. Ghost Peaks

3.1. Late elution of peaks from a previous run can appear as unexpectedly broad peaks in isocratic separations. For isocratic separations, the longer the retention time, the broader the peak should be, but all peaks in a narrow region of the chromatogram should have approximately the same peak width. When a broad peak appears among narrow ones, as in Figure 11 (arrow), a late-eluted compound from a previous injection is the most likely problem source. This is easy to check – just make a normal injection, but extend the run time by two- to three-fold. If the peak appears after the end of the normal run time, you have identified the problem source. Either extend the normal run time to include the elution of this peak or add a strong-solvent flush at the end of each run to wash strongly retained materials from the column.

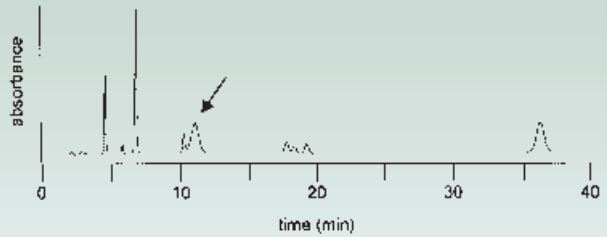


Figure 11. Late eluted peak normally eluted at 38.5 min appears at 12.0 min (arrow) in the next chromatogram of a shortened isocratic run. Adapted from [6].

3.2. Ghost peaks in gradient runs can be isolated by running a non-injection blank gradient and observing the baseline. When an excessive number of peaks appear in the blank gradient, as in Figure 12a, dirty reagents are one likely cause of the problem. In this case, the peaks in the run of Figure 12a are quite small (1-3 mAU), and would be of little concern for a major component assay of peaks in the 0.8-1.0 AU size range, but for stability-indicating or impurity methods, peaks in the 1-2 mAU size range may require quantification. In such cases, further investigation is warranted. During equilibration between gradient runs, non-polar impurities in the mobile phase tend to concentrate at the head of the column. Then, during the gradient, these impurities are eluted just as any other peak would be eluted in a gradient run. Check for the source of the problem by extending the equilibration period three-fold. If the peaks in the blank gradient increase by approximately three times, the aqueous solvent is the most likely source. Replace the water and/or additives with higher-purity components to eliminate the problem, as is the case for Figure 12b.

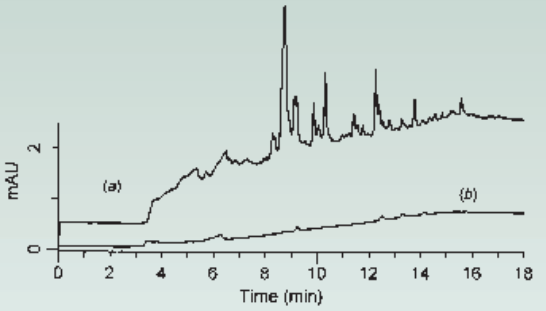


Figure 12. Blank gradient runs performed (a) with contaminated and (b) higher purity water in the A-reservoir. Column: 150x4.6 mm C18; 1.5 mL/min; 35°C; UV detection at 255 nm. Gradient: 0-83% ACN/water in 13 min with 5 min hold. Adapted from [7].

3.3. Negative peaks in isocratic or gradient runs are less common than positive peaks, but they can occur. Negative peaks are more common with ion pairing or other methods in which mobile phase reagents have significant UV absorbance at the selected detection wavelength. In such cases, the background absorbance may be significant (perhaps 0.5 AU or more) but it is not noticed because the system autozeros the detector signal at the beginning of each run. Any compound that has less absorbance than the mobile phase background will show up as a negative peak. Identification of the source and elimination of such peaks follows in the same manner as positive peaks – check the water, reagents or sample preparation process.

4. Column Backpressure

A rise in column backpressure is unavoidable as the column ages and high backpressure is one of the most common causes of column failure. However, some simple practices will help extend column life, including use of in-line filters (see 4.2), guard cartridges (4.3) and regular column flushing (5.2).

4.1. Locating pressure problems is a simple procedure. If you use an in-line filter, it is the most likely source of pressure increase, so checking it first can save troubleshooting time. Otherwise, the easiest way to find the cause of increased pressure is to systematically loosen tube fittings, starting at the outlet of the HPLC and moving upstream toward the pump. You should expect a negligible change in the pressure drop as each successive element is removed from the system, with the exception of the column. For example, removing the detector, then the tubing connecting the detector to the column, should not cause a significant reduction in backpressure unless one of these is blocked. When the column is removed, of course the pressure will drop – use historic information about the normal column pressure to figure out whether the column is the source of the problem. Once the source of the pressure problem is identified, replace the tubing or back-flush the offending part (see 5.2 for column flushing procedures).

4.2. In-line filters are one of the least expensive and most effective tools to extend column lifetimes. Typically, these filters contain a 0.5 µm porosity frit and are mounted just downstream from the autosampler to trap any particles originating from the mobile phase, pump, autosampler or sample. The frit at the head of the column generally is a 2 µm porosity frit for a 5 µm particle column, so the in-line frit helps prevent column blockage. The in-line filter should not be used to replace solvent filtration or other preventive practices, but rather serves as a backup to protect the column. When the system backpressure begins to rise, check and/or replace the in-line filter frit. We recommend using an in-line filter on every system, even when a guard column is used.

4.3. Guard cartridges provide two-fold protection of the column. If an in-line filter is not used, the frit at the head of the guard cartridge will trap particles that might otherwise foul the inlet frit on the column. The stationary phase in the guard cartridge should be matched to the analytical column so that it will trap substances that would be irreversibly adsorbed to the analytical column. Thus, the guard cartridge acts as a sacrificial element in the system. A 10% rise in system pressure, that is not corrected by replacement of the in-line filter, is a good indication that the guard cartridge should be replaced, as is a 10% drop in column efficiency or resolution. Figure 13 shows an example of the effectiveness of guard cartridges in protecting the analytical column.

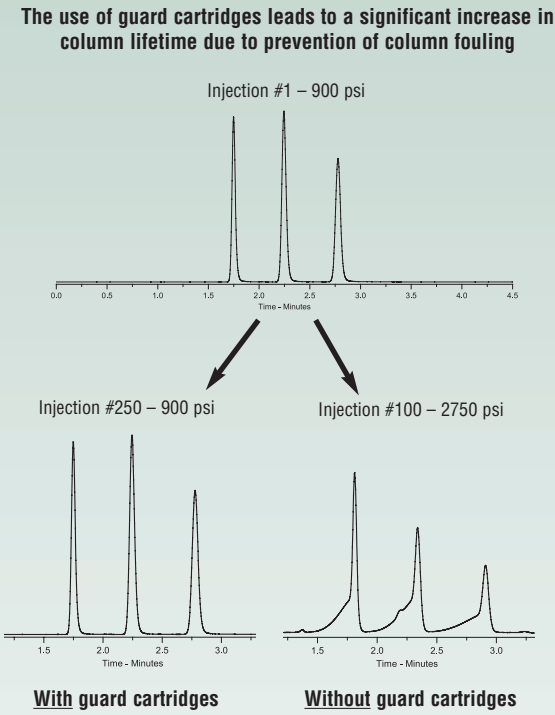


Figure 13. The use of guard cartridges can significantly increase column lifetime due to prevention of column fouling.

4.4 Buffer precipitation is a common cause of increased backpressure, especially in reversed-phase chromatography with high concentrations of organic solvent in the mobile phase. To try to remove buffer precipitated within a column, reverse the column and pump 20-40 column volumes of 100% water through the column, initially at a reduced flow (note: if buffer has been precipitated throughout the whole column this may not be possible). If this is successful, restore the column to the correct direction of flow and flush for 10-20 column volumes with 50:50 water /organic mobile phase, followed by a further 10-20 column volumes of 100% organic mobile phase, then finally re-equilibrating under the desired conditions.

To prevent reoccurrence, check that the mobile phase is compatible with the buffer concentration used and reduce the ionic strength if necessary. Consider increasing the percentage water and also premixing the mobile phase. With reversed-phase separations, avoid rapid changes from mobile phase containing buffer to 100% organic solvent (e.g. MeOH, ACN).

5. Column Care

Columns should be considered a consumable item and as such will have a limited lifetime. For most applications, columns should last 500-2000 injections, but this will vary with the cleanliness of the samples, the pH of the mobile phase and the use of guard cartridges. The practices listed here should help to maximize the useful life of silica-based columns.

5.1. Equilibration of the column when changing from one mobile phase to another, or when recycling a gradient, should take 10-20 column volumes. The column volume for various column dimensions is shown below. The less drastic a change in solvent (e.g. from 80% to 20% ACN/water vs. from ACN to THF), the less volume should be required. The easiest way to check for equilibration is to make two injections of sample – if the retention is the same, the column was equilibrated adequately; if retention shifts, increase the equilibration volume and try again. Equilibration is related to the volume of solvent, not the time, so higher flow rates can reduce equilibration times.

Approximate Column Volumes (mL)

Column i.d.	Column length		
	50 mm	150 mm	250 mm
2.1 mm	0.1	0.3	0.5
3.2 mm	0.3	0.7	1.2
4.6 mm	0.5	1.5	2.5
10.0 mm	2.4	7.1	11.8
21.2 mm	11.6	34.7	57.8

5.2. Column flushing is a simple procedure that can extend column lifetime by washing strongly retained material from the column. At the end of each day's use of the column, remove any buffer (see 4.4.), then flush the column with 100% of the strong solvent (generally ACN or MeOH for reversed-phase methods). The more extensive flushing procedures listed on the next page can be effective at restoring column performance, but remember that the column should be considered a consumable item, so it should not be expected to last forever! Avoid flushing reversed-phase columns with 100% water (except for embedded-polar-group or "AQ" columns), because phase dewetting will prevent good cleaning and column re-equilibration with mobile phase may be very slow.

Follow this general procedure for column flushing with the specific solvents mentioned below for your type of column. It is always good to check the manufacturer's recommendations prior to flushing so that you don't damage the column.

1. Disconnect and reverse the column
2. Connect the column to the pump, but not the detector
3. Flush with 10-20 column volumes of solvent at a flow rate no higher than that used for the QC chromatogram
4. If altering the procedures below, be sure to use miscible solvents for each successive step (see table page 24)

5.2.1. Reversed-Phase Columns (C18, C8, C4, Phenyl, CN, 'AQ' type)
- a. Mobile phase without buffer
- b. MeOH or ACN

If metal ions are thought to be causing contamination, flush with aqueous 0.05M EDTA, then water, followed by the above sequence. Columns which use ion-pairing reagents should be dedicated to ion-pairing applications.

5.2.2. Reversed-Phase Protein/Peptide Columns
- a. Mobile phase without buffer
- b. Gradient of 10-90% B; A = 0.1% TFA/water; B = 0.1% TFA/ACN

5.2.3. Unbonded Silica Columns (SIL)
- a. IPA
- b. MeOH
- c. Ethyl acetate

5.2.4. Bonded Normal-Phase Columns (CN, NH_2, Diol)
- a. Chloroform
- b. IPA
- c. Methylene chloride
- d. Hexane

5.2.5. Anion-Exchange Columns (SAX, WAX)
- a. Water
- b. Methanol
- c. Chloroform
- d. Methanol
- e. Water

5.2.6. Cation-Exchange Columns (SCX, WCX)
- a. Water (inject 4x 200 µL DMSO during flush)
- b. THF

5.2.7. Size-Exclusion Columns for Proteins
For weakly retained proteins
a. 0.1M phosphate buffer, pH 3
For strongly retained proteins
a. Gradient of 100% water to 100% ACN in 60 min

5.3. Column storage practices will help extend the lifetime of the column. The simplest storage procedure is to remove any buffer from the column (see 4.4.), then wash the column with 10-20 column volumes of strong mobile phase solvent (e.g. MeOH or ACN for reversed-phase, as detailed in 5.2) to remove strongly retained material from the column. Then flush the column with a further 10-20 column volumes of the storage mobile phase specified by the manufacturer (this information should be detailed on the QC test chromatogram originally supplied with the column). Finally, cap the column securely to prevent mobile phase evaporation.

Except for specific cases for which the column manufacturer recommends otherwise, (e.g. some ion-exchange columns), do not store the columns with buffer or less than about 25% organic solvent so as to avoid microbial growth.

6. Summary

Several common causes of peak shape problems, retention time variation, ghost peaks and column backpressure have been examined. Some of these problems originate from the sample, others from the mobile phase and still others from the column or other instrument components. A few good habits will help to minimize the occurrence of such problems.

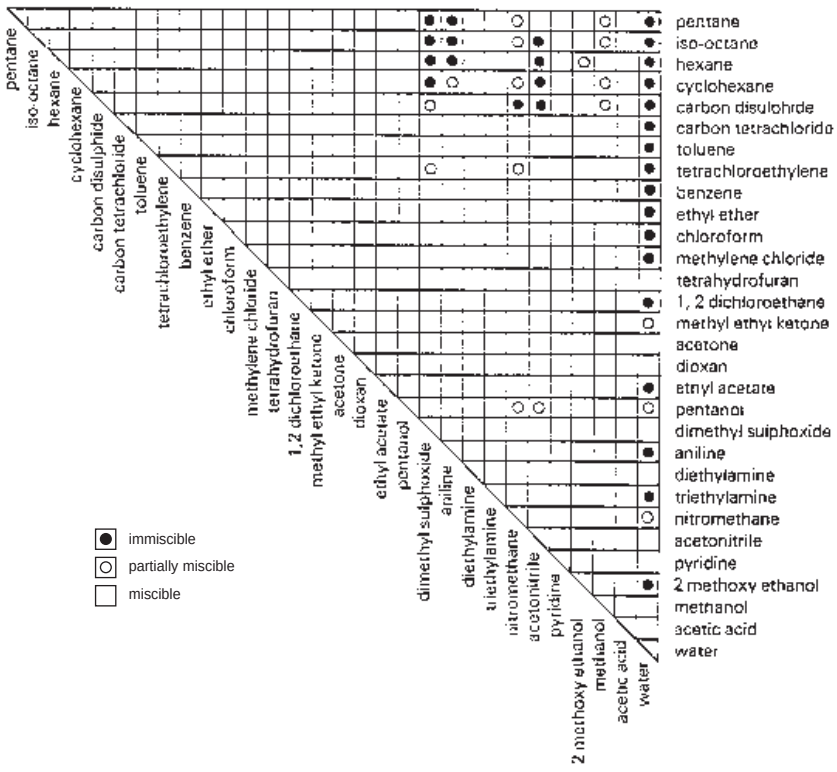
- Use a new, Type-B, high-purity silica-based column for each new project and couple this with the highest quality HPLC-grade reagents.
- Flush the HPLC system regularly to remove salts and buffers and service the system on a periodic basis to minimize check-valve and pump-seal problems. The more thorough the sample clean-up process, the cleaner the sample will be and the likelihood of sample-related problems will be lessened.
- At the completion of a series of runs (or after every run, in some cases), a strong-solvent flush will help to remove strongly retained materials from the column, minimize interferences in future runs and extend column lifetimes.

Columns won't last forever, but with proper care, you should be able to get a good return on your investment.

7. References

1. C. Hawkins and J.W. Dolan, LCGC 21 (2003) 1134-1138.
2. R.D. Morrison and J.W. Dolan, LCGC 23 (2005) 566-574.
3. J.W. Dolan, LCGC 14 (1996) 294-299.
4. J.J. Gilroy and J.W. Dolan, LCGC 22 (2004) 982-988.
5. D.H. Marchand, P-L. Zhu, and J.W. Dolan, LCGC 14 (1996) 1028-1033.
6. J.W. Dolan, LCGC 7 (1989) 822-826.
7. J.W. Dolan, J.R. Kern, and T. Culley, LCGC 14 (1996) 202-208.

8. Solvent Miscibility Chart



John Dolan is best known for his monthly *LC Troubleshooting* column in *LCGC* and *LCGC Europe*. In addition to over 250 installments of his column, Dr. Dolan has published more than 100 papers related to HPLC. His research interests are method development, column characterization and gradient elution. He has worked in all aspects of HPLC from instrument design, to writing software, to managing a contract laboratory and to teaching HPLC techniques. Currently John is a principal in LC Resources, a company dedicated to training chromatographers and providing consultation for chromatographic problems. He shares some of his expertise with us in this guide for HPLC troubleshooting. Further information on courses taught by John may be found on the LC Resources web site: www.LCResources.com

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