Ghost Peaks and Column Problems

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After the standard corrective measures are taken, sometimes LC column problems persist. Once the quick-fix solutions are exhausted, we must dig a little deeper to find the source of the problem.

This month's "LC Troubleshooting" addresses some less common problems encountered with liquid chromatographic (LC) columns and methods. We've all observed column back-pressure problems, but usually changing the column inlet frit fixes them — here is a case in which it didn't help. Or consider the predicament of a ghost peak in the gradient; when the ghost peak doesn't go away, we have to figure out where it originated. Finally, although most LC separations are accomplished using C8 or C18 reversed-phase columns, sooner or later we'll encounter a situation that requires a less commonly used column. In a discussion of problems related to a phenyl column, we find that, although these columns are different, most of the principles that we use with C8 and C18 columns apply to the phenyl columns as well.

HIGH BACK PRESSURE

Q: My silica-based size-exclusion column exhibits high back pressure that I have not been able to correct. The pressure had risen to about 2500 psi at a flow rate of 1 mL/min using tetrahydrofuran as the mobile phase. I replaced the frit at the head of the column, but this had a negligible effect on the pressure. Next, I tried backflushing the column. When the flow direction was reversed, the pressure dropped to 500 psi, but when I re-

turned the flow to the normal direction, the pressure was back up to 2400 psi. What could be causing these symptoms?

JWD: I suspect that the problem is associated with the outlet frit of the column. You have taken the obvious steps of replacing the inlet frit and backflushing the column, so it is unlikely that the column bed is causing the high pressure. I have observed similar symptoms when an inlet frit was partially blocked. When the column was reversed, the pressure dropped, but it increased when the column was used in the normal direction. When I replaced the inlet frit the problem was corrected. It appeared that there was some material stuck in the frit that acted as a one-way valve. When the flow direction was reversed, the contaminants were still attached to the frit but allowed free flow through the frit. In the forward direction, however, the material covered a sufficient proportion of the porous surface to restrict flow and thus increase pressure.

A similar thing could happen at the upstream end of the outlet frit. Contaminants could have accumulated from many sample injections, or they may have originated within the column. The column frits are described as having a nominal 2-μm porosity, but this is only the average porosity, which means that larger particles occasionally can pass through the frits. If a sufficient amount of >2-μm material has gone through the frit, the material could collect and block the outlet frit. I have also seen particulates much larger than 2 μm collected at the head of the column, under the frit. These particles, which appeared hair-like under the microscope, occurred when I was having problems with pump-seal and injector-rotor wear, so I assumed that they were a fluorocarbon material from one of these sources. Apparently, these particles were pliable enough to be forced through the frit under pressure and then were trapped at the head of the column. If such particulate matter reached the outlet frit, where the back pressure is much lower than at the column inlet, the frit could become blocked. Alternatively, the outlet frit may be blocked by small silica particles ("fines") resulting

from incomplete particle sizing during manufacture or from degradation of the particles during use.

Replace the column outlet frit, and I believe the pressure will return to normal. Take additional precautions to prevent future problems. First, be sure to filter any samples that appear cloudy, contain visible particulates, or are otherwise suspect. Next, be sure to use an in-line filter between the injector and the column. The filter frit should have 0.5-μm porosity to trap any particulates that might get to the column head. The use of a filter is especially important in size-exclusion applications because a guard column is rarely used. If the in-line filter becomes blocked fairly quickly, change the pump seals and check the injector for wear. Particulates released from worn seals will cause an unending series of blocked frits. Finally, be sure to use mobile phases that are compatible with the column. Check the column insert sheet or contact the manufacturer about acceptable conditions. Silica-based size-exclusion columns have different mobile phase restrictions than do polymeric columns. For example, polymeric columns can be used over a wide pH range, whereas silica columns generally are limited to the pH range from 2.5 to 7.0. On the other hand, silica columns tolerate certain solvents that cause polymeric columns to swell or shrink. And, as with any LC column, remove any buffers or salts from the system when it is not in use so that problems of buffer crystallization and microbial growth are avoided.

GHOST PEAKS IN GRADIENT

Q: I have been using a method for analyzing a pharmaceutical preparation on a C18 column. The gradient uses a phosphate buffer as solvent A and acetonitrile as solvent B. The gradient proceeds from 10% B to 80% B in 20 min at a flow rate of 1 mL/min, then reequilibrates for the next run. This method worked well for several months on the C18 column. Then, one day I noticed an extra peak in the chromatogram. After rerunning several samples, I ran a blank gradient and found that the peak was in the blank, even though the autosampler had not made an injection. The peak is approximately the same size in each run, including the blank. Where could this peak be coming from?

JWD: There are two likely sources of the ghost peak: the samples and the mobile phase. Generally, it's simplest to replace both solvent A and solvent B with fresh mobile phase, flush the column with strong solvent, and rerun the blank gradient. Most often this procedure will eliminate the ghost peak, and the result indicates that your solvent was contaminated. Identifying the exact problem source may not be worth the effort unless it is a recurring problem. If this quick-fix does not correct the problem, you will need to proceed with a more methodical problem-isolation strategy.

Even though the peak is observed in a blank gradient, you should not eliminate the sample as a possible problem source. Some-

times a minor sample component can build up on the column and elute in later gradient cycles when the mobile phase conditions are right. This is not commonly observed, but it can happen. The easy way to check for this buildup is to wash the column with strong solvent (100% acetonitrile in this case) to remove any strongly retained materials. A wash with 20 column volumes of solvent should be sufficient. If the ghost peak disappears, sample buildup is the likely cause. You can eliminate the problem by regularly flushing the column with strong solvent. If you are fortunate, you can get by with flushing the column once each day. Incorporate this flush with the system shut-down procedure at the end of each day's work. Be sure to flush the buffer from the system before switching to pure acetonitrile to avoid precipitation problems. If daily flushing is not sufficient, you may need to modify the method so that each analysis ends with a strong-solvent flush. For a problem such as this, program a steep gradient or a step gradient at the end of the normal analysis. For example, you might run your normal method, then go from 80% B to 95% B in 1 min, hold for 4 min, and return to the initial conditions for the next run. With a little experimentation, you should be able to determine the conditions that provide effective washing without unacceptably increasing the run time. As with any flushing procedure, remember that the flushing volume, not the time, is important. Thus, by increasing the flow rate during the final flush and reequilibration phases, you may be able to add the flushing steps without adding any time to your present method.

Although sample contaminants, as discussed above, may be the source of your problem, it is more likely that the ghost peak arises from one of the mobile phase components. The first step is to determine which mobile phase solvent (solvent A or B) is the problem source. Start by extending the reequilibration time (for example, to 30 min from an original 5 min); then rerun the blank gradient. If the peak size increases in proportion to the increase in reequilibration time ($6 \times$ in this case), solvent A is the problem. If no change in peak size is noticed, extend the solvent-B hold at the end of the gradient, then reequilibrate and run another blank gradient. If the peak grows in this case, solvent B is at fault. Although it may seem illogical for materials from a strong solvent B to collect and then elute under weaker conditions, it does occur.

Once you have identified the solvent that is causing the problem, replace it with fresh solvent. If the problem persists, one of the starting reagents is probably contaminated. Methodically replace each reagent with fresh reagent from a different batch. At this point, often the easiest approach is to use reagents from a different manufacturer to help identify the problem source. If you prepare your own HPLC-grade water in the lab, purchase a gallon of HPLC-grade water from a solvent vendor (alternatively, the distilled water in your local grocery store will be pure enough to use for testing and is not likely to have the same contaminants as your in-house water).

In most cases, the problem-isolation techniques discussed above will allow you to pinpoint the source of the ghost peak. In rare cases, other parts of the LC system are the problem source. For example, a dirty solvent reservoir can contaminate otherwise pure solvents. If the autoinjector rinse mechanism is not working properly, carryover from previous samples or general contamination can occur. A manual injector can become contaminated if the waste tube is allowed to siphon contents of the waste container back into the injection valve. I have seen a case in which oil from a piece of copper tubing used in a sparging apparatus contaminated the mobile phase. When the simple solutions are not effective, remove or substitute various system components until the problem disappears. When you isolate the problem source, clean or replace it and take precautions so that the problem does not recur.

PHENYL COLUMN CHANGES

Q: I am using an ion-pairing method on a phenyl column for the analysis of a pharmaceutical product and four of its associated metabolites. The mobile phase is 45:55 (v/v) acetonitrile/10 mM trimethylammonium (TMA). As the column ages, I see a gradual reduction in retention time, but retention returns to normal when I use a new column. The most recent column replacement was from a new batch of packing material, and the retention times for the peaks have increased. For example, the first band moves from 4.0 to 4.3 min and the last band from 15 to 22 min. I've tried adjusting the mobile phase by adding a little more acetonitrile to reduce the retention, but when I do this, the selectivity has changed sufficiently that two of the peaks are not resolved. What is going on?

JWD: I don't see a single obvious cause of the problem, but there are several areas in which your method might be adjusted to improve its reproducibility. First, phenyl columns are not as stable as the more commonly used C8 or C18 reversed-phase columns. Workers who study column stability have found that a small, but continuous, bleed of the phenyl phase from the column occurs throughout the column life (1). This bleed probably accounts for your observation of retention-time reductions. I don't think there is a good understanding of exactly why this happens, but it is a column characteristic that must be tolerated.

Another potential problem area has to do with the TMA you are using as an ion-pairing reagent. Most workers choose tributylammonium (TBA) ion as an ion-pairing reagent for acids. The more hydrophobic nature of the TBA, which is caused by the butyl side chains, provides stronger retention. The recommended starting concentration for TBA is ~40 mM (2). Retention in ion-pair separations is caused mainly by the molar concentration of the reagent taken up by the column packing, and higher molecular weight reagents require lower concentrations in the mobile phase to have an equivalent effect (2). Therefore, if 40-mM TBA is a good working concentration, a higher concentration of TMA is suggested for the same effect.

The use of mobile phase additives at too-low concentrations is a common fault of LC separation methods. For most reagents (ion-pairing, buffers, triethylamine [TEA], and so forth), there is a concentration vs. retention curve that shows a regular change in retention with fixed changes in concentration up to a particular concentration. Above this concentration, however, the curve levels out, so that small changes in additive concentration have little effect on sample retention. Methods will be much more stable if the additives are used in concentrations corresponding to this plateau region. For most LC reagents, 10 mM reagent concentrations in the mobile phase are about as weak as one should use; generally, 20–50 mM is a better first choice. In your case, it appears that the TMA concentration is marginal at best.

Finally, when column-to-column variations are seen, try adding a masking agent to the mobile phase to see if the column-to-column differences can be minimized. The most common difference between nominally identical columns from the same manufacturer is a difference in surface coverage by the bonded phase. Small differences in the percentage of residual unbonded silanol groups at the surface can have dramatic effects on the retention and selectivity for some compounds. The most popular additive used to mask these effects is TEA, which competes with basic sample components for the silanol sites. A TEA concentration of 20 mM is a good place to start (2) and should help to reduce the differences that you see between columns. Of course, when a new column is used, the best way to keep from being surprised by such changes is to test the method with columns from two or more packing batches before putting it into routine use. Sometimes in the haste to get a method into service, we forgo this precaution of method-ruggedness testing — and it comes back to haunt us when we least expect it.

So, although there are no cure-alls for the problems you have observed, some adjustments in the phenyl column method may improve its tolerance to changes as the columns age and when columns are replaced.

REFERENCES

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