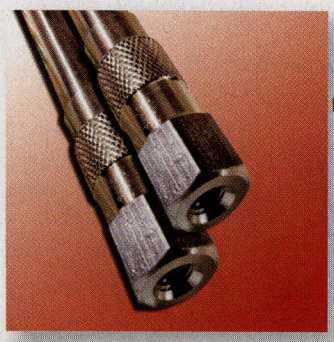


LC TROUBLESHOOTING

Column Care

How to most effectively clean your column.

John W. Dolan
LC Troubleshooting Editor

As I write this column, I have just completed two weeks of teaching short courses in Israel, and as I have observed before, the chromatographic problems know no national boundaries. Questions related to how to clean and store a reversed-phase liquid chromatography (LC) column are among the most common queries in the classes I teach, and my recent experience is no different. This month's "LC Troubleshooting" discussion will center on three of these: how to condition a new column, the effect of washing a column with water, and recommendations on cleaning a column after it has been used to analyze samples.

What to Do When New

So you've just received a new reversed-phase LC column. What now? Nearly all columns are shipped containing the solvent in which they were tested. Usually this is 60–80% methanol and water. This mobile phase is compatible with just about every mobile phase used for reversed-phase separations, so you can switch directly to the mobile phase you plan to use. As an added precaution, you might want to wash the column with 20–30 mL of the strong solvent of your mobile phase — methanol, acetonitrile, or tetrahydrofuran. Then switch over to the mobile phase and you should be ready to use the column. Remember, when flushing or equilibrating a column, it is the volume of solvent that is most important, not the time, so if you can increase the flow rate, you can shorten the wash-out time.

For methods that use organic solvent and water or buffer, 10 column volumes of mobile phase should be sufficient to equilibrate it for use. If you are using an ion-pairing method, it can take 20–50 column volumes of mobile phase before the column is equilibrated. The column volume, V_m, for 4.6-mm i.d. columns can be estimated as

$$V_m \approx 0.1\ L \qquad [1]$$

where V_m is in milliliters and L is the column length in centimeters. So a 15 cm × 4.6 mm column would have approximately 1.5 mL of volume. Columns 2.1 mm in diameter have approximately 20% of this volume, so a 5 cm × 2.1 mm column would contain ≈100 µL of solvent.

With today's high-purity, type-B, silica-based columns, reproducibility from one column to the next is the norm. This is in sharp contrast with the less pure silica, type-A columns commonly in use 15 years ago. However, many of the older, less pure columns are still in use for "legacy" methods that have been supporting the analysis of products or raw materials for many years. Sometimes a new column will require several injections of sample before it settles down to giving a constant retention time and peak area for the analyte. This is less common with the latest columns than with the older type columns, but a period of break-in is not a rare event. Sometimes initial column conditioning can be sped up by injecting several high-concentration injections of sample or standard. For example, if you see retention or area drift for several injections of 50 ng of standard, try injecting 1 µg of standard a couple of

times, then after the sample has been eluted, try the 50-ng standard again. Often this approach will speed up the initial column conditioning process.

Washing with Water

I often get a question that goes something like this, "My sample is water soluble, and my mobile phase contains buffer and methanol, so shouldn't I wash the column with water to remove all the water-soluble contaminants?" The answer is an emphatic, "NO!" Then the originator replies, "But the sample is not soluble in acetonitrile."

There are two flaws to the argument to wash a C8 or C18 column with water. First, the columns generally are not compatible with 100% water, and second, the sample is almost always soluble in acetonitrile or methanol.

Although we might think of a C18 packing particle as looking something like a tennis ball with all the C18 phase looking like fuzz or on the surface, this is not a good description. The particle is more like a sponge with almost all the surface area inside the particle, within pores in the 6–15 nm diameter size. The C18 surface is bonded inside the pores, with very little of it on the outside surface of the particle. This makes the pores very hydrophobic. During normal operation, a water–organic mobile phase fills the pores and fully wets the bonded phase. Analyte molecules must diffuse in and out of the pores to be retained by interaction with the stationary phase. When the mobile phase is replaced by water, the water diffuses into the pores, diluting the organic solvent in the mobile phase as well as the prior aqueous portion of the mobile phase — buffer or water. However, the water is too polar to penetrate the bonded phase itself, so it doesn't do a good job of washing the bonded phase. When all the organic solvent is removed from the pores, the mobile phase (100% water) is now so much more polar than the bonded phase, it can be pushed back out of the pores. I think of it as beading up on the nonpolar surface of the particles like drops of water on an oily spot in the parking lot. When this happens, you can wash all day with water and the column won't get any cleaner. And when organic solvent is added back to the mobile phase, it is slower to reequilibrate with the column than if some organic solvent remained.

This process of having the water excluded from the pores in the column packing material is called "dewetting." We used to call it "phase collapse," but the bonded phase doesn't collapse, it just excludes water from the pores. Some columns can be wetted by 100% water and are the exception to this dewetting behavior. These columns are the embedded polar phase columns, sometimes referred to as "AQ" or water-compatible columns. Such columns contain a sufficiently polar bonded phase that it remains wetted with 100% water.

A Better Way

A much better approach to washing the column is to first replace the buffer component of the mobile phase with water. For example, replace 60:40 acetonitrile–buffer with 60:40 acetonitrile–water. Wash the column with 5–10 mL of this — just enough to wash most of the buffer out. Then switch to 100% of the organic solvent, acetonitrile in this case. Further washing with 10–20 column volumes of the organic solvent should be sufficient to remove strongly retained materials. The column can then be stored in this solvent, either on the LC system or if the column is removed, with the column end-plugs in place. This washing procedure is appropriate for all reversed-phase columns, whether they are C8, C18, embedded polar phases, phenyl, cyano, or some other bonded phase.

The initial wash with nonbuffered solvent might not be necessary for many methods, but it is a good precaution to take, especially if the mobile phase contains a buffer, such as phosphate, that is not soluble in high concentrations of acetonitrile. For example, you don't want to switch directly from 30:70 acetonitrile–20 mM phosphate to 100% acetonitrile, because the phosphate might precipitate in the pump or column.

Another precaution I recommend is not to shut off the LC system for more than an hour or so if it or the column contains buffer. If the organic solvent evaporates when the system is standing idle, it can leave abrasive buffer residues on the pump pistons and other exposed parts. This will shorten pump seal lifetimes and cause premature failure of other parts. A better choice is to program the controller to switch to a wash sequence after all the samples are run. The wash sequence would replace buffer with water for a few minutes, as recommended previously, then wash the system with the strong solvent of the mobile phase before system shutdown. If the LC system does not have the capability of changing solvents in this manner, just program a low-flow method that keeps the pump running at a minimum flow rate, such as 0.1 mL/min, until you can flush out the system manually.

Insoluble Samples?

What about that argument that the sample is not soluble in the organic solvent of the mobile phase? It can be true that your sample is not freely soluble in methanol or acetonitrile according to the solubility tests by the synthetic chemist. However, at the concentrations used for analysis, it is unlikely that the sample is not soluble in the same solvents. If the sample were not soluble at the analytical concentrations, how would it be eluted from the column during analysis? In some rare cases, there might be a critical concentration of water–organic necessary for sample solubility, but for successful chromatography, it is likely that this will be close to the mobile phase concentration and the sample will be eluted by the mobile phase. Most of the column washing activities are targeted not at removing the sample from the column, but at removing contaminants from the sample matrix from the column. The strong solvent of the mobile phase is the best candidate for this.

More Aggressive Washing?

Sometimes I hear of workers who like to wash the column with solvents or solutions that are more aggressive than the strong solvent of the mobile phase. For example, after flushing with acetonitrile, they can wash with methylene chloride or methyl *tert*-butyl

ether or dimethyl sulfoxide. Yes, these might remove some very strongly retained materials, but you have to ask yourself if the effort is worthwhile. Consider the economics: a typical LC–UV analysis costs approximately $50 in the pharmaceutical industry. If a $500 reversed-phase column lasts 500 injections, this is $1 per injection, or 2% of the overall cost. If you can spend an hour cleaning the column and double the column life, you've saved only 1% of the cost of analysis — is this a good investment of your time? I figure that if the column lasts at least 500 injections, I have received good value from it — and in my experience, even with plasma-based samples, columns often last 2000 samples or more. Remember that the column is a consumable item, not a capital expenditure, even if it does cost a lot of money.

For the Next Use

If you always end a method by flushing the column with the strong solvent of the mobile phase and using it as the storage solvent, then starting up for the next application of the method is quite simple. You know that the column was washed with a strong solvent, so any strongly retained materials should have been flushed from the column. For the next use, just change over to the mobile phase and allow it to equilibrate — usually 10–20 column volumes of mobile phase will be sufficient. If you are not sure how long the equilibration takes, make a couple injections of standards — if the retention times for two consecutive runs are the same, the column is equilibrated.

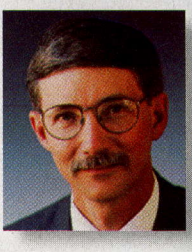
And a Word from Our Sponsors

Any discussion of column washing would be incomplete without the standard caution: "If all else fails, read the directions!" Every column is shipped with a list of care-and-use instructions. If you cannot find them, they should be posted on the column manufacturer's web site. These generally contain suggested conditions of use, pH limits, any prohibited solvents, and a cleaning procedure. The procedures discussed previously should be applicable to nearly all silica-based reversed-phase LC columns. Chiral columns, columns that use polymeric beads instead of silica for support materials, some normal-phase columns, and certain specialty columns can have restrictions in terms of solvents or other suggested cleaning procedures. In all cases, the manufacturer's instructions should be followed.

Conclusions

If you make it a standard practice to wash the mobile phase from the column and leave it filled with 100% of the strong solvent of the mobile phase, you can use this procedure for breaking in a new column, washing strongly retained materials from the column after use, or preparing a column for storage. If all your columns are stored in methanol, acetonitrile, or tetrahydrofuran, when it is time to run more samples, all you have to do is put the mobile phase on the system and equilibrate the column with 10–20 column volumes of mobile phase — 15–20 min at 1–2 mL/min should be sufficient. Then make sure the column is equilibrated by making two injections of standards — if the retention times and areas are the same, the column is ready for sample analysis.

For more information on this topic, please visit www.chromatographyonline.com/dolan



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For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at http://www.chromforum.com.