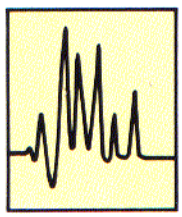
TROUBLESHOOTING

Readers' Questions

JOHN W. DOLAN



This month, readers' questions on microbore high performance liquid chromatography (HPLC), fitting assembly, and extra peaks in the chromatogram are addressed. Readers are invited to contribute their troubleshooting tips or to submit questions to be answered in future "Troubleshooting" articles. Write to: The Editor, *LC•GC*, P.O. Box 50, Springfield, OR 97477.

MICROBORE COLUMNS

Q: I have just started using a 25 cm × 1 mm, 5-μm microbore column with a C18 stationary phase for separations of biological samples. Even with my best efforts at minimizing the system volume, I can only get 10,000 plates for toluene ($k' = 4$) when using a flow rate of 30 μL/min, and the pressure is twice that reported on the column manufacturer's test sheet. The test sheets, which I've enclosed, show 22,000 plates for toluene on two different columns. What am I doing wrong?

JWD: Microbore systems require an extreme amount of care to minimize the extracolumn volume. Calculations (1) using the column test sheets show that the manufacturer has an LC system with about 1 μL (1 σ) of extracolumn band broadening, and yours has about 3 μL. That alone can account for the differences in the observed plate number. A recent article in *LC Magazine* discussed the three most important considerations in preventing band broadening in microbore HPLC: injection technique, tubing connections, and detector design (2). An example of the influence of connecting tubing on microbore separation is seen in Figure 1. In Figure 1a, the column is connected directly to the injector and detector. In Figure 1b, however, the column is connected using 5 cm × 0.005 in. i.d. tubing. The tubing accounts for a loss of 25% in peak height, a change that would not be noticeable for peaks from an LC system equipped with a conventional column. Very good performance can be obtained from a well-designed and properly operated microbore system, but it does take a great deal of care.

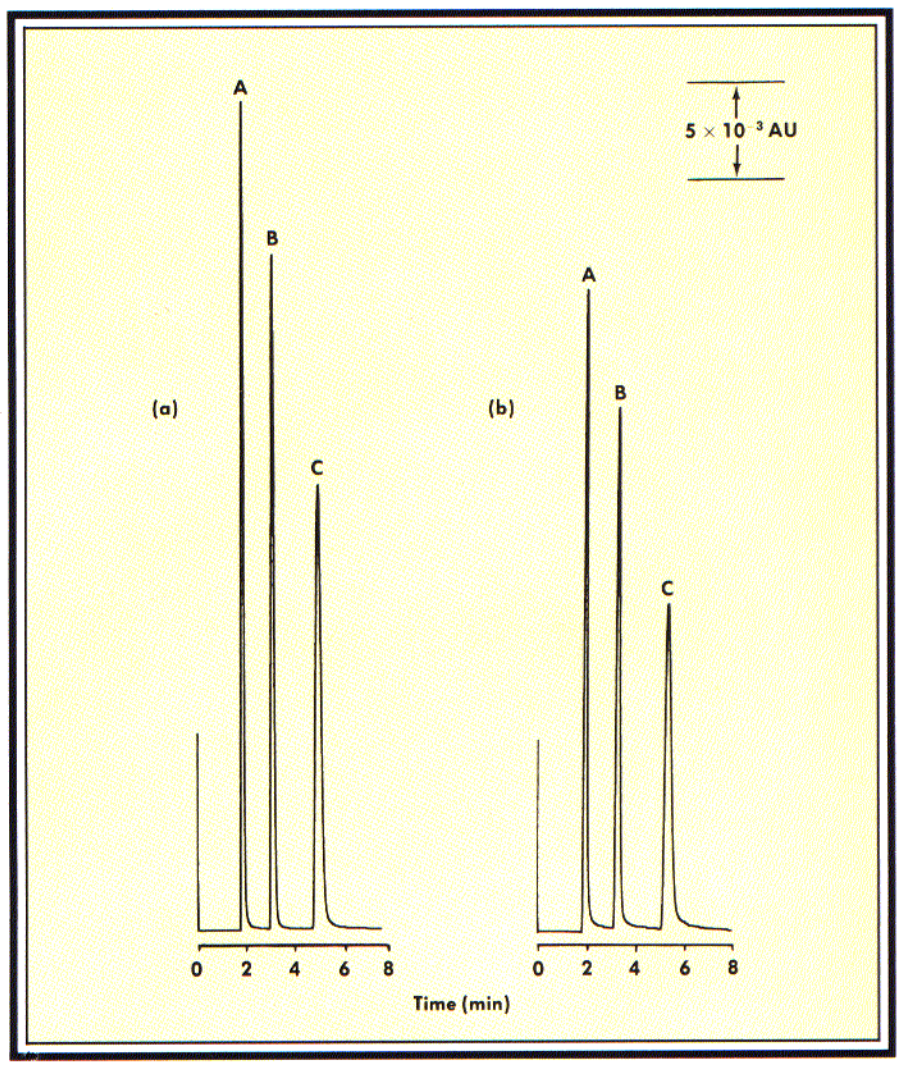


FIGURE 1: Effect of extracolumn volume on peak height for a microbore HPLC system. (a) Direct connection of column to injector and detector. (b) Column connected with 5 cm × 0.005 in. i.d. tubing at each end. Column: 10 cm × 1 mm, 3-μm C18; flow rate: 25 μL/min at 630 psi. (Reprinted from reference 2 with permission.)

Another reason you had trouble reproducing the manufacturer's results is that the plate numbers reported on the test sheets do not tell the entire story. All the peaks for both columns tail badly: only one peak has an asymmetry factor (As) of 1.15; all other peaks show $As > 1.4$. If plate numbers are calculated at half height, tailing is not adequately taken into account, so plate numbers are overstated. Furthermore, when you see a neutral compound, such as toluene, which has $As > 1.4$, you should suspect that some-

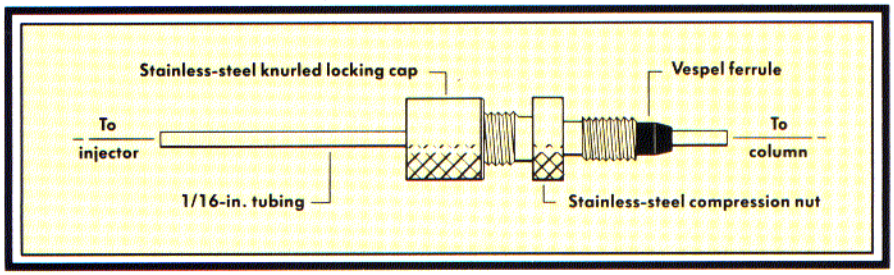


FIGURE 2: A finger-tightened fitting that combines the convenience of polymeric ferrules with the security of stainless-steel compression fittings. (Courtesy of Keystone Scientific.)

thing is wrong with the preparation and packing of the column. With a possible packing problem indicated, it's not surprising that the back pressure is higher than expected. This is not to say that commercial microbore columns aren't any good; it's just that they are much more difficult to manufacture with the same performance characteristics as conventional (for example, 15 cm × 4.6 mm) columns. The same kinds of variability were seen with conventional columns 10 years ago when slurry-packing techniques were being developed.

With these two problems in mind (equipment design and column-packing technology), it is wise to consider alternatives to the use of microbore columns. In your case, you could generate the same 10,000 plates in 2 min instead of 18 min on a 10 cm × 4.6 mm, 3-μm column in an LC system with conventional plumbing. Granted, the peak heights would be about one-tenth as large, but the larger detector cell of a conventional LC system will make up part of the difference. If you could increase the injection volume by a factor of five, you would probably get a similar response from the two systems. Another alternative is to use a "fast" LC column (for example, 3 cm × 4.6 mm with 3-μm particles), which can generate about 5000 plates in a 1-min separation (3). These short columns use conventional packing technology and, therefore, probably give more reproducible separations; however, they use small samples and produce very small peak volumes (which also requires minimizing the extracolumn volume). Still another alternative is to use a 2-mm i.d. column, which is easier to pack and has less stringent plumbing requirements, but uses smaller sample sizes than conventional columns.

In conclusion, I'm not surprised that you are having trouble with microbore HPLC; many workers do because the technology is not as mature as it is for separations performed on larger columns. With conventional columns and systems, we can be somewhat sloppy in our technique and still get acceptable separations; this isn't true for microbore. Microbore columns are particularly useful if sample size is limited, if expensive solvents are used, or for LC–MS. In most cases, however, alternatives should be explored first.

SLIPPING FITTINGS

Q: I would like to use finger-tightened fittings for connecting my LC columns, but I'm worried about the fittings slipping loose when the temperature changes or when the column is bumped. Are there any connectors that offer the safety of stainless-steel fittings but are as convenient as the polymeric fittings?

JWD: First of all, I think that the concern about fittings slipping apart is overstated. It arises from the fear of high pressure within the LC system (remember when it was called "high pressure" LC?), in addition to manufacturers' legitimate concerns about product liability. Nylon ferrules were mentioned in an earlier column as an inexpensive alternative to commercial polymeric fittings (4). I have used those to pressures of 6000 psi and have never had one slip. The commercially available finger-tightened fittings are claimed to withstand 2000–4000 psi. Many workers set an upper pressure limit of 2000–2500 psi for most assays to save wear and tear on the LC equipment, so any of the finger-tightened fittings should work well in these cases.

What are the hazards if the fitting does slip? First, and most important, chromatographic performance may deteriorate because of the dead volume created in the fitting if a tube is not seated properly (5). Second, and of less concern, is the problem of leakage. Even if the fitting "pops" off, there is no great hazard; nothing explodes or sprays like a runaway fire hose. Rather, only a small stream of solvent (for example, 1 mL/min) is emitted, and a puddle forms where the solvent leaks.

There are alternatives. One recently introduced product appears to address both of your concerns. This is the "slip-free" fitting from Keystone Scientific (State College, Pennsylvania) shown in Figure 2. The actual seal is made with a Vespel ferrule in the conventional manner, allowing the fitting to be easily disassembled and reformed for another column type. Next, a backup nut is finger-tightened against a preswaged stainless-steel ferrule that serves two functions. First, the backup nut forces the tube against the bottom of the female port, which ensures a proper connection every time, even if the initial assembly was incorrect. Second, because the stainless-steel ferrule is swaged on the tube in the conventional manner, the fitting is protected against blowout in case of temperature changes or other loosening influences. Because this fitting requires no tools for assembly and the seal is made with a polymeric ferrule, the fitting is easily disassembled for use with another column type or union.

I would appreciate feedback from readers on your experiences with those and other finger-tightened fittings. Are they as good as the manufacturers claim? How many times can you use the fittings before they no longer work well?

ONE FOR THE READERS

Q: I have developed an assay for a protein sample on a Waters Protein Pak DEAE 5PW column (Waters Chromatography Division, Milford, Massachusetts). When the sample is dissolved in water or starting buffer (5 m*M* Tris–acetate, 5 m*M* calcium acetate, pH 7.5), the system works well; however, when the sample is dissolved in a diluent (75 m*M* sodium chloride, 1 m*M* calcium chloride), an extra peak elutes with a retention time greater than the sample. This behavior is consistent but not quantitative and occurs without the protein sample. Unfortunately, the "contaminant" does not completely elute within the gradient, requiring 2–3 column volumes of the high-salt buffer (500 m*M* sodium acetate, 5 m*M* Tris acetate, 5 m*M* calcium acetate, pH 7.5) for complete elution.

Diluent obtained from three different companies (in addition to my own mixture) and sodium chloride itself give the same result. A UV scan of the 1 *M* sodium chloride that I use shows no absorbance at a detection wavelength of 280 nm. All samples and mobile phases are filtered through 0.45-μm membranes. Do you know what could be causing the extra peak and how I could get rid of it?

JWD: You seem to have covered all the bases in systematically tracking down a reagent as the source of a contamination. The salts in the diluent should be unretained in your system, so they should not have any influence on a late-eluting peak. The results are puzzling. I would like to invite readers who have suggestions to send them to me, and perhaps we can find a solution to your problem.

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