LC TROUBLESHOOTING

Column Stability: A Case Study

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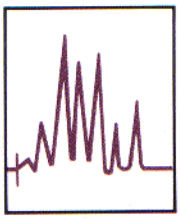

A reader has contributed a case study on the reaction of sample components with a column packing. Questions on sample preparation, pressure problems, and retention reproducibility in gradient elution are also addressed.

REACTIVITY OF POLYMER-BASED SEC COLUMNS

[The following discussion was submitted by Dan Miller of ICN Radiochemicals in Irvine, California. Dr. Miller experienced unexpected problems with a size-exclusion chromatography (SEC) column while he was attempting to purify iodinated nucleotides. This serves not only as an interesting case study, but also reminds us that column packings are chemical in nature and are susceptible to chemical reactions with sample components.]

While attempting a novel approach to purifying radioactive iododerivatives of cyclic nucleotides, I encountered a problem with the chemical reactivity of the column packing material. The problem was traced to a chemical reaction between a reaction initiator and the packing material.

A Bio-Gel TSK-10 LC column (30 cm × 7.5 mm) and a Bio-Gel TSK guard column (Bio-Rad Laboratories, La Jolla, California) were used with an isocratic system for liquid chromatography (LC). Eluting bands were monitored with UV detection at 280 nm and with radioactivity detection. Highest-purity reagents and HPLC-grade solvents were used throughout.

2-*O*-Succinyl cyclic adenosine monophosphate tyrosine methyl ester (sCAMP-TME) was iodinated with cold iodine according to a modified Hunter and Greenwood method (1) to produce nonradioactive iodine-labeled standards to compare with the unlabeled material. 2-*O*-Succinyl cyclic guanosine monophosphate tyrosine methyl ester (sGMP-TME) was produced in the same manner.

The first attempts at purification used a 50-m*M* sodium acetate buffer (pH 4.75) at a flow rate of 1.0 mL/min. Standards were run of the unlabeled sCAMP-TME, iodo-sCAMP-TME, and the reaction initiators (chloramine-T, NaI, and sodium metabisulfite). Peak identity was determined by injecting increasing amounts of the known solutes and recording their respective retention times. Only a buffer peak was detected when the nucleotide derivatives were injected. High-salt conditions and elevated pH (pH 8.3) were required to elute the adsorbed nucleotides. Adsorption may have resulted from a protonated amine on the adenine ring at low pH. The reaction initiators appeared to elute in one large peak at 15.5 min. It appeared, however, that the NaI reagent was not eluting under any of the conditions tested.

At this point, an experiment was carried out to confirm the adsorption of free iodine. A standard reaction was run as before to prepare iodo-sCAMP-TME, but this time with ~500 μCi of $Na^{125}I$. The analytical column was removed from the LC system and a sample of the reaction mixture was injected onto the guard column. After ~60 mL of 50 m*M* sodium acetate buffer (pH 4.75) was pumped at 1 mL/min, no radioactivity was detected in the eluent. Increasing the ionic strength (to 300 m*M*) by adding NaCl, changing the pH to 8.3, or adding 15% methanol to the eluent had no effect on the elution of ^{125}I. The guard column was then disassembled and a small amount of the packing material was taken from each end and counted in a gamma counter. The packing material from the inlet end of the guard column stalled the gamma counter ($>10^6$ cpm), while the material from the outlet end showed approximately two times the normal background level. This confirmed that the iodine was being irreversibly adsorbed to the column packing.

The interaction between the NaI and the column packing can be postulated. TSK's literature describes the packing material as a stable hydroxylated polyether, useful for purifying neutral polycationic polymers in a wide pH range (pH 2–12). There is no indication of instability toward iodine. It is possible, however, that iodine (or bromine) will cleave the ether bond, giving *n*-iodo products (C–O–C + NaI $\rightleftharpoons$ C–I + Na–O–C). This splitting would break down the matrix, changing its original character. These changes could be significant if, as in the present case, large volumes of reaction mixtures containing excess NaI were injected onto the column in a semipreparative mode.

In conclusion, it appears that these hydroxylated polyether-based packing materials are unsuitable for use with samples containing free iodine (or bromine). Exposing the packing to these halogens may quickly degrade the matrix, reducing the useful lifetime of the column. In any event, it is wise to use a guard column to protect the analytical column from damage.

SAMPLE PREPARATION

Q: How does the pH of the extraction solvent affect the separation of a sample? I am extracting a crop matrix at low pH to remove my sample of interest. This solution is back-extracted with hexane, evaporated to dryness, and redissolved in isopropanol. The isopropanol solution is then injected and eluted from a C18 column with a methanol–water mobile phase. Bad peak shapes have appeared, especially with the early peaks, and I suspect that this a result of the pH of my initial extraction solvent. Can you help?

JWD: Although you haven't given me the complete details of your method, I suspect that the pH of the initial extraction solvent is not causing the problem. The acid from the original extraction should remain in the aqueous phase when the sample is extracted into hexane. More than likely, the problem lies with the isopropanol that you are using as an injection solvent. When you inject a sample in solvent that is as strong as or stronger than the mobile phase, you may cause peak broadening in the chromatogram. This is because the injection solvent acts like a strong mobile phase and causes the sample to migrate down the column until it is diluted in the regular mobile phase. This results in a broad band of sample at the top of the column and, thus, a broader band at the column outlet. These early-eluting bands migrate farther under the influence of the (strong) injection solvent than do later bands and, therefore, are more affected.

There are three ways to get around the problem of a strong injection solvent. First, use mobile phase (methanol–water) or dilute mobile phase to dissolve the sample. It may be possible to dissolve the sample in methanol and then dilute it to the same concentration as the mobile phase. Try to keep the injection volume at 25 μL or less, if you use mobile phase to dissolve your sample. Second, often you can inject the same mass of sample in a

larger volume of diluted injection solvent and have fewer band-broadening problems. In this case, dissolve the sample in isopropanol, then dilute it, for example, three times with water; now inject three times the previous injection volume. This weakens the injection solvent so that little or no sample migration occurs before the injection solvent is washed off the column. When sufficiently dilute solutions are injected, several hundred microliters of sample can be injected without significant band broadening. Third, if you must use pure isopropanol as the injection solvent, inject as little sample as is needed, preferably <10 μL. When a small volume of strong injection solvent is used, it can be diluted quickly by the mobile phase, minimizing band-broadening problems.

CHECK-VALVE PROBLEMS

Q: I experience inconsistent pressures and pressure pulses with my LC system. It seems as if I am always rebuilding check valves, and I filter all solvents before use and prime the pump. Is there something else I should be doing?

JWD: There are several parts of the system that you may be overlooking. First of all, check the solvent inlet filter (the one in the reservoir) to be sure it is not partially blocked. The easiest way to do this is to remove the filter; if the pulsation problem disappears, replace the filter with a new one. Otherwise, put the old filter back. Second, the pressure in the inlet line may be too low to allow the inlet check valves to function properly. This can be caused by a reservoir lid that is too tight, allowing a partial vacuum to be formed when solvent is withdrawn. Be sure that the cap is loose or has a 1-mm vent hole to prevent this problem. Elevating the reservoir so that the mobile phase inlet is above the level of the pump also will improve pump reliability by adding a bit of head pressure to the pump inlet. Third, you will find that most pumps are more reliable if you degas the solvent before use, even if it isn't required. Vacuum degassing usually is sufficient; helium sparging is more effective.

Finally, it may be that you are not adequately rebuilding the check valves. My success rate at check-valve rebuilding is not much better than 50%, and I wondered why until I visited a manufacturer's check-valve assembly area. At the factory, check valves are assembled in a dust-free clean room, an environment seldom found in typical LC labs. The results are much more reliable check valves. In your case, I'd try a set of new or factory-rebuilt check valves. They may solve the problem; if not, continue to rebuild your own.

GRADIENT RETENTION-TIME PROBLEMS

Q: In our lab, we do routine gradient elution assays. Our problem is that we use different brands of LC instruments, and each brand of instrument has a different dwell volume; as a result, the retention time varies from instrument to instrument under "identical" assay conditions. Is there a way to get around this problem?

JWD: The dwell volume, sometimes called the gradient delay or gradient lag, is the volume of the LC system from the point where the solvents are mixed to the head of the column. With low-pressure mixing systems, this is the volume of the mixer, the pump heads, and the connecting tubing, often 5–6 mL. High-pressure mixing systems have just the volume of the mixer and the tubing, generally in the range of 2 mL. Some systems have dwell volumes that are <1 mL.

As an example, assume a low-pressure mixing system (5-mL dwell volume) operated at 1 mL/min. If the sample is injected onto the column at the same time the gradient is started (which is common practice), the sample will migrate through the column for 5 min (5 mL at 1 mL/min) under *isocratic* conditions before the gradient reaches the column. Sample components with low capacity factors (k') may elute from the column during this time, whereas components with high k' will not migrate until the gradient reaches the head of the column 5 min later. Compare this with the same sample and mobile phase used with an LC system that has a dwell volume of <1 mL. Both the early- and late-eluting sample components will begin to migrate under the influence of the gradient almost immediately — and thus will have different retention times than were observed for the previous case. This is why you observe different retention times for different LC systems.

There are two solutions to the problem. First, you can delay sample injection until the gradient reaches the head of the column. For the low-pressure mixing system discussed above, you would inject 5 min after the gradient was started; the delay would be <1 min for the high-pressure–mixing system. Alternatively, in some cases, you can adjust the initial gradient conditions so that no sample migration occurs during the isocratic hold before the gradient reaches the column. That is, if you make the starting gradient composition sufficiently weak, all the bands will remain at the head of the column until the gradient reaches the column. The first solution will work for all samples, but some systems using autosamplers are not able to make injections after the gradient is started. If you are making manual injections, be careful to time the injection precisely each time. The second method will work only for compounds that are sufficiently retained under initial gradient conditions; if the technique can be used, however, it is easy to automate.

REFERENCES

(1) W.M. Hunter and F.C. Greenwood, *Nature* **194**, 495 (1962).

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